County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:11 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 5 of 9
Attachments: 51 Axelstad Effects of pre and postnatal exposure.pdf; 52 Klammer Effects of a 5-day treatment.pdf; 53 Klammer et al ED Toxicology.docx; 54 Ponzo Evidence of reproductive disruption.pdf; 55 Carbone Exp Clin Endocrinol Diabetes.docx; 56 Szwarcfarb Exp Clin Endocrinol Diabetes.docx; 57 Seidlová-Wuttke Toxicol Appl Pharmacol 2.docx; 58 Seidlová-Wuttke Toxicol Appl Pharmacol.docx; 59 Schmutzler Toxicology 2004b.docx; 60 Schneider OMC Two generation reproduction toxicity2.pdf; 61 Hamann 4MBC and OMC and thyroid.docx; 62 Manova Aggregate consumer exposure.pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

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Effects of pre- and postnatal exposure to the UV-filter Octyl Methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring

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ABSTRACT

Octyl Methoxycinnamate (OMC) is a frequently used UV-filter in sunscreens and other cosmetics. The aim of the present study was to address the potential endocrine disrupting properties of OMC, and to investigate how OMC induced changes in thyroid hormone levels would be related to the neurological development of treated offspring.

Groups of 14–18 pregnant Wistar rats were dosed with 0, 500, 750 or 1000 mg OMC/kg bw/day during gestation and lactation. Serum thyroxine (T_4), testosterone, estradiol and progesterone levels were measured in dams and offspring. Anogenital distance, nipple retention, postnatal growth and timing of sexual maturation were assessed. On postnatal day 16, gene expression in prostate and testes, and weight and histopathology of the thyroid gland, liver, adrenals, prostate, testes, epididymis and ovaries were measured. After weaning, offspring were evaluated in a battery of behavioral and neurophysiological tests, including tests of activity, startle response, cognitive and auditory function. In adult animals, reproductive organ weights and semen quality were investigated.

Thyroxine (T_4) levels showed a very marked decrease during the dosing period in all dosed dams, but were less severely affected in the offspring. On postnatal day 16, high dose male offspring showed reduced relative prostate and testis weights, and a dose-dependent decrease in testosterone levels. In OMC exposed female offspring, motor activity levels were decreased, while low and high dose males showed improved spatial learning abilities. The observed behavioral changes were probably not mediated solely by early T_4 deficiencies, as the observed effects differed from those seen in other studies of developmental hypothyroxinemia. At eight months of age, sperm counts were reduced in all three OMC-dosed groups, and prostate weights were reduced in the highest dose group. Taken together, these results indicate that perinatal OMC-exposure can affect both the reproductive and neurological development of rat offspring, which may be a cause of concern, as humans are systematically exposed to the compound through usage of sunscreens and other cosmetics.

Introduction

The UV-filter Octyl Methoxycinnamate (OMC), also known as Ethylhexyl Methoxycinnamate, is a very frequently used chemical in sunscreens and cosmetics worldwide. OMC is absorbed though the skin and is detectable in human blood and urine samples after topical application (Janjua et al., 2004). OMC has also been found in milk samples of women who have used OMC containing products (Schlumpf et al., 2008a), which indicates that humans are systemically exposed to this compound. Findings in several studies indicate that the substance acts as an endocrine disruptor (Schlumpf et al., 2001, 2008b; Schmutzler et al., 2004; Seidlova-Wuttke et al., 2006; Klammer et al., 2007).

Some *in vitro* studies have shown OMC to act as an estrogenic compound, as it enhanced proliferation of breast cancer cells (Schlumpf et al., 2001) and activated transcription in human cell lines via the estrogen receptor (Schreurs et al., 2002; Gomez et al., 2005). In an ecotoxicology study, Inui et al. (2003) demonstrated that in male medaka, OMC caused increased production of vitellogenin, a classical marker of estrogenic action in fish. In several studies in rats, OMC exposure has lead to increased uterine weight. This has been observed in an uterotropic test with immature female rats after 4 days of dosing with 1035 mg OMC/kg bw/day (Schlumpf et al., 2001), in ovariectomized (OVX) adult rats treated for 5 days with 1000 mg

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OMC/kg bw/day (Klammer et al., 2005), and in OVX rats fed OMC for 12 weeks, at a dose of approximately 1100 mg/kg bw/day (Seidlova-Wuttke et al., 2006). These results indicate that OMC possesses estrogenic activity, as estradiol causes similar effects on uterine weight. However, OMC also significantly increased serum LH levels (Seidlova-Wuttke et al., 2006) and upregulated expression of the estrogen receptor beta (Klammer et al., 2005) which are effects opposite those observed after estradiol treatment. Thus, OMC seems to exert both estrogenic and estrogen-independent activities *in vivo*.

In a large two-generation study, pregnant dams were treated with OMC in doses of 150, 450 or 1000 mg/kg bw/day in the feed. Exposure began before mating and continued throughout gestation, lactation, adolescence, mating of the F1 generation and until weaning of the F2 generation. Exposure caused no adverse effects on reproduction and development. The authors concluded that OMC displayed no estrogenic potential *in vivo* (Schneider et al., 2005), but no behavioral or thyroid endpoints were included in this study.

There is however good reason to assume that OMC does interfere with the hypothalamo-pituitary-thyroid (HPT) axis, and therefore could also affect brain development. Schmutzler et al. (2004) found that treatment of OVX rats for 12 weeks with 1100 mg OMC/kg bw/day, reduced thyroxine (T_4) levels in the blood as well as activity of 5′-deiodinase (the enzyme that converts T_4 to triiodothyronine (T_3)) in the liver. Reduction of deiodinase activity in peripheral tissues is also one of the mechanisms by which the potent anti-thyroid drug Propylthiouracil (PTU) exerts its effects (Cavalieri and Pitt-Rivers, 1981; Leonard and Rosenberg, 1978; Visser et al., 1979). Decreased T_4 levels were also observed in OVX rats treated with 200 and 1100 mg OMC/kg bw/day for 12 weeks, although a statistically significant effect was only observed in the low dose group (Seidlova-Wuttke et al., 2006). In OVX females treated with 10, 33, 100, 333 or 1000 mg OMC/kg/day for 5 days, the two highest doses also reduced T_4 levels in serum and deiodinase activity in the liver (Klammer et al., 2007).

Altogether, many studies indicate that OMC possesses endocrine disrupting properties, and especially the reductions in T_4 levels seem consistent across studies. The aim of the present study was to address the potential endocrine disrupting properties of OMC on the developing reproductive and thyroid hormone systems, and to investigate how OMC induced changes in thyroid hormone levels would be related to the neurological development of the offspring. Altered hormone levels during shorter periods of adulthood may not lead to adverse effects, but during early pre- and postnatal development, such alterations can affect both the physiological and neurological development of the offspring, as developmental hypothyroidism can cause delayed development, reduced growth, hearing loss and persistent neurobehavioral effects, in both experimental animals and humans (Zoeller and Crofton 2005; Miller et al., 2009; Pop et al., 1999; Haddow et al., 1999). Previously our group has shown that hearing loss, hyperactivity and impaired maze learning in rats could be directly correlated to decreases in early developmental T_4 levels—when these decreases were induced by developmental exposure to the anti-thyroid drug PTU (Axelstad et al., 2008). In order to gain more knowledge on how hypothyroxinemia in the pre- and postnatal period is related to subsequent neurotoxicity in rats, when caused by an environmentally relevant chemical with more possible modes of action, we wanted to examine if correlations between reduced T_4 levels and altered behavior would also exist after developmental OMC exposure.

Materials and methods

Test compound

The test compound was 2 Ethylhexyl 3-(4-Methoxyphenyl)-2 Propenoate, also called 2-ethylhexyl-4-methoxycinnamate or simply Octyl Methoxycinnamate (OMC). CAS no 5466-77-3, product number ACR291160250, purity 98.0% (VWR Bie & Berntsen, Herlev, Denmark). Corn oil (VWR Bie & Berntsen, Herlev, Denmark) was used as vehicle.

Animals and treatment

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the Inhouse Animal Welfare Committee. Seventy-two time-mated, nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were supplied at day 3 of pregnancy. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions: semitransparent plastic cages ($15 \times 27 \times 43$ cm) with Aspen bedding (Tapvei, Gentofte, Denmark) situated in an animal room with controlled environmental conditions (12 h reverse light–dark cycles with light starting at 9 p.m., temperature 22 ± 1 °C, humidity 55 ± 5%, ventilation 10 air changes per hour). Food (Altromin Standard Diet 1314) and acidified tap water were provided ad libitum.

The day after arrival, i.e. gestation day (GD) 4, the animals were weighed and assigned to four groups of 18 animals each, with similar weight distributions. They were given 3 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. The study was run in two blocks, with 2 weeks in between blocks and an equal representation of each dose group in each block. Dams in the four experimental groups were weighed and gavaged once daily at approximately the same time, from GD 7 to postnatal day (PND) 17 (day of delivery excluded) with 0 (vehicle), 500, 750, or 1000 mg OMC/kg bw/day. Vehicle control and OMC solutions were continuously stirred during the dosing period, and fresh solutions were prepared for each of the two study blocks. The dams were treated at a constant volume of 2 ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing. The dams were pair-housed until GD 17 and individually hereafter. They were observed daily for signs of toxicity, and body weights were recorded daily from GD 4 and during the entire dosing period.

Delivery, postnatal development and weaning

The day after delivery, weights of dams and individual pups were recorded. The pups were counted, sexed, checked for anomalies, and anogenital distance (AGD) was measured using a stereomicroscope. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 22, was designated PND 0 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as the animals gave birth on GD 22–23.

At PND 7 and 13 all pups were weighed and on PND 13 they were also examined for the presence of nipples/areolas, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Body weight of offspring was recorded again on PND 21, 28, 36 and 50. After PND 50 body weights were recorded when behavioral and physiological tests were performed in postnatal weeks (PNW) 9, 13–16 (females), 17, 22–25 (males), 31 and 35 (necropsy). At PND 16, litter size was standardized to 1–2 males and 3 females, when possible. From these offspring, 1–2 males and 1–2 females from each litter were weaned on PND 28, and kept for assessment of onset of puberty and for later behavioral testing. Remaining pups were euthanized on PND 28 (see the following discussion). Of the 72 time-mated dams, 57 gave birth to viable litters, which after weaning resulted in four groups of 14–18 male and 14–18 female pups, representing 12–18 litters per group. The weaned offspring was housed in pairs of the same sex and exposure status.

Onset of puberty

Onset of puberty was assessed by determining day of vaginal opening (VO) in female offspring and preputial separation (PPS) in males. All weaned females were scored on PND 29, 31 and daily from PND 34–38, while all weaned males were scored on PND 41, 43, 45, 48 and 49. The weight of the animals was measured when VO and PPS was observed.

Organ weights, histopathology and gene expression analysis PND 16 and 28

On PND 16, 1–10 pups in each litter were euthanized depending on the original size of the litter. All euthanized pups were weighed and decapitated. Trunk blood was collected for analysis of T_4, testosterone, progesterone and estradiol levels (blood samples were pooled for all males and all females within each litter). From 3 males and 2 females per litter the liver, adrenals and thyroid gland were excised, weighed and used for histopatological examinations. Adrenals from two pups per litter were placed in RNAlater for gene expression analysis. Thyroid glands intended for histopathology (from one male and one female per litter) were not weighed, but were excised on the thyroid cartilage in order to obtain optimal histological preservation. In all euthanized offspring the reproductive organs were examined macroscopically for anomalies. From 3 male and 2 female offspring per litter the testes, epididymides, ventral prostates and ovaries were excised, weighed, fixed in formalin and processed for paraffin embedding. From one male per litter prostate and testes were placed in RNAlater for gene expression analysis.

Dams and one female in each litter were euthanized on PND 28. The animals were weighed and decapitated after CO_2/O_2 anesthesia, and trunk blood was collected for measurement of T_4, progesterone and estradiol in serum. The uteri of the dams were excised, and the number of implantation scars was registered. The thyroid glands were dissected, weighed, and prepared for histopathological investigation. In the female pups, the thyroid gland, the uterus and ovaries were excised, weighed, and prepared for histopathology.

All organs intended for histopathological examinations were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histological evaluation was made of maternal thyroids and of all organs in which statistically significant changes in organ weights were seen.

All organs for gene expression analysis were kept in RNAlater (Qiagen) for later RNA purification and cDNA production according to the procedure from the manufacturer and as described in Laier et al. (2006). Expressions of the genes: P450scc, P450c17 and StAR in the adrenals, StAR, P450c17, P450scc, PEM, Bzrp and Scarb 1 in testis and TRPM-2 and PBP C3 in the ventral prostate were all taken relative to the endogenous control 18S rRNA and evaluated by real-time RT-PCR on a Taqman 7900 HT (Applied Biosystems) as described in Laier et al. (2006) where all abbreviated gene names are explained and primers and probes are listed as well.

Hormone analysis

On GD 15 and PND 15 dams were anesthetized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam) and blood was drawn from the tail vein. From offspring on PND 16 and PND 28, dams on day 28 and from eight months old offspring, trunk blood was collected for the hormone analysis. T_4 was examined in all blood samples (except offspring PND 28), while testosterone was measured in males on PND 16, and estradiol and progesterone were measured in dams on GD 15, PND 15 and 28 and in female offspring on PND 16 and 28. Plasma level of T_4 was analyzed using a modified Delfia T_4 (as described in Axelstad et al., 2008). Testosterone, estradiol and progesterone were extracted from the serum as previously described (Vinggaard et al., 2005) and the hormones were measured by time-resolved fluorescence using commercially available fluoroimmunoassay (FIA) kits (PerkinElmer Life Sciences, Turku, Finland).

Behavioral testing

The investigations were performed between 9.00 a.m. and 4 p.m. during the animals' dark cycle, i.e. their active period. The behavioral studies and hearing tests were recorded by experimenters who were blinded with respect to exposure groups. Exposed and control animals were tested alternately and except from tests in the radial arm maze, so were females and males.

Motor activity and habituation capability. Motor activity was measured twice in all weaned rat offspring—in postnatal week (PNW) 9 and again in PNW 17 (4 months). At testing, the animals were placed individually in clean plastic cages without bedding, food or water, and the cages were placed in activity boxes with photocells which measured activity for 30 min (as described in Axelstad et al., 2008). A computer automatically recorded output from photocells and collected data. Total activity during the 30 min observation period was used as a measure of general activity. In order to assess habituation, the 30 minute period was divided into shorter time intervals (3×10 min and 2×15 min) when analyzing the data.

Radial arm maze. At the age of 3 months for females (PNW 13–16), and 5 months for males (PNW 22–25) the weaned animals were tested in a standard 8-arm Radial Arm Maze (RAM) from Viewpoint (Sandown Scientific, Middlesex, England). The maze was built of transparent plexiglas with 8 arms (55 cm long) radiating from an octagonal central area (50 cm wide), and was situated on a light-sensitive board which was elevated 68 cm above the floor. The walls in the room were decorated with a number of spatial cues (geometric shapes and posters), in order for the rats to triangulate their position. At the end of each arm a reward (peanut) was placed. Through the light-sensitive board, a computer automatically recorded movements of the rats in the maze and performance could be viewed on a monitor placed in the adjoining room.

One week before testing, the animals were housed one per cage and given a restricted amount of food (12 g of food/day for females and 15 g/day for males) and one peanut to get accustomed to the taste. The food restriction was expected to lead to a decrease in body weight of approximately 10%, at the end of the testing period (based on previous studies). The animals were tested in one daily session in a total of 15 sessions during 3 consecutive weeks (5 trials per week), as described in Axelstad et al. (2008). In the daily test session, each rat was placed in the central maze area, and at this moment the monitoring system was started through a remote control. The experimenter left the room and the rat was allowed to explore the maze until all arms were visited, or 10 min had elapsed. Latency to visit the end of all 8 arms and the choice of arms was registered by the computer. The number of errors, defined as visiting an arm that had already been visited, was calculated from the data.

Acoustic startle reaction. Acoustic startle reaction (ASR) and prepulse inhibition (PPI) were tested in 56 female and 57 male offspring (representing all litters), at the age of 6½ months (PNW 28), as earlier described (Hougaard et al., 2005) in two chambers (San Diego Instruments, San Diego, USA) with 70 dB(A) white background noise. Each chamber contained a Plexiglas test tube (diameter 8.2 cm and length 25 cm), mounted on a platform with a piezoelectric accelerometer attached beneath. The accelerometer detected and transduced displacement of the tube, in response to movements of the rat. Animals were acclimatized 5 min in the test tube before sessions started and ended with 5 startle trials of 40 ms 120 dB(A) bursts of white noise. In between, 35 trials were delivered in semi-randomized order (10 trials of 120 dB(A); 5 each of 4 prepulse + startle trials (20 ms prepulses of 72, 74, 78, and 86 dB(A); 5 trials with only background noise)). Tube

movements were averaged over 100 ms following onset of the startle stimulus (AVG). For each prepulse intensity, the five AVGs were averaged and used in calculation of PPI, which was expressed as percent reduction compared to the average of the 10 middle startle trials: %PPI = 100 − ((AVG at prepulse + startle trial)/(AVG at 10 middle startle trials))*100%.

Test of hearing

At seven months of age (PNW 31), hearing function was evaluated in most of the animals previously tested in the acoustic startle reaction test. 48 male and 48 female rats were tested (12 males and 12 females per group, representing 11–12 litters per group). Hearing was assessed by measurements of distortion product oto-acoustic emissions (DPOAE) and by determination of hearing thresholds (HT) at 4 kHz, assessed by measurements of auditory brain stem response (ABR), with the animals in general anesthesia (Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam)), as described in Axelstad et al. (2008).

Organ weights, histopathology and semen quality analysis in adult animals

At eight months of age (PNW 35–36), all male and female offspring were anesthetised by CO_2/O_2 and decapitated. Trunk blood was collected and analyzed for testosterone and T_4 levels. Thyroid gland, liver, prostate, testes, vesiculas, ovaries and uterus were excised, weighed, fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin. Organs displaying statistically significant weight changes were evaluated histologically.

From all males the epididymides were removed and the cauda of the right epididymis was used for sperm motility analysis. The cauda of the left epididymis was frozen in liquid nitrogen for later sperm count.

Sperm motility. Spermatozoa were obtained from the distal cauda and sperm samples were prepared and analyzed by computer assisted sperm analysis (CASA) as described in Jarfelt et al. (2005). The parameters evaluated in this study were: percent motile and percent progressive spermatozoa, curvilinear velocity, amplitude of lateral head displacement which describes the vigor of the spermatozoa, and some progressive parameters, i.e. average path velocity and straight line velocity.

Sperm count. Cauda of the left epididymis was thawed at room temperature and prepared for sperm count analysis as described by Jarfelt et al. (2005). Samples were analyzed using 10× UV fluorescent objective and IDENT OPTIONS set A. Ten fields were analyzed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda.

Statistical analysis

For all analyses, the alpha level was set at 0.05. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as

Table 1
Pregnancy and litter data, including body weight (bw) of dams and offspring exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Dams and litters	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of dams (litters)	n = 18 (18)	n = 18 (12)	n = 18 (14)	n = 18 (13)
Dam bw gain, GD 7–21	87.2 ± 3.3	80.4 ± 4.3	77.2 ± 3.4*	69.8 ± 2.1***
Dam bw gain, GD 7–PND 1	21.7 ± 1.6	12.7 ± 3.2**	12.5 ± 1.9**	5.23 ± 3.1***
Dam bw gain PND 1–PND 21	24.2 ± 2.3	35.5 ± 2.3*	33.6 ± 4.0*	37.9 ± 3.4**
Gestation length (days)	22.8 ± 0.1	23.0 ± 0.1	22.9 ± 0.1	23.1 ± 0.1
% postimplantation loss	10.2 ± 3.4	7.95 ± 4.2	5.82 ± 2.2	7.51 ± 1.8
% perinatal loss	13.7 ± 3.8	10.9 ± 4.2	10.2 ± 2.6	13.6 ± 3.2
Litter size	10.9 ± 0.8	11.3 ± 1.1	10.5 ± 0.7	11.9 ± 0.6
% perinatal deaths	3.97 ± 2.0	3.00 ± 1.8	4.65 ± 1.6	6.55 ± 2.9
% males	51.4 ± 3.5	57.1 ± 5.2	48.5 ± 4.4	48.2 ± 4.0
Offspring				
Mean birth weight	6.08 ± 0.1	6.04 ± 0.2	5.76 ± 0.1*	5.14 ± 0.1**
AGD males (mm)	3.50 ± 0.03	3.40 ± 0.04	3.47 ± 0.04	3.45 ± 0.05
AGI males (mm/cu. root bw)	1.90 ± 0.02	1.86 ± 0.02	1.92 ± 0.03	1.98 ± 0.03
AGD females (mm)	1.82 ± 0.04	1.70 ± 0.03	1.77 ± 0.02	1.72 ± 0.04
AGI fem. (mm/cu. root bw)	1.01 ± 0.02	0.95 ± 0.01	0.99 ± 0.01	1.00 ± 0.02
Nipples (areolas) males	0.03 ± 0.01	0.05 ± 0.04	0.10 ± 0.04	0.12 ± 0.05
Nipples (areolas) females	12.4 ± 0.1	12.6 ± 0.2	12.6 ± 0.1	12.7 ± 0.1
Mean bw PND 7	14.8 ± 0.4	13.2 ± 0.3*	13.5 ± 0.3*	11.6 ± 0.2**
Mean bw PND 14	28.7 ± 0.9	25.1 ± 0.8**	26.6 ± 0.7*	23.1 ± 0.6**
Mean bw PND 21	47.8 ± 1.2	44.4 ± 1.2	46.2 ± 0.7	41.7 ± 0.6**
Mean female bw PND 28	74.5 ± 1.7	69.2 ± 1.0*	71.3 ± 1.2	62.7 ± 1.2**
Mean male bw PND 28	81.2 ± 1.4	73.3 ± 1.6**	74.8 ± 1.1**	68.7 ± 1.5***
Mean female bw PND 36	110.4 ± 2.0	104.6 ± 1.3*	109.1 ± 2.0	98.8 ± 1.9**
Mean male bw PND 36	129.3 ± 2.5	118.3 ± 2.3**	121.6 ± 2.4*	114.4 ± 2.4***
Mean female bw PND 50	150.2 ± 3.0	147.4 ± 2.4	154.6 ± 2.9	139.8 ± 2.6*
Mean male bw PND 50	212.4 ± 3.8	195.0 ± 3.3**	203.8 ± 3.9	195.5 ± 3.6**
Mean age vag. opening (vo)	33.7 ± 1.5	34.5 ± 1.2	34.5 ± 1.0	34.6 ± 0.9
Mean weight at vo.	100.4 ± 10.1	98.4 ± 6.5	102.2 ± 7.5	93.7 ± 6.8*
Mean age, preputial sep. (pps)	43.1 ± 1.3	44.1 ± 1.9	44.1 ± 1.4	44.4 ± 2.4
Mean weight at pps.	171 ± 13	163 ± 10	168 ± 11	164 ± 11
Mean male bw PNW 9	257 ± 21	237 ± 13**	243 ± 17*	238 ± 12**
Mean male bw PNW 17	411 ± 42	370 ± 43**	383 ± 25*	377 ± 31*
Mean male bw PNW 22	447 ± 52	407 ± 30*	411 ± 34*	407 ± 33*
Mean male bw PNW 25	414 ± 34	382 ± 23**	388 ± 27*	380 ± 25**
Mean male bw PNW 35	499 ± 44	462 ± 36*	453 ± 32**	463 ± 34*

an independent, random and nested factor in ANOVA, or analysis was done on litter means. Litter size was included as a covariate in analyses of body weight of the pups until PND 16. Body weight was included as a covariate in analyses when relevant, e.g. for terminal organ weights. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskal–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Statistical analyses of the effects on macroscopic lesions and histopathology were done using Fisher's Exact Test. Startle data were analyzed by use of Systat Software Package v. 9. All other analyses were performed using SAS Enterprise Guide 3.0 (2004), SAS Institute Inc, Cary, NC, 274 USA.

Results

Pregnancy data and postnatal growth

Maternal body weight gain was significantly reduced during the gestation period in the OMC exposed groups (Table 1). Body weight gains from GD 7 to the day before birth (GD 21) were significantly lowered in the two highest dose groups compared to controls ($p = 0.032$ and $p = 0.0004$). When adjusted for weight and number of offspring (BW gain GD 7-PND 1), the effect on maternal weight gain was even more marked and statistically significantly different from controls in all three dose groups ($p = 0.0092$, $p = 0.0057$, and $p = 0.0001$). During the lactation period, dams in all three OMC groups gained significantly more weight than controls ($p = 0.010$, $p = 0.024$, and $p = 0.002$), which eliminated differences in body weights between dosed and control animals at the time of weaning. Gestation length, litter size, postimplantation loss, neonatal death, and gender distribution were similar in the four groups (Table 1). Neither anogenital distance at birth, nor nipple retention on PND 13 differed by exposure, in either gender.

Offspring body weights were significantly reduced by OMC treatment (Table 1). The body weight of male offspring was significantly reduced in the two highest dose groups at birth and in all three dose groups on PND 7, 14, 28 and 36. Body weights in female offspring were significantly reduced in the highest dose group at birth, in all three groups at PND 7 and 14, and in the lowest and highest dose groups on PND 28 and 36 (Table 1). After PND 50 the weights of OMC-dosed females no longer differed significantly from that of controls (data not shown). Male body weights remained significantly lower in all OMC dose groups compared to controls from PND 50 and throughout the rest of the study (Table 1).

Mean age and body weight at sexual maturation, measured as day of vaginal opening (VO) and preputial separation (PPS) are shown in Table 1. Both male and female offspring from the high dose OMC group reached sexual maturation a little later than control offspring, though the difference was not statistically significant. This tendency towards later sexual maturation may likely be due to lower body weights in this group.

Hormone levels in dams and juvenile offspring

T_4 levels measured in blood from dams and offspring are shown in Fig. 1 and Table 2. T_4 levels decreased markedly with higher doses of OMC in dams on both GD 15 and PND 15 and were significantly affected in all dose groups ($p < 0.0001$). Male pup T_4 levels on PND 16 were also significantly decreased in all three dose groups ($p < 0.0001$) (Fig. 1c), while female T_4 levels on PND 16 were unaffected by OMC exposure (Table 2). All control T_4 levels in dams and pups were comparable to historical control levels from our laboratory. Progesterone, estradiol and testosterone levels in serum from dams and offspring are shown in Table 2. No statistically significant differences in progesterone and estradiol levels were observed in the dams. In the female offspring on PND 16 estradiol levels were unaffected by OMC treatment. Progesterone levels appeared lower in all dosed groups, but the difference from controls was not statistically significant. On PND 28, progesterone levels were decreased in female offspring from all dosed groups ($p = 0.021$, $p = 0.002$, and $p = 0.017$), whereas estradiol levels were decreased in the two lower ($p = 0.008$ and $p = 0.012$) but not in the highest OMC dose group. In male pups,

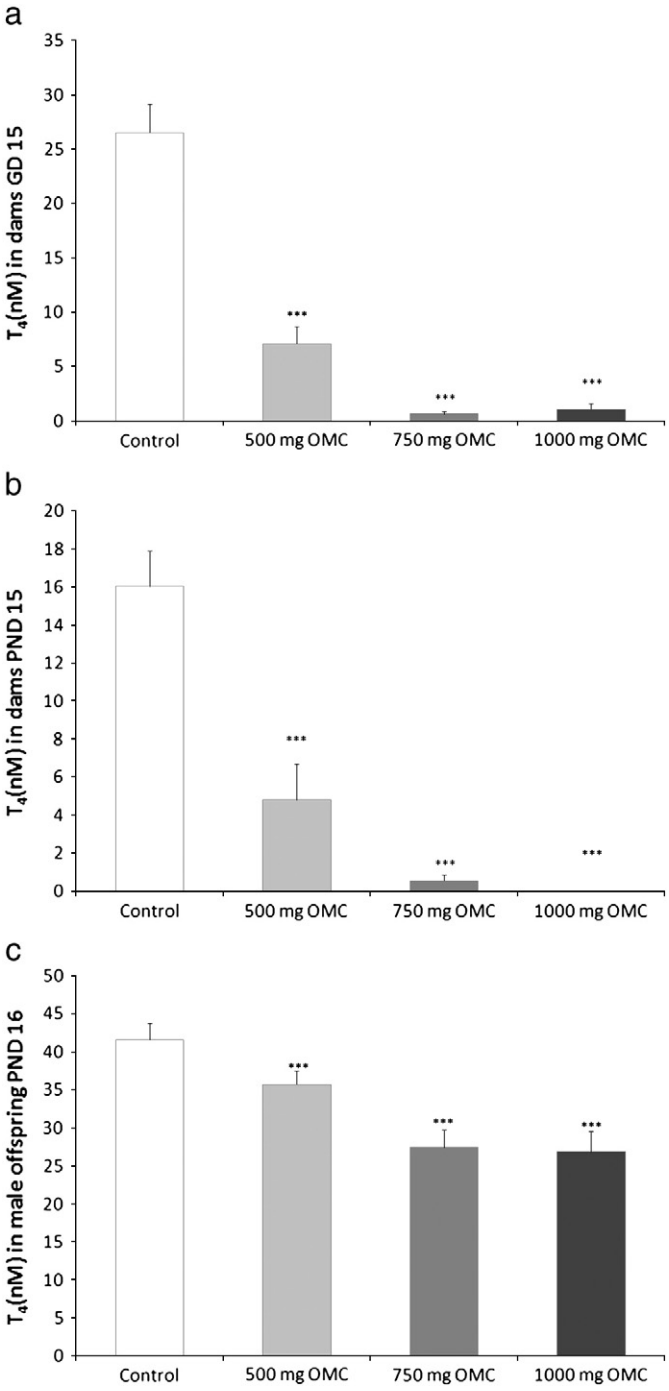

Fig. 1. Thyroxine (T_4) levels (nM) in dams on GD 15 (a) and PND 15 (b), and male offspring on PND 16 (c) after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means, based on litter means + SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to controls *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$.

Table 2
Effect of perinatal OMC exposure on the reproductive and thyroid hormone systems. T_4 levels in all dams and offspring, progesterone and estradiol levels in dams and female offspring and testosterone levels in male offspring euthanized at GD 15, PND 15, 16, 28 or in adulthood, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. N = 11–18. Data represent group means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Hormone measurements (nM)	Control	500 mg OMC	750 mg OMC	1000 mg OMC
Dams GD 15				
Progesterone	192.1 ± 44	135.7 ± 69	174.1 ± 68	133.0 ± 61
Estradiol	0.030 ± 0.01	0.030 ± 0.01	0.033 ± 0.01	0.031 ± 0.01
T_4	26.5 ± 10.9	7.12 ± 5.6***	0.61 ± 1.0***	1.02 ± 1.9***
Dams PND 15				
Progesterone	64.47 ± 7.8	68.88 ± 6.0	70.33 ± 4.1	70.58 ± 4.9
Estradiol	0.0066 ± 0.001	0.0073 ± 0.001	0.0079 ± 0.003	0.0095 ± 0.003
T_4	16.03 ± 7.71	4.80 ± 6.66***	0.56 ± 1.17***	0.00 ± 0.00***
Dams PND 28				
Progesterone	29.87 ± 26.6	40.25 ± 38.2	48.95 ± 38.2	36.77 ± 28.8
Estradiol	0.051 ± 0.06	0.032 ± 0.03	0.031 ± 0.02	0.079 ± 0.08
T_4	26.57 ± 8.04	25.68 ± 8.09	28.47 ± 7.32	36.39 ± 10.73
Offspring PND 16				
Progesterone	10.48 ± 6.6	5.41 ± 2.8	6.33 ± 2.7	7.23 ± 1.6
Estradiol	0.035 ± 0.016	0.037 ± 0.009	0.035 ± 0.007	0.029 ± 0.007
Testosterone	0.843 ± 0.61	0.389 ± 0.24**	0.337 ± 0.25***	0.242 ± 0.19***
T_4 male	41.60 ± 9.3	35.72 ± 6.3***	27.44 ± 8.5***	26.85 ± 9.7***
T_4 female	35.79 ± 7.1	35.41 ± 7.9	34.75 ± 8.4	30.68 ± 7.9
T_4 litter mean	39.19 ± 7.5	35.56 ± 6.6	31.27 ± 6.2*	28.77 ± 7.2***
Offspring PND 28				
Progesterone	11.25 ± 8.2	6.56 ± 8.2*	3.74 ± 1.6**	4.82 ± 2.9*
Estradiol	0.011 ± 0.005	0.005 ± 0.002***	0.006 ± 0.003*	0.009 ± 0.006
Adult offspring				
T_4 male	28.3 ± 8.4	28.4 ± 4.9	31.9 ± 8.5	27.9 ± 9.3
T_4 female	33.2 ± 14.1	31.4 ± 8.1	31.3 ± 9.5	41.6 ± 13.9
Testosterone	1.98 ± 1.8	1.95 ± 2.5	3.40 ± 2.8	2.27 ± 1.7

testosterone levels were significantly decreased in all three dose groups on PND 16 ($p = 0.006$, $p = 0.0005$, and $p = 0.0001$) (Fig. 2), but were unaffected at termination of the study (Table 2).

Organ weights, histopathology and gene expression in juvenile animals

Body and organ weights from offspring euthanized on PND 16 and PND 28 are shown in Table 3. As body weights were lower in the OMC-dosed animals compared to controls, absolute organ weights that seemed similar between control and high dose groups, proved statistically different when body weight was included in the statistical analysis of the organ weights (as covariates). There was, however, great compliance between results from the statistical analysis of absolute organ weights (with body weight as a covariate) and relative organ weights, therefore significant differences in organ weights between control and OMC-dosed groups will only be referred to as "relative to body weight", in the remainder of the paper.

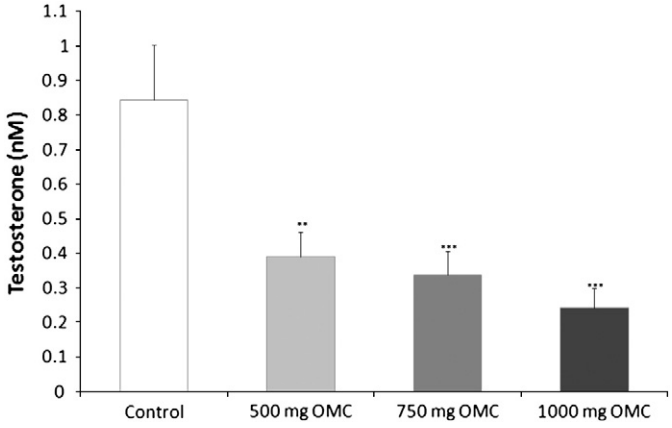

Fig. 2. Testosterone levels (nM) in male offspring on PND 16 after exposure to 0, 500, 750 or 1000 mg OMC /kg/day from GD 7 to PND 17. Data represent group means, based on litter means + SEM, n = 11–15. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$.

On PND 16, the relative weight of the thyroid gland was significantly higher in the two highest dose groups, when litter means including both male and female values were analyzed ($p<0.0001$). This increase was more pronounced in male than in female offspring, though not significantly different from controls in any dose group, when data from the two sexes were analyzed separately (data not shown). Histological examination of the thyroid glands from PND 16, showed no alterations by OMC treatment (Table 4).

On PND 16 the relative testis weights were significantly decreased in the two high dose groups ($p = 0.031$ and $p = 0.0007$), as can be seen in Table 3. The testes were evaluated for degree of lumen formation in the seminiferous tubules, as an indicator of the degree of testicular maturation. In all animals from the highest dose group, less than 1/3 of the tubular cross-sections had lumen formation, whereas animals with more than 1/3 and more than 2/3 tubules with lumen formation were frequently observed in the other dose groups (Table 4). In the highest dose group, 10 of 12 animals showed no tubule formation versus only 2 of 16 animals in the control group, indicating delayed testis development. Epididymal histopathology on PND 16 did not appear altered by OMC treatment.

Relative prostate weights were significantly decreased in the high dose group ($p = 0.0003$) (Table 3). Evaluation of prostate histopathology on PND 16 revealed dose-related changes in development. In the highest dose group, no animals had the large, fluid-filled acini,

Table 3
Effect of OMC on terminal body weights (bw) and organ weights in male and female rat offspring euthanized at PND 16 and in female offspring euthanized PND 28, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Liver, thyroid and adrenal weights are litter mean values from male and female offspring combined. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; ***: $p<0.0001$.

Organ weights PND 16	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of litters	16	11	14	13
Bw male (g)	31.8 ± 3.5	29.1 ± 2.1*	30.7 ± 4.0	26.6 ± 2.9***
Bw female (g)	29.8 ± 3.7	28.6 ± 2.0	28.8 ± 2.2	25.5 ± 2.5***
Adrenals (mg)	9.17 ± 1.5	8.80 ± 1.0	9.20 ± 1.1	7.61 ± 0.8
Adrenals (mg/100 g bw)	29.4 ± 3.3	30.5 ± 2.2	30.6 ± 3.1	29.1 ± 2.4
Liver (mg)	775 ± 91	734 ± 47	797 ± 89[a]	727 ± 87[a]
Liver (mg/100 g bw)	2484 ± 83	2539 ± 82	2640 ± 149***	2758 ± 95***
Thyroid gland (mg)	4.67 ± 0.8	4.18 ± 0.8	5.40 ± 1.8[a]	4.62 ± 0.6 [a]
Thyroid gland (mg/100 g bw)	15.1 ± 1.9	14.5 ± 2.5	18.0 ± 4.8***	17.6 ± 2.3***
Ovaries (mg)	11.8 ± 1.6	10.8 ± 1.5	11.1 ± 1.4	9.55 ± 0.8
Ovaries (mg/100 g bw)	39.1 ± 5.0	37.5 ± 5.1	39.0 ± 5.5	38.4 ± 4.2
Testes (mg)	111 ± 14	99.7 ± 7.5	101 ± 7.8[b]	85.1 ± 8.9[b]
Testes (mg/100 g bw)	349 ± 21	344 ± 26	331 ± 22*	320 ± 17***
Prostate (mg)	12.4 ± 2.0	10.4 ± 1.5	11.3 ± 2.4	8.49 ± 1.8[b]
Prostate (mg/100 g bw)	39.0 ± 4.5	36.0 ± 4.3	36.3 ± 5.7	31.8 ± 4.6***
Epididymides (mg)	23.6 ± 3.3	21.7 ± 1.7	23.1 ± 2.9	20. ± 2.1
Epididymides (mg/100 g bw)	74.2 ± 8.4	75.4 ± 8.7	75.8 ± 10	75.7 ± 9.0
Organ weights PND 28	**Control**	**500 mg OMC**	**750 mg OMC**	**1000 mg OMC**
No. of litters	15	11	13	13
Bw in female offspring	74.4 ± 8.89	68.9 ± 4.59	70.3 ± 3.47	63.5 ± 3.69
Thyroid gland (mg)	8.54 ± 2.34	8.54 ± 2.32	7.57 ± 1.77	7.36 ± 1.36
Thyroid gland (mg/100 g bw)	11.6 ± 3.09	12.3 ± 2.98	10.7 ± 2.43	11.6 ± 1.93
Ovaries (mg)	33.7 ± 3.19	33.6 ± 4.62	31.9 ± 4.18	31.1 ± 3.65
Ovaries (mg/100 g bw)	45.6 ± 4.96	48.8 ± 6.70	45.5 ± 6.74	49.2 ± 5.67
Uterus (mg)	46.5 ± 9.22	41.5 ± 11.1	43.8 ± 5.82	37.7 ± 8.31
Uterus (mg/100 g bw)	63.0 ± 12.	60.5 ± 16.9	61.8 ± 7.08	59.3 ± 12.2

[a] Indicates an absolute organ weight that was significantly larger than in the control group, when body weight was included in the statistical analysis as a covariate ($p<0.05$).
[b] Indicates an absolute organ weight that was significantly smaller than in the control group, when body weight was included in the statistical analysis as a covariate ($p<0.05$).

characteristic of control prostates (Table 4). Among the 750 mg OMC males, half the males had large acini, but these were not fluid-filled as seen in controls. These histological changes reflect the lower prostate weights seen in the highest dose group.

Relative liver weights were significantly increased in the two highest dose groups ($p<0.0001$), while the relative weights of the adrenals, the epididymides and the ovaries were unaffected by OMC treatment (Table 3). No differences between exposure groups were seen in the evaluation of liver histology (data not shown). Gene expression analysis was done on PND 16 testes, prostate and adrenal glands. No significant changes in expression were found for any of the genes in neither of these organs (data not shown).

Table 4
Histopathology of dams and offspring after exposure to 0, 500, 750, or 1000 mg OMC/kg bw/day from GD 7 to PND 17. Data are percentages of affected animals followed by the number of animals with the listed characteristics/total number of animals evaluated. Figures in bold are statistically significantly different from controls, *: $p<0.05$, ***: $p<0.001$ with a two-sided Fisher's exact test.

Histopathology	Control	500 mg OMC	750 mg OMC	1000 mg OMC
Dam thyroids PND 28				
No hyperplasia	53%, 9/17	38%, 3/8	31%, 4/13	45%, 5/11
Unilaterally hyperplasia	18%, 3/17	13%, 1/8	23%, 3/13	18%, 2/11
Bilaterally hyperplasia	29%, 5/17	50%, 4/8	46%, 6/13	36%, 4/11
Uni- or bilaterally hyperplasia	47%, 8/17	63%, 5/8	69%, 9/13	55%, 6/11
Pup thyroids PND 16 (female)				
No hyperplasia	85% (11/13)	70% (7/10)	80% (8/10)	64% (7/11)
Hyperplasia	15% (2/13)	30% (3/10)	20% (2/10)	36% (4/11)
Testis PND 16				
0% tubules with lumen	13%, 2/16	27%, 3/11	14%, 2/14	**83%, 10/12*** **
<1/3 tubules with lumen	62%, 10/16	73%, 8/11	64%, 9/14	**100%, 12/12***
1/3 to 2/3 tubules with lumen	19%, 3/16	27%, 3/11	29%, 4/14	0%, 0/12
>2/3 tubules with lumen	19%, 3/16	0%, 0/11	7%, 1/14	0%, 0/12
Cellular debris in a few tubules	13%, 2/16	27%, 3/11	14%, 2/14	8%, 1/12
Prostate PND 16				
Alveolar lumen size				
Presence of large, fluid-filled acini	50%, 8/16	50%, 4/8	**8%, 1/12***	**0%, 0/9***
Presence of large, flattened acini	6%, 1/16	13%, 1/8	**50%, 6/12***	0%, 0/9
Intermediate size acini	31%, 5/16	25%, 3/8	25%, 3/12	**78%, 7/9***
Small acini	13%, 2/16	13%, 1/8	17%, 2/12	22%, 2/9
Prostate adults				
General acinar atrophy	0%, 0/18	7%, 1/14	**27%, 4/15***	**50%, 7/14*** **

On PND 28, female offspring body weights were still significantly reduced in the 500 and 1000 mg OMC/kg bw/day groups ($p = 0.021$ and $p = 0.0001$), while relative weights of the uterus and ovaries showed no variation with OMC exposure. The relative weights of the thyroid glands were no longer significantly affected by the OMC exposure (Table 3). In the dams, body and thyroid weights were not affected by OMC exposure on PND 28, nor was the thyroid histology (data not shown).

Motor activity levels in young and adult male and female offspring

Results from the activity test in PNW 9 and PNW 17 are shown in Fig. 3. In the younger animals, female control animals showed significantly higher activity levels than control males, as expected ($p<0.0001$). Female offspring exposed to OMC were less active compared to controls, significantly so in the 750 and 1000 mg OMC/kg groups ($p = 0.0004$ and $p = 0.041$), while male activity levels seemed unaffected by OMC exposure. Fig. 3b shows the total activity levels from when the animals were tested in PNW 17. Again, female control animals showed significantly higher activity levels than control males ($p = 0.0006$). Female animals exposed to OMC were again less active compared to controls, but this time only significantly so in the highest dose group ($p = 0.05$). Male offspring from the 750 mg OMC/kg bw/day group displayed elevated activity levels compared to control males ($p = 0.014$). As this effect was not present in the 1000 mg OMC/kg bw/day males, it may be a chance finding. When the 30-minute test period was divided into shorter time periods, both male and female animals in all dose groups displayed distinctly higher activity levels in the beginning compared to the end of the test. Females were approximately 3 times more active in the first 10 min of the test compared with the last 10, while males were 4–5 times more active in the beginning of the test than in the end. This was the case for both control and OMC-dosed animals, indicating that habituation was not affected by OMC exposure. This pattern was seen both in PNW 9 and PNW 17 (data not shown).

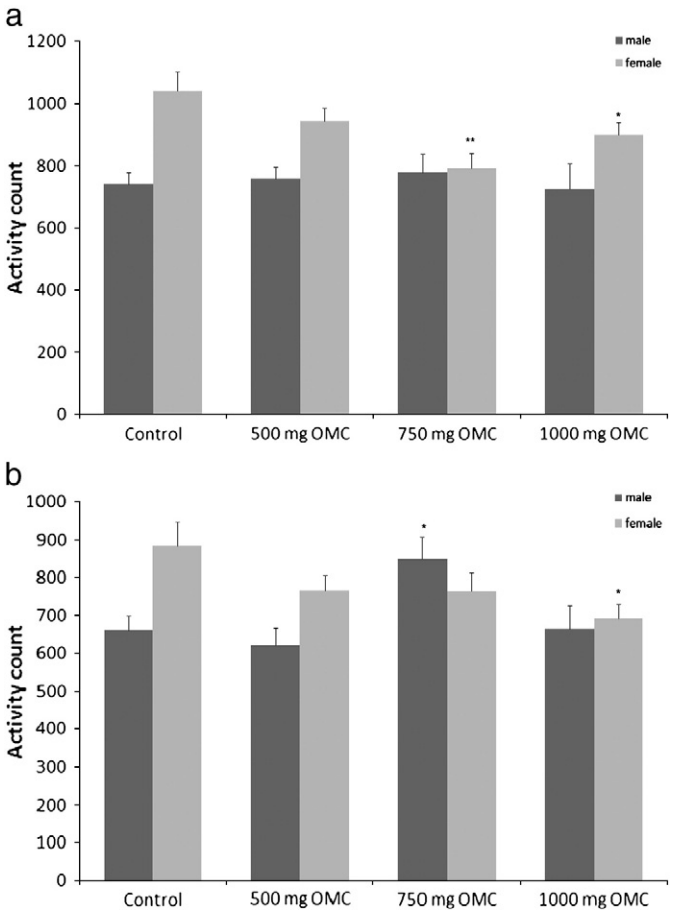

Fig. 3. Total motor activity level during 30 min of testing of male and female offspring at 9 (a) and 17 (b) weeks of age, after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means, based on litter means ± SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to same sex controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$. Difference between male and female control animals is statistically significant, $p<0.01$.

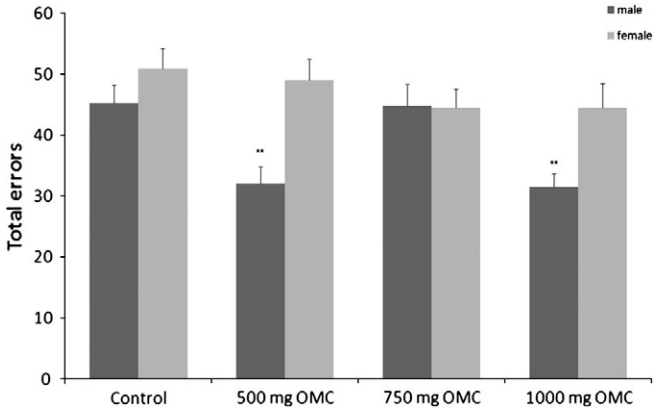

Fig. 4. Total number of errors in the radial arm maze during three weeks of testing, in adult male and female rats, exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means + SEM, n = 11–18. Asterisks indicate a statistically significant difference compared to same sex controls *: $p<0.05$; **: $p<0.01$; ***: $p<0.0001$.

Radial arm maze

The total number of errors made during three weeks of testing in the RAM is presented in Fig. 4. In OMC treated males a reduction in the number of errors was registered for both the 500 and 1000 mg OMC/kg groups ($p = 0.0076$ and $p = 0.0024$) compared to controls, while performance in the 750 mg/kg bw/day males was not significantly different from controls. The effect in OMC-dosed males was only evident during the first week of testing (data not shown). At no time point did latency or frequency of choosing adjacent arms differ between dosed and control males. In females, behavior was similar in control and exposed offspring on all tested parameters. There was no significant difference between male and female control values for the total number of errors. Food restriction caused a small reduction in male body weights (at end of the testing period, the reduction was around 7%), while female body weights were unaffected.

Acoustic startle reaction

When the acoustic startle reaction was tested, control and exposed males startled at comparable levels. Control females generally displayed a lower level of startle than did exposed females. However, statistical analysis indicated no significant differences in exposed compared to dosed offspring for basal reactivity, habituation to the initial 5 noise pulses, or PPI, in either males or females (data not shown).

Auditory function

Hearing as assessed by both oto-acoustic emissions and auditory brainstem response at 4 kHz was closely similar in all groups, and

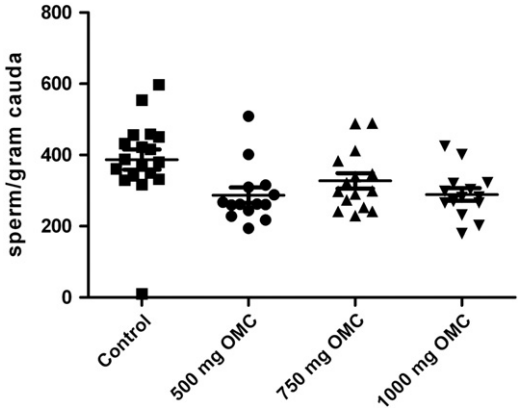

Fig. 5. Number of sperm per gram cauda in adult male rats exposed to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Individual male values are shown, together with group mean and SEM, n = 14–18. If the outlier in the control group is included in the statistical analysis, the 500 and 1000 mg OMC/kg groups are significantly different from control, while all three OMC dose groups are significantly lower than controls if the outlier is excluded from the statistical analysis.

there were no differences between dosed and control groups in either males or females (data not shown).

Hormone levels, organ weights and semen quality in adult offspring

No effect was seen on T_4 or testosterone levels, when measured in blood from 35 week old animals (Table 2). In Table 6, body and organ weights in adult rat offspring are shown. Male body weights were lower in all OMC groups ($p = 0.022$, $p = 0.0021$, and $p = 0.013$), while female body weights were unaffected by exposure. Relative weight of thyroid gland, liver, ovaries, uterus, testes and vesicula was unaffected by OMC treatment. In the control group, one male had very low testis weight, and histological evaluation revealed general degeneration of seminiferous chords in this animal. Testes weights were analyzed both with and without the outlier, but in both cases no treatment-related effects on testes weights were seen. Relative prostate weights were significantly reduced in the 1000 mg OMC/kg bw/day group ($p = 0.0073$), and histological examination showed a significantly increased number of animals with general acinar atrophy in the two highest dose groups, 27% and 50% respectively, while none was seen in the control group.

The sperm count was significantly lowered in the OMC exposed groups compared to controls (Fig. 5 and Table 5). The sperm count results are shown as number of sperm per gram cauda epididymis, and the observed dose-dependent reduction was not due to effects on the weight of the epididymis, as this was unaffected by OMC exposure (Table 5). The one male from the control group having low testes weights, also had almost no sperm cells in the epididymis. When this outlier value was included in the statistical analysis, the reduced sperm count was statistically significant in the 500 and 1000 mg/kg bw/day groups ($p = 0.009$ and $p = 0.0039$ respectively), whereas all three dosed groups differed significantly from the control group ($p = 0.0002$, $p = 0.0085$ and $p < 0.0001$) if this outlier was excluded from the results. Mean values and standard deviation with and without the outlier from the control group are shown in Table 5. None of the investigated sperm motility parameters was significantly affected by OMC exposure (Table 5).

Table 5
Effect of OMC on terminal body weights (bw), organ weights and semen quality in male and female rat offspring euthanized at PNW 35 (8 months), after exposure to 0, 500, 750 or 1000 mg OMC/kg/day from GD 7 to PND 17. Data represent group means based on litter means ± SD. Asterisks indicate a statistically significant difference compared to controls *: $p<0.05$; **: $p<0.01$, ***: $p<0.0001$.

Organ weights adult males	Control	500 mg OMC	750 mg OMC	1000 mg OMC
No. of males	18	14	15	14
BW (g)	499 ± 43	462 ± 36*	453 ± 32**	462 ± 34*
Liver (g)	13.93 ± 1.33	12.69 ± 1.23	12.74 ± 1.01	13.10 ± 1.15
Liver (g/100 g bw)	2.79 ± 0.19	2.74 ± 0.18	2.81 ± 0.12	2.83 ± 0.16
Thyroid gland (mg)	24.81 ± 4.4	24.11 ± 4.6	21.63 ± 3.1	23.32 ± 5.3
Thyroid gland (mg/100 g bw)	5.04 ± 0.9	5.25 ± 0.9	4.77 ± 0.6	5.04 ± 1.1
Testes (mg)[a]	3917 ± 662	3945 ± 285	3960 ± 272	3795 ± 413
Testes (mg/100 g bw)[a]	790 ± 141	857 ± 77.5	877 ± 76	821 ± 69
Prostate (mg)	963 ± 146	728 ± 192	694 ± 160	603 ± 104**
Prostate (mg/100 g bw)	168 ± 28	158 ± 42	153 ± 35	131 ± 28**
Vesicula (mg)	1790 ± 286	1702 ± 294	1791 ± 243	1887 ± 283
Vesicula (mg/100 g bw)	360 ± 64	368 ± 61	395 ± 54	408 ± 54
% motile sperm	46.2 ± 17.9	46.5 ± 14.4	42.5 ± 12.8	47.4 ± 13.5
% progressive sperm	27.1 ± 11.0	27.7 ± 8.9	25.3 ± 8.2	28.1 ± 11.0
Curvilinear velocity	344.3 ± 37.3	356.9 ± 23.5	357.6 ± 34.3	341.4 ± 31.7
Ampl. of lateral head displ.	17.9 ± 1.7	17.9 ± 1.7	18.6 ± 1.8	18.1 ± 1.6
Average path velocity	186.9 ± 21.3	194.0 ± 11.3	189.5 ± 17.9	180.3 ± 15.8
Straight line velocity	122.0 ± 17.9	127.2 ± 10.9	125.2 ± 14.07	114.8 ± 12.4
Cauda epididymis (mg)	282.3 ± 46.1	269.4 ± 32.3	276.4 ± 30.1	269.6 ± 29.9
Sperm/g cauda (all data)	387.3 ± 119	287.3 ± 80.3**	327.4 ± 84.1	289.2 ± 66.5**
Sperm/g cauda (without outlier)	409.5 ± 78.3	287.3 ± 80.3***	327.4 ± 84.1**	289.2 ± 66.5***
Organ weights adult females	**Control**	**500 mg OMC**	**750 mg OMC**	**1000 mg OMC**
No. of females	18	14	16	14
BW	268 ± 25	262 ± 21	273 ± 18	256 ± 23
Liver (mg)	7316 ± 840	7170 ± 939	7542 ± 666	7070 ± 815
Liver (mg/100 g bw)	2727 ± 152	2733 ± 265	2768 ± 178	2760 ± 277
Thyroid gland (mg)	17.9 ± 2.5	22.3 ± 10	18.0 ± 5.9	19.4 ± 6.4
Thyroid gland (mg/100 g bw)	6.70 ± 1.1	8.63 ± 4.0	6.56 ± 2.0	7.30 ± 2.2
Uterus (mg)	631 ± 182	615 ± 209	553 ± 144	784 ± 428
Uterus (mg/100 g bw)	237 ± 72	238 ± 93	203 ± 51	313 ± 183
Ovaries (mg)	82.2 ± 13	87.7 ± 24	94.9 ± 20	89.4 ± 17.4
Ovaries (mg/100 g bw)	31.2 ± 5.0	33.5 ± 9.0	35.0 ± 6.7	35.1 ± 7.5

[a] Testes weight means are shown for all data. Excluding the outlier in the control group from the data analysis, gave mean values for testes weight of 4043 ± 405 mg, and 816 ± 901 mg/100 g bw respectively, but did not alter the fact that no significant effect of OMC exposure was seen on adult testes weights.

Discussion

In the present study, pre- and postnatal OMC exposure was associated with changes in both reproductive and thyroid hormone levels, and altered development of some reproductive organs, semen quality as well as some behavioral endpoints.

Table 6 summarizes the endpoints that were significantly affected by OMC treatment. Serum T_4 levels in dams were almost completely reduced on both GD 15 and PND 15, while T_4 levels in the offspring were only reduced about 30% in the males, and were unaffected in the females on PND 16. In a study of PTU, using a similar study design (Axelstad et al., 2008), rather different effects were seen. Here, offspring levels of T_4 decreased severely on PND 16 (75% reduction in the high dose group) and furthermore a marked increase in thyroid weights and adverse effects on thyroid histology were seen. At the same time, reductions in dam T_4 levels on GD 15 were much less marked than in the present study. These results indicate that the mechanisms by which the two chemicals affect the thyroid and reduce T_4 levels probably differ substantially, even though both have been shown to reduce deiodinase activity (Cavalieri and Pitt-Rivers, 1981; Visser et al., 1979; Schmutzler et al., 2004; Klammer et al., 2007). The striking difference in OMC's effects on T_4 levels in dams and offspring respectively, was surprising. A possible explanation could be that OMC was only transferred to the offspring in very low concentrations, and therefore only affected T_4 levels slightly. Alternatively, the offspring for some reason were less susceptible to the thyroxine diminishing effects of OMC than the dams. No published rat studies have investigated the internal OMC levels in milk or serum after experimental exposure. However, studies in rats dosed with two other UV-filters, 4-methylbenzylidenecamphor (4-MBC) and 3-benzylidene camphor (3-BC), which have been shown to have similar effects on the developing reproductive and thyroid hormone systems as OMC, are transferred to rat breast milk in a dose-dependent manner (Schlumpf et al., 2008a). Furthermore, an epidemiological study of UV-filter exposure has shown that several UV-filters, including OMC, are transferred to human breast milk, as a clear correlation between exposure to OMC containing cosmetics and presence of OMC in human breast milk samples was found (Schlumpf et al., 2008a). It is therefore plausible that OMC is also transferred to breast milk in rats. Furthermore, a clear and dose-dependent reduction in testosterone level was seen in male offspring on PND 16, which also indicates that OMC did reach the offspring. Taken together, these results indicate that OMC probably affected the thyroid status of adult female rats more than seen in the offspring. In future studies, measurements of T_4 levels soon after birth would be useful for investigating if placental OMC transfer alone impacts on neonatal thyroid status. Furthermore, investigating effects of OMC given by gavage directly to neonatal rats rather than through the dams, would further elaborate on the mechanisms by with OMC causes thyroid hormone disruption. In order to compare experimental doses with human exposure more efficiently, including measurements of OMC concentrations in rat milk would be helpful.

Table 6
Endpoints that in the present study show association with OMC treatment. The LOAEL is given in mg OMC/kg/day.

Endpoint	OMC dose
T_4 levels in dams GD 15 and PND 15	Reduced at 500
T_4 levels male offspring PND 16	Reduced at 500
Testosterone levels male offspring PND 16	Reduced at 500
Progesterone levels female offspring PND 28	Reduced at 500
Estradiol levels female offspring PND 28	Reduced at 500 (but not 1000)
Body weight female offspring PND 7–14	Reduced at 500
Body weight female offspring PND 21–50	Reduced at 1000
Body weight male offspring (all study)	Reduced at 500
Testes weight PND 16	Reduced at 750
Testes histology PND 16	Affected at 1000
Prostate weight PND 16	Reduced at 1000
Prostate histology PND 16	Affected at 750
Thyroid gland weight PND 16	Increased at 750
Liver weight PND 16	Increased at 750
Motor activity PNW 9 female	Reduced at 750
Motor activity PNW 17 female	Reduced at 1000
Motor activity adult male	Increased at 750 (but not 1000)
RAM performance male	Improved at 500 (but not 750)
Sperm count	Reduced at 500
Prostate weight adult	Reduced at 1000

Reduced growth is a well known consequence of developmental hypothyroidism (Akaike et al., 1991; Kobayashi et al., 2005; Gilbert and Sui, 2006; Noda et al., 2005). However in most studies of hypothyroidism, growth retardation is not observed until the offspring are about two weeks of age, and is primarily seen when offspring T_4 levels are very severely reduced. In the present study, female offspring body weights were diminished from birth until PND 50, while body weights in male OMC offspring were low throughout the study. These effects may therefore not have been a consequence of developmental hypothyroidism at all, but rather a direct effect of OMC exposure. This was probably also the case for the effects on liver weight, as alterations in liver weight generally suggest treatment-related induction of enzymes or peroxisome proliferation (Sellers et al., 2007). The increase in relative liver weight seen in the present study was comparable to the effects seen in a two-generation study (Schneider et al., 2005), where OMC exposure caused increased liver weights in adulthood in both the parental and the F1 generations. In that study the increased weights were accompanied by slight cytoplasmic eosinophilia, which was not seen in offspring on PND 16 in the current study.

The observed dose-dependent decrease in testosterone levels on PND 16 (Fig. 2) corresponded well with the reductions in relative testes and prostate weights, as well as with the histopathological changes seen in these organs. The gene expression data indicate that the lower prostate weights were not related to antagonism of the androgen receptor. This conclusion is reached as both PBP C3 and TRPM-2 are regulated by AR mediated pathways, and their expression did not change after exposure to OMC. The reduction in testosterone observed in the male pups at PND 16 was not due to effects on the expression of the investigated steroidogenic enzymes, as these were not altered by OMC.

In the adult animals, prostate weights remained low in the high dose group, and histopathological changes were seen in the two highest dose groups. The observed acinar atrophy, is a typical finding in rats exposed to estrogenic chemicals. Interestingly, histopathology of prostates was a more sensitive endpoint than prostate weight changes in the current study. Furthermore, OMC exposure lowered sperm counts in all the exposed groups (Fig. 5). In the Schneider et al. (2005) study, the total number of spermatids/g cauda epididymis in the F1 generation was also significantly reduced in animals receiving 1000 mg/kg bw/day. The authors inferred that the effects were caused by anomalously high control values, exceeding historical controls. In the present study, control values did not differ from historic controls and our findings therefore indicate that the lowered sperm counts are caused by developmental OMC exposure. This is a quite alarming result in relation to the low sperm counts and declining sperm quality in humans reported during the last decades (Carlsen et al., 1992; Jørgensen et al., 2006). No effect on sperm motility parameters was seen in the OMC exposed animals in the present study or in the Schneider et al. (2005) study. This indicates that OMC affects the number but not the function of the sperm cells.

Developmental exposure to another UV-filter 4-MBC, has also caused adverse effects on the male reproductive system, as decreased

testes weights in young animals and decreased prostate weights in adulthood have been seen (Schlumpf et al., 2008b). So far no investigations of sperm counts have been performed in rats developmentally exposed to 4-MBC or other UV-filters, so it is not clear whether they exert similar effects on sperm production.

The reproductive system of the female offspring was also affected by OMC exposure, as both estradiol and progesterone levels were significantly lowered in PND 28 offspring. We did not see any effects on uterine weight, an endpoint that has been affected in some previous studies (Schlumpf et al., 2001; Klammer et al., 2005; Seidlova-Wuttke et al., 2006). However in these studies the effects were observed in females still undergoing exposure to OMC. Whether OMC affected uterine weight in the present study would have been better assessed in the female offspring on PND 16 rather than on PND 28.

In the two-generation study by Schneider et al. (2005) no effect on uterine weight on PND 21 was reported. However, in this paper only results from significantly affected organs were shown, and these were defined as statistically significant changes in both absolute and relative weights, showing a dose–response relationship. If this was not the case with uterine weight, this might explain the differing results. Another explanation could be the use of different rat strains, as Schneider et al. (2005) used Wistar rats, while Sprague–Dawley and Long–Evans rats were used in the studies where an effect was seen (Schlumpf et al., 2001; Klammer et al., 2005; Seidlova-Wuttke et al., 2006) and furthermore studies of OVX females could be more sensitive to changes in uterine weight than studies in intact animals.

Endocrine disruption can also affect timing of sexual maturation, however this endpoint was not affected in the present study. Pre- and postnatal exposure to estrogenic chemicals usually delays sexual maturation in male offspring, and causes precocious sexual maturation in female offspring. These effects have been seen after estradiol treatment (Biegel et al., 1998; Rodriguez et al., 1993), and have also been seen in males exposed to the UV-filters 4-MBC and 3-BC. The UV-filter exposure did however not affect sexual maturation in the female offspring, even though it caused significant alterations in uterine and ovary weights (Schlumpf et al., 2008b).

OMC exposure also affected the neural development in the present study. A number of animal studies have shown that transient thyroid hormone insufficiency during development is associated with structural abnormalities in the brain (Auso et al., 2004; Sharlin et al., 2008; Gilbert and Sui, 2008). We have previously hypothesized that the degree of hypothyroxinemia can be directly correlated to auditory function, learning abilities and motor activity levels in the offspring (Axelstad et al., 2008). However, this was not the case in the present study. T_4 levels were significantly decreased in both dams and male offspring during OMC dosing, but most of the behavioral endpoints were affected differently than would have been expected after developmental hypothyroxinemia, and did not correlate to T_4 levels in dams or offspring. This can probably be explained by the fact that OMC is a chemical with many properties, including estrogenic, anti-thyroid and possibly more modes of action.

Based on previous studies, we expected to see hyperactivity in the adult offspring that suffered from hypothyroxinemia during early brain development (Axelstad et al., 2008; Kobayashi et al., 2005). Instead, motor activity levels were reduced in females, and no consistent effects were evident in males. Based on these results we would hypothesize that in rats, only severe postnatal reductions in T_4 levels lead to hyperactivity, while reductions in maternal T_4 levels are of less importance. Furthermore, a possible explanation for the observed effects on female activity levels could be a masculinisation of this sexually dimorphic behavior. Male rats are usually less active than females and in the present study activity levels in the high dose females resembled those of the control males. Perinatal exposure to exogenous estrogens can induce male-specific behaviors in females (Sharpe, 2010), and a masculinising effect on female behavior after developmental exposure to estrogenic compounds has been reported in a number of recent publications. Developmental estradiol exposure altered female saccharin preference and mating behavior in the male direction (Ryan et al., 2010) and developmental exposure to the UV-filters 4-MBC and 3-BC strongly impaired female sexual behavior (Schlumpf et al., 2008b). However, motor activity levels in general can also be interpreted much more globally than as a type of sexually dimorphic behavior, and since the changes in female activity levels did not correlate with the observed reductions in estradiol, a direct effect of OMC on female activity levels may be a more plausible explanation of the results.

In the radial arm maze we had expected to see impaired spatial learning abilities (Axelstad et al., 2008; Akaike et al., 1991; Noda et al., 2005). The observed improvements in learning and memory ability in the males are therefore difficult to explain based on the anti-thyroid properties of OMC. However, this type of polarization of behavior (i.e. a further masculinisation of male behavior) has been seen in other studies from our lab where males have been treated neonatally with endocrine disrupting chemicals such as flutamide, procymidone or vinclozolin (Hass et al., 2001; Christiansen, 2009). These chemicals are anti-androgens, but apparently result in effects on male maze learning which resemble the ones observed in the present OMC study.

The acoustic startle reaction was unaffected by OMC exposure. This is in accordance with findings after perinatal exposure to the PCB-mixture Arochlor 1254, which also reduced circulating levels of T_4 in dams, fetuses and offspring. However, startle testing on both PND 28 and 70 did not reveal treatment-related effects on the amplitude of the startle response, nor were any effects on habituation seen (Crofton et al., 2000). Two studies of PTU do report increased startle amplitude in adult animals neonatally exposed to PTU (Goldey et al., 1995; Kobayashi et al., 2005). In both studies, exposure reduced levels of T_4 massively and to a greater extent than Arochlor 1254. Provided that hypothyroxinemia is the cause of change in startle amplitude, postnatal levels of T_4 probably need to be severely reduced for effects on startle amplitude to appear.

In the hearing test, no differences were present between dosed and control animals, and again our data indicate that postnatal T_4 levels in the offspring are the determining factor. This is much in line with findings in the cross fostering study of Arochlor 1254 (Crofton et al., 2000). In this study, rat offspring were exposed to Arochlor via the mother either during gestation or during lactation. Offspring that were exposed solely during gestation did not present with hearing deficits, but so did lactationally exposed offspring. This is also consistent with the fact that the greater part of development of the hearing organ takes place during the first postnatal weeks in the rat (Crofton et al., 2000).

In summary, the present study addressed the potential endocrine disrupting properties of OMC on the developing reproductive and thyroid hormone systems, and investigated how changes in thyroid hormone levels would affect the neurological development of the offspring. We have shown that OMC possesses endocrine disrupting properties, as severely decreased T_4 levels were seen in exposed dams from all three dose groups. These changes did however not cause the expected behavioral effects in the offspring and no correlations with T_4 levels were seen. Our results indicate that in rats, only severe postnatal T_4 decreases are determining for adverse brain development. Furthermore, our working hypothesis that the degree of pre- and postnatal hypothyroxinemia correlates directly with reduced auditory function, decreased learning abilities and increased motor activity (Axelstad et al., 2008) was not corroborated in the present study, and is probably not suitable for all compounds that adversely affect the thyroid hormone system, especially if these have several modes of action. The same

conclusions were drawn by Miller et al. (2009) who investigated the effect of developmental PCB exposure on the thyroid hormone system, and found that if the compounds causing T_4 reductions also have other mechanisms of action, then changes in thyroid hormone levels are not necessarily causative of downstream neurotoxic outcomes (Miller et al., 2009).

Based on the present study only a LOAEL could be set for OMC, as adverse effects were seen on male body weights and sperm counts at all dose levels. Behavioral changes were less sensitive than changes in thyroid hormone levels, which indicate that setting a NOAEL for OMC based on reductions in thyroid hormone levels, will also be protective against behavioral effects caused by developmental hypothyroxinemia. Furthermore, our results show that behavioral testing of thyroid disruptors with multiple modes of action provides useful complementary information and contributes to a broader understanding of the toxicity of the tested chemicals, than studies that only take hormonal measurements and development of reproductive organs into account.

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Effects of a 5-day treatment with the UV-filter octyl-methoxycinnamate (OMC) on the function of the hypothalamo-pituitary–thyroid function in rats

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Abstract

Octyl-methoxycinnamate (OMC) is one of the most frequently used UV-filters in sunscreens to protect the skin against the noxious influence of UV radiation. Recently, OMC was suspected to act as an "endocrine active chemical" (EAC) with estrogenic actions. While EACs have been investigated thoroughly for interference with reproductive function in mammalians, surprisingly little efforts have been made to investigate an interference of EACs with the hypothalamo-pituitary–thyroid (HPT) axis despite the expression of estrogen receptors in all parts of this axis. Therefore, we conducted an *in vivo* study with ovariectomised rats treated for 5 days with different doses of OMC or 17β-estradiol (E2) as a control. Determined parameters comprised serum levels of TSH, T4 and T3, hypothalamic TRH mRNA expression, protein-expression of the sodium–iodide-symporter (NIS) and the TSH receptor and the activities of thyroid peroxidase (TPO) in the thyroid and the T3-responsive hepatic type I 5′deiodinase (Dio1) in the liver.

While E2 did not affect TSH-, T4- or T3-levels, OMC caused a dose-dependent decrease of serum concentrations of all of these hormones. TRH expression remained unaffected, while in the thyroid, expression of the TSH receptor but not of NIS was stimulated by OMC. TPO activity was unaltered but Dio1 activity was reduced by OMC. Thus, our results demonstrate a non-estrogenic interference of OMC within the rodent HPT axis with inadequate feedback response to impaired thyroid hormone status, indicated by decreased serum thyroid hormone and hepatic Dio1 levels.

Keywords: OMC; Rat; Thyroid; Estrogen; TRH; TSH; T3; T4

1. Introduction

Ethylhexyl-methoxycinnamate (formerly known under the trivial name octyl-methoxycinnamate, OMC), is one of the most frequently used chemical UV-filters worldwide. It is contained in high amounts (up to 1 of 10 parts per weight) in sunscreens to protect the skin against the noxious influence of UV radiation. Recent studies indicated, that OMC may have significant undesirable effect as an "endocrine active chemical" (EAC) (Schlumpf et al., 2001; Schlumpf et al., 2004) and regulatory authorities like the Scientific Committee of Cosmetic Products and Non-Food Products (SCCNFP) of the European Union have

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released statements of concern on use of OMC. The term EAC addresses those chemicals, which possess the ability to interfere with endocrine systems, like the hypothalamo-pituitary–gonadal axis which might be of major concern during perinatal development and differentiation of vertebrate organisms. In the last decades research on adverse effects of EAC focussed on putative estrogenic and anti-androgenic affects in wildlife populations and laboratory animals (for review, see (Gelbke et al., 2004; Gray et al., 2006; Safe, 2004). With regard to OMC, an estrogenic multi-organic risk assessment has been reported which revealed that OMC exerts both estrogenic as well as non-estrogenic activities in uterus, vagina and liver (Klammer et al., 2005; Seidlova-Wuttke et al., 2006b).

Similarly to 17β-estradiol (E2) and both its receptor subtypes, the thyroid prohormone T4 and its biological active metabolite T3 affect the function of virtually all organs during development and in adulthood since the various forms of the T3-binding thyroid hormone receptors are broadly expressed throughout the body (O'Shea and Williams, 2002; Shahrara et al., 1999). However, despite the pivotal function of thyroid hormones in the organism, little attention has been paid to interference of EACs with the hypothalamo-pituitary–thyroid axis (HPT) in mammals. Likewise, the literature regarding effects of E2 on the HPT axis has been mainly compiled before the molecular characterisation of T3 receptors and the data reported are contradictory (Boado et al., 1983; Franklyn et al., 1987; Lisboa et al., 1997; Thomas et al., 1986). In a recent study with ovariectomised (ovx) rats which were treated with E2 and various EACs for 3 months via the food, OMC at a dose of approximately 200 mg/kg bodyweight caused a reduction of T4-levels without affecting T3- or TSH-levels (Seidlova-Wuttke et al., 2006a). In contrast, E2 did not affect these parameters, further supporting the assumption that OMC might be an EAC with both, estrogenic but also non-estrogenic properties.

To further investigate the activity of OMC on the HPT axis, dose-response relationships of several parameters of the HPT axis were investigated following a treatment protocol with ovx rats previously used to conduct a multi-organic risk assessment study (Klammer et al., 2005). Following parameters of the HPT axis were analysed: TRH expression in the hypothalamus was measured at mRNA level, TSH-, T4- and T3-serum levels were determined by radioimmunoassay. Expression of two-key factors of thyroid function, the sodium–iodide-symporter (NIS) and the TSH-receptor (TSHR), was analysed on protein-level by western blot. In addition, the enzyme activities of the thyroid peroxidase (TPO) in the thyroid, the key enzyme catalysing iodination of tyrosyl-residues in thyroglobulin and their coupling to iodothyronines and the T3-responsive type I 5′deiodinase (Dio1) in the liver were determined.

2. Methods

2.1. Test substances

OMC (ethylhexyl-methoxycinnamate, EUSOLEX 2292®) was obtained from Merck KG (Frankfurt, Germany) and 17β-estradiol-valerate was purchased from Sigma–Aldrich GmbH (Taufkirchen, Germany). The test substances were dissolved in olive oil, which also served as solvent control.

2.2. Animals and treatment

Female rats, used in this study, were housed and treated in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123). The experiments were approved by a permit issued by the Landesamt für Verbraucherschutz, Braunschweig, Germany.

For all experiments female Sprague–Dawley rats, purchased from Winkelmann (Borchen, Germany) were used. Animals were kept under standard conditions (soy-free formulation of diet, ssniff SM R/M, 10 mm, supplied by ssniff Spezialdiäten GmbH, Soest, Germany and water *ad libitum*, lights on from 5:30 am to 6:00 pm, room temperature 23 °C and a relative humidity of 55%). At the age of 3 months, all rats were bilaterally ovariectomised (ovx). After a recovery phase of 17 days, the animals were treated via gavage once per day with either 1 ml pure olive oil (Ctl), 600 μg/kg bodyweight 17β-estradiol-valerate (E2) or 10, 33, 100, 333 or 1000 mg/kg OMC (OMC-10 to OMC-1000). The treatment was conducted each day between 5:30 and 6:00 am. The animals were observed for daily clinical signs of impairment of health or systemic toxicity. Bodyweight was determined daily. At day 5 of treatment, the animals were decapitated under deep CO_2 anaesthesia 3–4 h after the last application. Blood was collected from the trunk and serum samples were stored at −20 °C. The collected organs were frozen in liquid nitrogen and stored at −70 °C until further processing. The mediobasal hypothalamus (MBH) was dissected from the frozen brain as described previously (Leonhardt et al., 2000)

2.3. Serum analysis

Thyroid hormones (total T3/T4) were measured using commercially available kits validated for rat serum following the manufacturers' instructions (Active® T3, Active® Thyroxin, DSL, Sinsheim, Germany). The TSH radioimmunoassay was performed, as described previously, with reagents kindly supplied by the Hormone Distribution Program of the NIDDK (Baur et al., 2000). The TSH tracer was labelled with ^{125}I (Hunter and Greenwood, 1962).

2.4. RNA isolation and RT-PCR

Total RNA was isolated with the RNeasy Total RNA kit (Qiagen, Hilden, Germany) including the removal of DNA with DNase following the manufacturers instructions. Reverse transcription was carried out using M-MLV reverse transcriptase RNase H minus point mutant including recombinant RNasin ribonuclease inhibitor (Promega, Mannheim, Germany) and random primers (Invitrogen, Karlsruhe, Germany). Real time-PCR was performed using the ABI Prism 7700 sequence detector (PE Applied Biosystems, UK) and the QPCR core kit (Eurogentec, Belgium). The sequences of proTRH primers and probe were derived from previously published data (Cote-Velez et al., 2005).

Forward: 5′-TTCTGGATTCCTGGTTCTCAGATG-3′
Reverse: 5′-GGATGTTGCCTCTTGGTGACA-3′
Probe: 5′-AGCACCCTGGCAGGCGATCCTTC-3′

2.5. Protein extraction and western blot analysis

One thyroid lobe was homogenised in 250 μl buffer (250 mM sucrose, 10 mM Tris–HCl, pH 7, 5 mM NaCl, 1 mM EDTA with 1 tablet Complete Mini, EDTA free per 10 ml (Roche, Mannheim, Germany). The suspension was sonicated for 1 min (Sonifier B12, Branson Instruments, Danbury, CT, USA) and centrifuged for 10 min, 10,000 × *g* at 4 °C. Protein concentration was determined with a commercially available Bradford-assay (BioRad, Munich, Germany). The supernatant was stored at −70 °C.

The samples were diluted to a protein concentration of 3.5 mg/ml and incubated for 5 min at 95 °C with loading dye (Roth, Karlsruhe, Germany). SDS-PAGE was carried out on a 7.5% gel (Laemmli, 1970) with a Mini Protean 3-Cell (BioRad, Munich, Germany) for 35 min at 200 V. Protein was transferred to NC-membrane using semi-dry FastBlot (BioRad) for 30 min at 3 mA/cm^2 gel according to the manufacturers' instructions. Loading was normalised by intensity of bands after Ponceau S staining (Salinovich and Montelaro, 1986). The membrane was blocked for 1 h at room temperature in TPBS (PBS with 1% Tween-20) with 7% dry milk, and incubated with the primary antiserum overnight at 4 °C (TSHR (1:500): Santa Cruz Biotech., Santa Cruz, CA, USA; NIS (1:500): Acris Antibodies, Hiddenhausen, Germany). The membrane was incubated with the secondary antiserum for 1 h at room temperature (goat anti-rabbit-HRP (1:2500): Dianova, Hamburg, Germany; Envision, DAKO Cytomation, Hamburg, Germany) and washed in TBPS. The blot was stained using DAB solution (DAKO Cytomation) for 45–60 s, subsequently scanned and analysed densitometrically.

2.6. Deiodinase assay

The assay was performed according to a protocol published previously (Schmutzler et al., 2004). Livers of four animals per treatment group were analysed for Dio-activity. Pulverised liver samples (∼50 mg) were homogenised in a buffer containing 250 mmol/L glucose, 20 mmol/L HEPES, 1 mmol/L EDTA and 1 mmol/L DTT by ultrasonification (20 pulses of 0.6 s at 200 W) and centrifuged at 10,000 × *g*. Supernatants were decanted and precipitates were resuspended in homogenisation buffer by another course of ultrasonification (10 pulses of 0.6 s at 200 W) and used for the assay. The protein contents of the homogenates were determined by a modified Bradford protein assay using γ-globulin as protein standard. Specific activity of 5′DI was determined measuring the release of $^{125}I^-$ from ^{125}I-labelled reverse rT3 (DuPont, Bad Homburg, Germany) in the presence of 100 nmol/L unlabelled rT3. The incubation volume was 50 μl and the incubation time was 1 h at 37 °C. Samples were measured in triplicates using 20 μg protein. Dio activity was expressed as pmol $^{125}I^-$ released per mg protein per min.

2.7. TPO assay

For the preparation of rat TPO, single thyroid lobes were homogenised in 1 ml of a buffer containing 5 mM potassium phosphate (pH 7.4), 200 mM sucrose, 500 units/mL catalase, and 1 mM EDTA. The homogenate was centrifuged at 600 rpm for 10 min at 10,000 × *g* at 4 °C, the precipitate was discarded, and the supernatant was centrifuged at 100,000 × *g* for 45 min at 4 °C. The supernatant was discarded and the precipitate was resuspended in 100 μl of 200 mM potassium phosphate buffer (pH 7.4) and stored at −80 °C. Protein concentrations were determined by a modified Bradford assay. The *ex vivo* TPO activity in the thyroids of treated animals was measured by a guaiacol assay using 50 μg of isolated protein in 50 mmol/L potassium phosphate buffer (pH 7.4), 40 mmol/L guaiacol and 220 μmol/L H_2O_2. Thyroids of seven–nine animals per treatment group were measured in triplicate. The reaction was followed by photometric determination of the increase in absorption at a wavelength of 470 nm at 25 °C.

2.8. Statistical analysis

Data were analysed by ANOVA followed by Dunnett's test or Mann–Whitney U-test where appropriate using the Prism software (Graph Pad4, San Diego, USA) and are presented as means + S.E.M. or as box and whisker plot. Data from mRNA and protein expression measurements were normalised to percentage values, *i.e.*, absolute values determined in the ovx group were averaged and the mean was set 100%. Data measured in the other experimental groups were calculated in relation to this mean. P values <0.05 were considered significant.

3. Results

During the entire experiment, the animals did not show any signs of impairment of health, deduced from behaviour, water consumption and food intake. Bodyweight in the E2 treated animals decreased slightly as

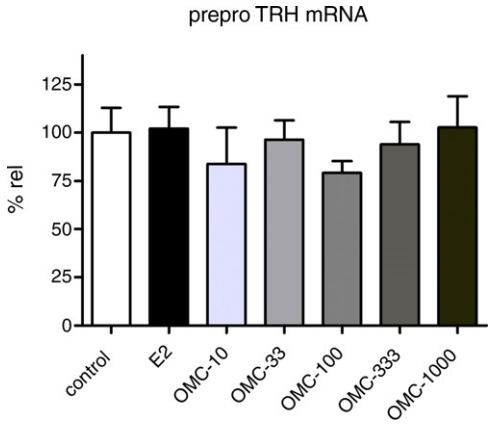

Fig. 1. PreproTRH expression in the MBH after 5 days of treatment. Values are given in relation to the average of the control which is set to 100%. Means + S.E.M., $n = 12$.

reported previously (Jarry et al., 2004) while OMC did not have any significant impact on bodyweight. Organ weight and macroscopic appearance of the liver, the spleen and kidney was unaffected by any of the treatment in comparison to the control group (data not shown).

The expression of proTRH transcripts in the mediobasal–hypothalamus (MBH) remained unchanged upon treatment with E2 or OMC by all applied dosages (Fig. 1) with no trends for a dose–response relationship.

TSH-serum levels were increased slightly, but not significantly, to 120% after E2 treatment, while OMC application induced a dose-dependent decrease down to 38% in the OMC-1000 group of the level determined in the control group (Fig. 2) reaching significance at the OMC-333 and OMC-1000 dose.

There were no statistically significant effects of E2 on either serum T4 or T3, in spite of what appeared to be a small decline in the mean concentration of T3 (83%

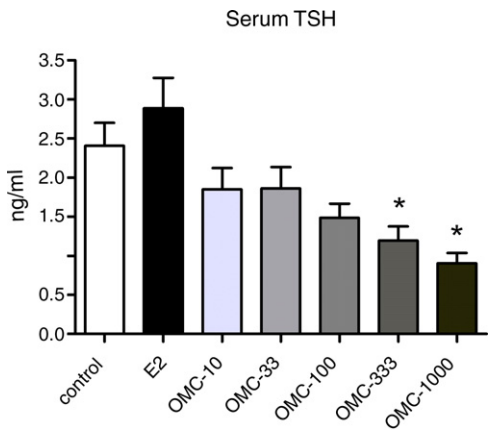

Fig. 2. Serum levels of TSH after 5 days of treatment. Means + S.E.M., $n = 12$, $^{*}P < 0.05$ vs. control group.

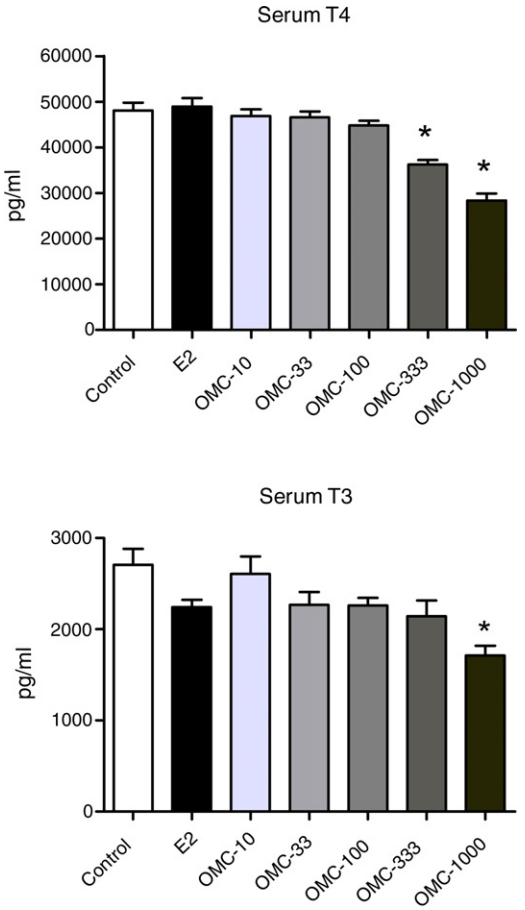

Fig. 3. T4- and T3-serum levels after 5 days of treatment. Means + S.E.M., $n = 12$, $^{*}P < 0.05$ vs. control group.

of control group) (Fig. 3). In contrast, mean T3-serum levels in the highest dose OMC-1000 group were reduced to 63% in comparison to the control group; T4-levels in the OMC-333 and OMC-1000 groups were lowered to 75 and 59% compared to the control group, respectively.

The expression of the NIS, determined by semi-quantitative western blot at the protein level, remained unaffected by treatment with OMC (Fig. 4). In contrast, as shown in Fig. 5, at the highest dose (OMC-1000) OMC increased the expression of the TSH receptor in the thyroid to about 144% compared to the control group. In the western blot analysis we investigated only the OMC-1000 group versus control, because only upon administration of this dose TSH-, T4-, and T3-serum levels were significantly altered.

Neither treatment affected thyroid TPO-activity (Fig. 6). While E2 treatment had no effect on hepatic Dio1 activity (Fig. 7), a dose-dependent decrease was observed after OMC application which reached significance in groups OMC-333 and OMC-1000 (72 and 64%

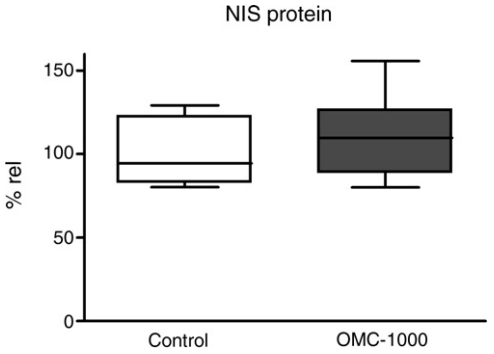

Fig. 4. Protein expression of the sodium–iodide-symporter (NIS) in the thyroid after 5 days of treatment with the highest dose of OMC. Densitometric analysis of signal intensity in semi-quantitative western blot, Values are given in relation to the control which is set to 100%. Medians plus whiskers to the smallest and largest values, $n = 5$.

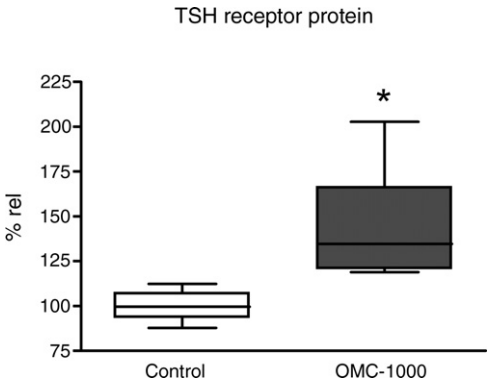

Fig. 5. Protein expression of the TSH-receptor after 5 days of treatment. Values are given in relation to the control which is set to 100%. Medians plus whiskers to the smallest and largest values, $n = 5$, $^{*}P < 0.05$ vs. control group.

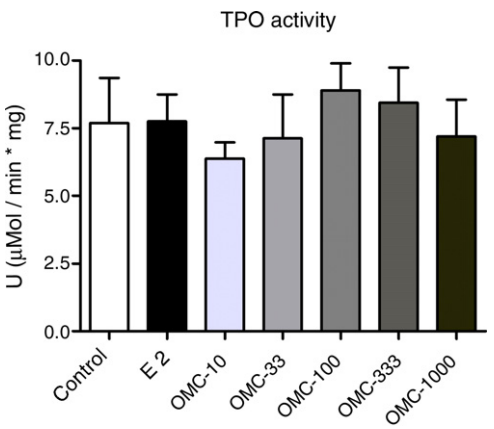

Fig. 6. Activity of thyroid peroxidase after 5 days of treatment. Means + S.E.M., $n = 7$.

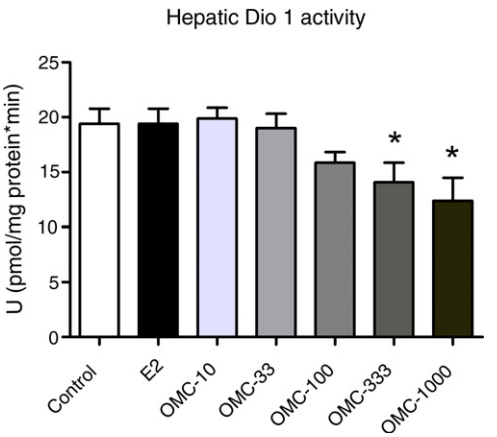

Fig. 7. Dio1 activity in the liver after 5 days of treatment. Means + S.E.M., $n = 9$–12, $^{*}P < 0.05$ vs. control group.

compared to controls, respectively). As expected, no Dio2 activity was detectable in these liver samples (data not shown).

4. Discussion

Two lines of evidence demonstrate that the thyroid directly or indirectly is a target of sex steroids: (a) thyroid tumors are about three times more frequent in females than in males and the incidence of benign thyroid diseases, such as subclinical and overt hypo and hyperthyroidism increases in postmenopausal women (Henderson et al., 1982; Schindler, 2003). Both autoimmune thyroid diseases, Graves' disease with hyperthyroidism and Hashimoto's disease leading to hypothyroidism are more prevalent in females and also congential hypothyroidism is more frequent in newborn girls, (b) normal and malignant thyroid tissues express both types of ER (Egawa et al., 2001; Kawabata et al., 2003; Schlecht et al., 2004).

To investigate a putative estrogenic action of the endocrine active compound OMC on the HPT axis, ovx rats were treated with the substance according to a protocol which in a previous study demonstrated a multi-organic effect of this UV-filter (Klammer et al., 2005). While the present data clearly show an interference of OMC with the function of the HPT axis, E2 was without any significant effect on the examined parameters after 5 days of treatment. Thus, the observed rapid and dose-dependent effect of OMC, in the present study, has to be attributed to a non-estrogenic endocrine activity which may not involve the interaction of the UV screen with ERs known to be expressed in either pituitary, thyroid or liver.

The literature on E2 effects on the rat HPT axis of the rat is inconsistent. Depending on the animal model and strain, route of application and duration of treatment with different E2-preparations various results have been reported. Therefore, we discuss mainly studies performed with ovx animals: Thomas et al. (Thomas et al., 1986) described no alterations of T4- and T3-levels in ovx, E2-treated animals while Chen and Walfish (Chen and Walfish, 1978) reported an E2-induced elevation of total T3-levels and a decrease in total T4. No effects of a long-term treatment with E2 on either TSH-, T4- and T3-levels were observed in young ovx rats while in middle-aged ovx animals T4 and T3 concentrations were decreased (Bottner and Wuttke, 2005). In our experiment animals were treated for 5 days with E2 via gavage and no effects on the HPT axis were observed. One might argue, that the dose applied was insufficient, however, this assumption is unlikely since the mean E2 value determined in our steroid treated animals was 85.5 ± 10.2 pg/mL, *i.e.*, at a slightly supraphysiological level which caused an about four-fold increase of uterus weight compared to control ovx rats (for the effects of both, E2 and OMC on uterine weight see (Klammer et al., 2005).

The biosynthesis of TRH in hypophysiotropic neurons of the paraventricular nucleus in the mediobasal hypothalamus is inversely regulated by feedback effects of circulating levels of thyroid hormones (Kakucska et al., 1992). In our study, we did not observe any alteration of proTRH expression by short-term administration of E2 which is in line with the unaffected TSH-, T4- and T3-levels. However, OMC caused a significant decrease of the concentrations of these three hormones. At present, the unchanged TRH expression remains puzzling, however, it can be speculated that the degree of changes of hormone concentrations and/or the period of lowered levels was to small and/or too short to significantly stimulate hypothalamic proTRH expression. Likewise, it may be possible that mRNA expression does not reflect peptide production, the post-translational processing and secretion. Synthesis of TRH is regulated via both gene transcription and processing of proTRH, as prohormone convertase PC1/3 is also up-regulated by low levels of thyroid hormones (Espinosa et al., 2007; Perello et al., 2006). Thus, as a consequence of the low T4- and T3-levels after OMC treatment one would expect increased proTRH processing. This is not in line with our observations which, instead, would predict a decreased processing of proTRH. Thus, in this case, one would have to assume a more or less direct effect of OMC on prohormone convertases in the PVN of the hypothalamus that would counteract the effects of the low thyroid hormone levels. Although hypothalamic TRH is also regulated by other factors than thyroid hormones, including leptin and glucocorticoids (Bruhn et al., 1998; Fekete et al., 2006), an effect of endocrine disrupters on hypothalamic prohormone convertases or on TRH has so far not been described.

Alternatively, a direct effect of OMC on the pituitary may also be conceived.

For a neurohormone such a discrepancy between levels of expression and secretion has been reported for the effects of interleukin 1beta (IL-1β) on GnRH expression in ovx rats: levels of GnRH mRNA remained unaffected by the treatment, however, gonadotropin secretion was reduced, because of the diminished translational efficiency of the GnRH mRNA (Kang et al., 2000). Thus, we cannot exclude the possibility that OMC acts in the hypothalamus resulting in reduced TRH release. Alternatively, we also cannot exclude the possibility that the decreased TSH-levels are caused by a direct effect of OMC on pituitary thyrotropes.

Further analyses were performed to investigate whether OMC alters thyroid function directly which would be another explanation for low T4- and T3-serum levels at high OMC doses. The sodium–iodide-symporter (NIS) transports iodide which is used for thyroid-hormone synthesis and is therefore, a key process in thyroid function (Kaminsky et al., 1993). The analysis of the NIS expression on the protein level revealed no consequence of OMC treatment, and can be excluded as a reason for the decreased thyroid hormone levels. However, NIS has to be integrated into the basolateral thyrocyte membrane to exert its iodide transport function, but so far we have no information yet, whether this step is affected by high dose OMC. Iodide, actively transported by the NIS, is organified into thyreoglobulin by the thyroidperoxidase (TPO) in a radical reaction at the luminal surface of the apical membrane of the thyroid follicles. Again, OMC treatment had no effect on thyroid function with regard to TPO activity. In contrast, western blot analysis revealed that the expression of the TSH receptor (TSHR) in the thyroid was increased. In the thyroid cell-line FRTL-5, which was transfected with a TSHR-promotor construct, it was shown that the promotor activity was inversely proportional to TSH concentration (Ikuyama et al., 1992). Thus, the OMC induced alteration of TSHR expression is probably a compensatory response to adjust sensitivity to the decreased TSH-levels.

OMC treatment resulted in decreased hepatic Dio1 activity which is associated with reduced thyroid hormone serum levels. Expression of Dio1 is stimulated by thyroid hormones on the transcriptional level via T3

responsive elements in the Dio1 gene promoter (Jakobs et al., 1997; Toyoda et al., 1995). Accordingly, in rats, hyperthyroidism generated by T4 or T3 injection resulted in increased, hypothyroidism induced by thyroidectomy in reduced Dio1 enzyme activities in the liver (Escobar-Morreale et al., 1997). Therefore, the lower Dio1 activity in the liver of the OMC-treated rats may be a direct response to the drop in thyroid hormone levels, particularly in T4, measured in these animals. These data are consistent with another experiment, where OMC administered for 12 weeks to ovx rats significantly decreased serum T4 (Schmutzler et al., 2004). However, the present investigation shows that the effects of OMC on thyroid hormone homeostasis are rapid, as significant changes in T4- and T3-levels are already seen after 5 days.

According to the classical negative feedback concept, low T3- and T4-serum levels should lead to higher TSH secretion from the pituitary. In the present study however, despite lower thyroid hormone levels, TSH concentrations were reduced. This hormonal constellation is reminiscent of changes observed in central tertiary hypothyroidism or thyroid hormone metabolism known as non-thyroidal illness (Fliers et al., 2006). Since our animals did not show any signs of illness or abnormalities of behaviour, the simultaneously reduced TSH-, T4- and T3-levels may result from a direct action of OMC on the thyrotropes resulting in reduced thyrotropin release.

In summary, our data demonstrate a non-estrogenic endocrine disrupting activity of OMC on the HPT axis which should be taken into account for a risk assessment of this commonly used UV-filter. In a recently published manuscript we presented a risk assessment from a pharmacodynamic dose-response study on the estrogenic properties of OMC on various endpoints within the HPG axis (Klammer et al., 2005). Deducing NOEL and LOEL values from the data presented in this manuscript, for which we used the same experimental design, one could conclude that the benchmark doses of OMC for effects on the HPT axis are very similar, leading to the same conclusion with regard to risk assessment.

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Multi-organic risk assessment of estrogenic properties of octyl-methoxycinnamate in vivo: A 5-day sub-acute pharmacodynamic study with ovariectomized rats

Klammer, H., Schlecht, C., Wuttke, W. & Jarry, H.

Abstract
Sun protection products contain a variety of UV-filters, among others, octyl-methoxycinnamate (OMC). Recently, an uterotrophic effect in immature rats has been reported, indicating that OMC might have estrogenic properties and thus is an endocrine active chemical (EAC). However, determination of an estrogenic activity in the uterus only is a restricted approach with the potential risk of missing undesirable actions in other organs regulated by estrogens. A pharmacodynamic experiment with 5 dosages of OMC in adult ovariectomized (ovx) rats was carried out to quantify the multi-organic estrogenic properties of OMC. As control compound, estradiol-valerate (E2) was included. Animals were treated per gavage for 5 days. The expression levels of markers of estrogenic action in several organs were measured by RT-PCR. Effects on metabolic parameters were assessed by determination of the serum concentrations of leptin, cholesterol, high and low density lipoproteins (HDL and LDL), glucose and triglycerides. Observed changes upon OMC treatment were analyzed using the NO(A)EL and the benchmark dose approach. **From the obtained pharmacodynamic data of the most sensitive parameter (truncated estrogen receptor protein 1 gene expression in the pituitary) we obtained threshold values that are exceeded by the recommended use of OMC containing formulations for skin protection in humans, therefore we propose to reduce the use of OMC in cosmetic products. In addition to estrogenic actions of OMC, non-estrogenic effects have been found for this chemical supporting the need of a multi-organic risk assessment of putative EACs.**

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Evidence of reproductive disruption associated with neuroendocrine changes induced by UV–B filters, phtalates and nonylphenol during sexual maturation in rats of both gender

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ABSTRACT

Endocrine disruptors (EDs) are exogenous substances or xenoestrogens natural or synthetic, capable o interacting with different systems and altering their normal hormonal regulation, being the reproductive system one of the most affected. EDs produce their effects not only by acting on nuclear steroid receptors, but also on membrane receptors, steroidal and non-steroidal synthetic enzymatic pathways and/or metabolism. The incorporation to the body depend on each EDs, which are liposoluble and easily deposited in the tissue; thus ensuring a prolonged accumulation and release, even when the exposure is not continuous. In addition to cross the placenta, EDs may act in the offspring during the reproductive system formation and maturation key stages and its regulatory mechanisms. The effects o EDs can be multiple, but most acts mediating estrogenic and/or antiandrogenic effect. Three groups of EDs are widely used: in plastics (phtalates), sunscreens (cinnamate and methylbenzylcamphor), and detergents (nonylphenol). In this paper we review the effects of the exposure to these environmental chemicals on the reproductive system and the possible mechanisms by which they occur, focusing in the hypothalamic–pituitary neuroendocrine mechanisms that regulate the reproductive system.

1. Introduction

Environmental pollution had and has a major impact on biological systems. In the last few decades huge amounts of chemicals of different origin, structure and use coming from industrialization have been released into the environment as chemical waste, causing changes to the exposed living organisms. These substances are generically considered endocrine disrupters (EDs) (Schug et al., 2011). They have been also called xenoestrogens or xenobiotics as they have the capacity to alter hormonal homeostasis through the same biological paths used by endogenous steroids (Akingbemi and Hardy, 2001; Sharpe, 2001), modifying the endocrine system, like the reproductive and the thyroid axis (Danzo, 1998; Heindel, 2006; Schmutzler et al., 2004; Zoeller, 2007) and causing adverse health effects in an organism or its progeny. One decade ago, Krimsky suggested a new hypothesis known as "Endocrine Disruption" (Krimsky, 2000), (see Table 1). At present, the US Environmental Protection Agency (EPA) defines them as: "Exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are responsible for homeostasis, reproduction and developmental process".

There is increasing interest and research on effects of EDs on neuroendocrine systems (Dickerson and Gore, 2007; Gore, 2010). Most studies have focused on the effects of chemicals known for several years as EDs (pesticides, phytoestrogens, dioxins, etc.) on gonadal and thyroid neuroendocrine systems. This article will primarily discuss the literature on neuroendocrine alterations induced by substances used to manufacture products used daily, such as plastics (phthalates), sunscreens and cosmetics (cinnamate and methylbenzylcamphor) as well as detergents (nonylphenol), focusing in their effects on the hypothalamic–pituitary-gonadal axis and the sequelae on reproductive function.

1.1. Characteristics and mechanism of action of EDs

EDs include substances with estrogenic and/or antiestrogenic androgenic and/or antiandrogenic actions (Carbone et al., 2010; Carou et al., 2008; Rivas et al., 2002) and mimetizers or antagonists of the thyroid hormones (Brucker-Davis, 1998; Schmutzler et al., 2004). Also EDs can interference with hormonal feedback regulation and neuroendocrine cells. Along with the direct influence of EDCs on estrogen or androgen actions, they can affect endogenous steroid production through negative and positive feedback

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Table 1
Endocrine disruption hypothesis.

The effects occur through mechanisms of different action, all of them related to the hormonal action
There is no exposure limit without effect. It is shown a relation dose-effect not necessary lineal, which in some cases is apparently paradoxical with U or inverted U curve types. May have effect in low doses (parts per billion) while not showing manifestation at high doses. While the limits of exposure are measured in parts per million (ppm), the body concentrations of some of them exceed that limit
Most of the effects are persistent and bioaccumulative and have a latency period of decades
Their effects are specifically evident in critical development periods or in moments of particular susceptibility
Their effects may be the result of the combine action of different compounds that may trigger a synergic, antagonic and/or additive response
The expression of the effects may appear late in life in the exposed individual, and even appear over generations

effects that may differ depending on developmental stage, leading to perturbations in neuroendocrine systems function (Gore, 2010).

At the molecular level EDs can act through different mechanism (De Coster and van Larebeke, 2012): (a) activation of the classical nuclear receptors ERα and ERβ, which bind estrogen responsive elements (EREs) in the promoters of target genes, regulating its expression (Levin, 2005), (b) activation of membrane-bound estrogen receptors: mERα, mERβ, and GPR30 (non nuclear steroid hormone receptors), inducing rapid nongenomic responses and acting through second messenger-triggered signal cascades (Watson et al., 2007), (c) binding to cytosolic ER receptors, activating the kinases and leading to the activation of the Src/Ras/ERK signaling cascade (Nadal et al., 2001), (d) cross-talk between genomic and nongenomic pathways (Silva et al., 2010), (e) activation of estrogen-related receptors (ERR), which are orphan nuclear receptors (Takayanagi et al., 2006), (f) changes in DNA Methylation or Histone-Modifications, that produced epigenetic modifications (Ho et al., 2006). Also early life exposures to EDs may alter gene expression in hypothalamic nuclei via nongenomic epigenetic mechanisms (Gore, 2008), modifying the phenotype expression and explaining the fact that multi and trans-generational exposure to EDs may promote the development of a disease over future generations (Hoshino et al., 2005; Anway and Skinner, 2006), (g) acting on the enzymatic pathways involved in steroid biosynthesis and/or metabolism of hormones, such as the enzymes 3b-HSDs and 17b-HSDs, aromatase, sulphatases (Whitehead and Rice, 2006).

EDs may be transferred through placental circulation and breast secretion (Campoy Folgoso et al., 2004; Nishikawa et al., 2010; Rogan et al., 1987;Wickizer and Brilliant, 1981; Schlumpf et al., 2010).), disturbing sexual differentiation and development of exposed offspring during early prenatal or postnatal stages (Colborn et al., 1993). Most of the EDs are lipid-soluble substances and they tend to be deposited especially in adipose tissue, in a dose-dependent manner, causing a very important cumulative effect (Schlumpf et al., 2004). In addition, simultaneous exposure to several EDs produced an additive or synergic effect (Crews et al., 2003). Exposure to EDs minimum doses may cause even more drastic effects than the ones appearing in high doses exposure. This occurs because EDs exert their effects following a non classic response dose curve: in inverted U or U shape (Vandenberg et al., 2012; vom Saal et al., 2007). This would explain the fact that EDs may alter the normal function of the endocrine system even at doses that could be considered, from toxicological point of view, as "safe dose" or "safety margin".

EDs are released in the environment and can be found in natural water courses and in fish (Poiger et al., 2004). In humans, exposure occurs through air, contaminated water or food intake, dermal contact and even through medical consumables and devices such as catheters, breathing and respiratory equipment and blood bags. It is also possible that the disruptive endocrine action may come from food ingestion with some hormonal action per second. For example, it has been demonstrated that urinary concentration of the estrogenic ED genistein was about 500 times higher in the soy milk formula-fed infants than in the cow milk formula-fed infants (Cao et al., 2009).

1.2. Impact of EDs on male and female reproductive peripheral organ

Many decades ago some researchers observed the loss of reproductive capacity, malformation in the reproductive organs and abnormal sexual behavior in animals of the ecosystem (Carson, 1962).

One of the more important variable to take into account to evaluate the impact of EDs on the reproductive system is the period of exposure, being more relevant during early fetal and neonatal developmental, when the programming of the endocrine system is carried out. In males, exposure to EDs during gestation and early stages of life has been related with the presence of genital malformations as cryptorchidism, hypospadia and decreased anogenital distance (Boisen et al., 2001; Gray Jr. et al., 2000; Skakkebaek et al., 2001; Swann et al., 2005), decreased sperm quality and increased testicular cancer incidence (Landrigan et al., 2003; Olea Serrano and Zuluaga Gómez, 2001; Paulozzi, 1999). Skakkebaek et al. (2001) described the testicular dysgenesis syndrome (TDS), characterized by the presence of testicular cancer, hypospadia, cryptorchidism and low semen quality as a consequence of exposure to environmental contaminants. The reduction of the anogenital, distance parameter used as biomarker of reproductive effects, has been related with, higher levels of phthalates in urinary excretion and in the amniotic fluid in exposed gestational mothers (Huang et al., 2009; Swann et al., 2005). The decrease in sperm count in human, would be related with gonadal atrophy attributable to chronic exposure from fetal development to xenoestrogens (Sharpe et al., 1993; Toppari et al., 1996), similar to that produced by dietary 17 β-estradiol exposure (10 and 50 ppm) in adult rats (Cook et al., 1998).

In females the early contact with EDs during development has been related to an acceleration of pubertal development, polycystic ovary syndrome and cystic endometrial hyperplasia, endometriosis, uterine fibroids, premature, thelarche, early menarche, irregular menstrual cycle and a higher risk regarding breast and cervical cancer (Crain et al., 2008). It is well known the high incidence of ovarian, breast and vaginal carcinoma observed in daughters born form women treated with diethylstilbestrol (DES) during pregnancy (Blatt et al., 2003; Titus-Ernstoff et al., 2010). Direct exposure to EDs during woman reproductive stage was related to implantation failure, miscarriages and premature birth (Crain et al., 2008).

1.3. Neuroendocrine control of sexual maturation and reproduction. Possible interference by EDs

During the last two decades, an earlier onset of puberty was described (Herman-Giddens et al., 1997; Lee et al., 2001). It was not related to improvement in health and nutritional status, because they not always are accompanied by an increase of fat mass and leptin (Aksglaede et al., 2009). Thus, other factors including EDs could be involved in this early puberty (Teilmann et al., 2002). Puberty is a phenomenon that is initiated and regulated at neuroendocrine level. The control of the reproductive neuroendocrine regulation involves neurons in the basal hypothalamus that synthesize and release the decapeptide GnRH, which drives reproduction throughout the life cycle and also is the primary stimulus to the pituitary and gonadal axis. It is known that sexual hormones regulate the

reproductive axis acting on the peripheral organs, but they also change hypothalamic GnRH and pituitary gonadotropin secretion (Herbison, 1998). In males, estrogen participates in the negative feedback mechanism at hypothalamic level (Rochira et al., 2006), repressing GnRH promoter in vitro at 1 nM concentration (Roy et al., 1999). In females, estrogens are involved in the maturity of the positive feedback mechanism and in ovulation (Herbison, 1998). On the other hand, testosterone produces a tonic inhibitory feedback on neuroendocrine system in males (Mooradian et al., 1987). It is important to underline that GnRH neurons could be directly involved in responding to estrogens (Matagne et al., 2003) from early development stage, when 17 β estradiol (100 nM) stimulates the neurogenesis of precursors to GnRH neurons in the olfatory placode (Agça et al., 2008). In adulthood these estrogenic effects may become by indirect mechanism, involving other estrogen responsive cells. In immature female rats 17 β estradiol (10^{-7} M) increases the frequency of pulsatile GnRH secretion in hypothalamic explants. Similarly, early postnatal exposure of female rats to estradiol or to the insecticide DDT (dichlorodiphenyltrichloroethane), substance with estrogenic action, results in early developmental acceleration of the GnRH secretion in vitro (estradiol 10^{-9} M and DDT10^{-6} M) (Raiser et al., 2007). Neuroendocrine control of reproduction and its alteration following exposure to sex steroids during fetal or perinatal life has been described (Gorski, 1968). Prenatal exposure to testosterone propionate (100 mg twice per week from day 30 to day 90 of pregnancy) caused alteration of pubertal timing and estrous cyclicity in ewes through neuroendocrine alteration of estradiol positive feedback (Unsworth et al., 2005).

In vivo and in vitro assays have shown EDs estrogenic activity. For example, the UV filters 4-methylbenzylidene Camphor (4-MBC) and octyl methoxycinnamate (OMC) increased proliferation in breast cancer cells lines (concentration values between 1.56–3.73 μM) and in uterine weight when they were administered to rats (4-MBC 309 mg/kg/day and OMC 935 mg/kg/day in chow) (Schlumpf et al., 2001). Also, 4-MBC (35.3 μM in vitro) was able to bind to the estrogen receptor alpha (Schlumpf et al., 2004). Therefore, the probable action of an endocrine disruptor with estrogenic/antiandrogenic effect would alter normal neuroendocrine pathway causing an abnormal development and function of reproductive axis in both sexes.

Neuroendocrine sexual differentiation of the reproductive system occurs during the last gestational stage and at early neonatal period (Vermeulen, 1993). Estrogens or estrogen mimetic substances act more efficiently when they are administered during gestation (Gray and Kelce, 1996). On the other hand, certain EDs might interfere in the regulation of the reproductive axis altering the gonadotropin pituitary secretion (Carou et al., 2008, 2009a, 2009b; Furuta et al., 2006). Estrogens regulate the neuroendocrine activity of the reproductive axis through receptors like the beta type estrogen expressed in GnRH hypothalamic neurons (Hrabovszky et al., 2001). Also, it is well known that at hypothalamic level, one mediator of the estrogenic action on GnRH neurons is the excitatory and inhibitory amino acids system (Donoso et al., 1990; Brann and Mahesh, 1992, 1994; Jarry et al., 1992; Gore and Roberts, 1994; Moguilevsky and Wuttke, 2001). We have demonstrated that some EDs with estrogenic, antiestrogenic and/or antiandrogenic actions on the hypothalamic-pituitary-gonadal axis (di-2-ethyl hexyl phtalate (DEHP) 30 mg/kg/day in drinking water, octyl methoxycinnamate (OMC) in vitro at 10^{-7} M and 4 methoxybenzyllidene camphor (4-MBC) 100 mg/kg/day sc.), were able to induce neuroendocrine changes modifying hypothalamic concentrations of aspartate, glutamate and gamma-aminobutyric acid (GABA) in rats exposed during gestation and lactation (Carbone et al., 2009a; Carou et al., 2008, 2009b; Szwarcfarb et al., 2008). Another indirect regulatory pathway involved in these neuroendocrine effects, could be persistent alterations of hypothalamic KiSS-1 system observed after exposure to estrogenic compounds like Bisphenol A (10 and 100 μg/rat) at critical periods of brain sex differentiation (Navarro et al., 2009).

Some evidence of the neuroendocrine reproductive effects of EDs in humans is the early hypothalamic-pituitary maturation and central precocious puberty associated with previous exposure to EDs (Krstevska-Konstantinova et al., 2001; Teilmann et al., 2007). In conclusion, EDs can interfere with steroid hormone actions altering the control of GnRH neurons, by indirect neurotransmitter system afferents (Dickerson and Gore, 2007; Gore, 2008).

2. Endocrine alterations induced UV–B filters

The use of sunscreens with UV-filters to protect the skin is increasing worldwide despite experimental animal and in vitro studies have shown that some UV-filters have adverse effects and act as EDs. The most common active ingredients used in sunscreens are 4-MBC and OMC, which at present are considered EDs (Schlumpf et al., 2001). The commercial forms of these substances are lotions or creams containing 4% of 4-MBC or OMC (Schauer et al. 2006). It has been demonstrated that the UV filters 4.MBC and OMC are absorbed through the skin. A toxicokinetic study showed that topical application of sunscreen formulation at 4% 4-MBC (w/w) resulting in a dermal dose of 22 mg/kg bw, was capable of increasing plasma concentrations of 4-MBC and its metabolites 6 h after application in humans. The same was observed in rats treated with this substance at doses of 400 and 2000 mg/kg, suggesting more intensive biotransformation of 4-MBC in rats as compared to humans after dermal application and a poor absorption of 4-MBC through human skin (Schauer et al., 2006). In other studies, 4-MBC and OMC could be detected in human plasma and urine after 1–2 h following daily whole-body application (2 mg/cm^2) of sunscreen formulation at 10% (w/w) for 4 days and for 2 weeks, being urine and plasma concentrations higher in male than in female (Janjua et al., 2008; Janjua et al., 2004) and indicating a gender difference in the metabolism distribution and possibly also in the accumulation of UV-filters in adipose tissue.

On the other hand, UV filters OMC and 4-MBC were found in more than 96% of 2517 urine samples collected throughout 1-year from the general US population in the NHANES study (Calafat et al. 2008). These UV filters can get into the body fluids by either drinking contaminated water or contaminated fish intake (Poiger et al. 2004). Also, 4-MBC and OMC are secreted by the mammary gland being present in 85% of Swiss human milk samples, so that breast-fed babies could be exposed to these substances (Schlumpf et al. 2010).

Few studies have focused on humans and have investigated the potential side effects of UV-filters, although an increasing number of experimental animal and in vitro studies indicated that some UV-filters have estrogenic action, causing endocrine and developmental adverse effects in immature female rodents (Krause et al. 2012).

2.1. 4-MBC

It was shown that 4-MBC (309 mg/kg/day in chow) produced an uterotrophic effect in rats and an increased proliferation in breast cancer cells (1.56–3.73 μM in vitro) (Schlumpf et al., 2001). Also, 4-MBC (35.3 μM in vitro) was able to bind to the estrogen receptor alpha (Schlumpf et al., 2004). These observations were confirmed by Tinwell et al., 2002, using 4-MBC 10 μM in a cell proliferation assay and doses of 500–800 mg/kg by oral gavage or 500–1000 mg/kg sc., in vivo studies. However, Maerkel et al., 2007 reported absence of the uterotrophic effect of 4-MBC when it was orally administered at doses of 7, 24 and 47 mg/kg/day. This fact

could be attributed to a decrease in the circulating levels of the 4-MBC metabolite which has a greater affinity for the alpha type estrogen receptor (Volkel et al., 2006), main mediator of the estrogenic effect on peripheral tissues (Harris et al., 2002) as well as to a lower affinity for the beta type estrogen (Volkel et al., 2006), mainly related to the neuroendocrine regulation of the reproductive axis (Klann et al., 2005; Mueller et al., 2003; Schlumpf et al., 2004). In addition, a very weak estrogenic effect of 4-MBC (57.5 and 250 mg via food), was demonstrated by Seidlová-Wuttke et al., 2006a, 2006b, in uterus and vagina of rodents. This estrogenic action of 4-MBC might interfere with the normal development of puberty and disturb the reproductive function, as it is described below.

Developmental toxicity of the UV filter 4-MBC was studied in rats born to parents exposed to this substance in food before mating, during pregnancy and lactation and also in the offspring exposed to 4-MBC into adulthood. It was observed that 4-MBC at the lowest observed adverse effect level (LOEAL) of 0.7 mg/kg/day, delayed male puberty and affected reproductive organ weights of adult offsprings, enhancing prostate growth (Schlumpf et al., 2008). We have demonstrated that exposure to low doses of 4-MBC (2 and 10 mg/kg during five days) can produce a decrease in LH and FSH serum and in GnRH hypothalamic release, indicating that low doses of 4-MBC inhibit the reproductive axis in adult male rats (Carou et al., 2008). Since it has been observed that 4-MBC (100 μM) is capable of generating an increase in the quantity of mRNA ER, similar to the one caused by 1 nmol/l of estradiol (Klann et al., 2005), this UV filter would facilitate the negative feedback mechanism on the reproductive axis. This fact could explain the decrease in gonadotropins observed by Carou et al. (2008) in male rats exposed to 4-MBC. In addition, the disruptive estrogenic action of 4-MBC would have its starting point at neuroendocrine regulation level and would be caused by a selective action of 4-MBC (at doses more than 1 μM) on the beta type estrogen receptors (Mueller et al., 2003; Schlumpf et al., 2004), which are expressed in the GnRH neurons (Hrabovszky et al., 2001). Taking into account that the beta estrogen receptors have an important role in the neuroendocrine regulation of the reproductive axis, 4-MBC would behave as a partial agonist of alpha and beta estrogenic receptors; but its action on the gonadal axis would be exerted mainly on the beta type receptors (Mueller et al., 2003; Schlumpf et al., 2004).

In adult rats of both sexes exposed to 4-MBC (100 mg/kg sc.,daily) from prenatal development, we have described that the 4-MBC may modify the neuroendocrine regulation of the gonadal axis, inhibiting serum GnRH hypothalamic release in males, and stimulating gonadotropin secretion in females. These changes were correlated with an important inhibitory effect on the release of the aspartate and glutamate neurotransmitters as well as a decrease in the GABA inhibitory amino acid. Therefore, these modifications in the neurotransmitter systems induced by prenatal exposure to 4-MBC would be responsible for the different effects observed in males compared to females (Carou et al., 2009b). In the same way, Maerkel et al., 2007 reported that the neuroendocrine disruptive action of 4-MBC (pre and postnatal to adulthood at doses of 7, 24 and 47 mg/kg) in male and female rats, would cause an effect consistent with a sexually dimorphic gene regulation in brain. Sexual maturation could be affected by exposure to 4-MBC during embryonic and fetal development as it was shown by Carou et al. (2009a). This UV filter administered at high doses of 100 and 500 mg/kg/day to pregnant rats was able to inhibit the testicular axis in male rats during the prepubertal stage and stimulate it during peripubertal one, producing a decrease in testicular weight, LH, GnRH and glutamate levels or an increase in serum gonadotropins and the hypothalamic aspartate concentration, respectively. These results would indicate that during embryonic and fetal development the hypothalamic sensitivity could be disturbed by 4-MBC due to its estrogenic effect, inducing changes in the expression of the beta estrogenic receptor and affecting the pattern of neuronal migration in critical periods of brain maturation (Dellovade et al., 2000). This 4-MBC (7 and 24 mg/kg bw/day) estrogenic effect on neuroendocrine brain structures can also be evidenced in female showing a reduced receptive behavior and an increased rejection behavior toward the male, accompanied by a reduction in progesterone receptor mRNA at the hypothalamus (Faass et al., 2009), that normally correlated with lordosis behavior (Ogawa et al., 1994).

Besides, the different responses of 4-MBC observed during sexual maturation, could be the result of qualitative and quantitative differences in the effect of neurotransmitters that regulate GnRH neurons. During the peripubertal stage, changes occur in the regulating mechanisms of the hypothalamic-pituitary-gonadal axis, which in turn originate changes from a prepubertal type regulation to an adult-type control mechanism. This change constitutes one of the main events involved in the pubertal development (Clarkson and Herbison, 2006; Moguilevsky and Wuttke, 2001). Excitatory amino acid glutamate and aspartate are known stimulators of the reproductive axis, during sexual maturation and in adult life; and in addition there is evidence of their involvement in the increase of the gonadotropins release at the onset of puberty (Moguilevsky et al., 1995; Losada et al., 1993. According to what was described above, prenatal exposure to 4-MBC sunscreen can alter neuroendocrine mechanisms involved in sexual maturation in an age, sex and dose dependent manner (see Table 2).

2.2. OMC

The estrogenic activity of OMC has been determined in vitro (1.56–3.73 μM) and in vivo (935 mg/kg/day to rats that received the chemicals for 4 days in powdered feed chow) studies (Schlumpf et al., 2001). In contrast, a very weak estrogenic effect in the uterus of adult rats was observed by chronic administration of OMC at lower and higher doses (57.5 or 275 mg) via pellet food (20.6 or 22.3 g) (Seidlová-Wuttke et al., 2006a). Effects of pre- and postnatal exposure to OMC (500, 750 or 1000 mg/kg/day) on the reproductive development of rat offspring were studied by Axelstad et al. (2011), finding reduced relative prostate and testis weights, and a dose-dependent decrease in testosterone levels in immature rats as well as reduced sperm counts and prostate weights in adult rats.

On the other hand, oral administration of OMC in high dose (250 mg per 20 g food) to ovariectomized adult female rats produced a mild stimulation on serum LH and in the uterine weight, suggesting that this stimulatory effect could be related to the existence of neurotransmitter-involving mechanisms exerted by OMC at hypothalamic level (Seidlová-Wuttke et al., 2006b).

In "in vitro" studies we observed changes in the release of hypothalamic GnRH, which correlated with changes in the release of excitatory and inhibitory neurotransmitters. OMC (10^{-7} M) significantly decreased GnRH release in normal male and female adult rats as well as in castrated rats with substitutive therapy. In addition, these changes correlated with a decrease in glutamate hypothalamic release and an increase in GABA inhibitory amino acid in males; while in females OMC decreased the excitatory amino acids aspartate and glutamate, but GABA was not modified. These results suggested that OMC acting in a sex-dependent manner could alter the relation between neurotransmitter-sexual hormones and GnRH in both sexes' adult rats (Carbone et al., 2010a).

The disruptive action of OMC was also observed in studies "in vitro" carried out with hypothalamic fragments of pre and peripubertal rats of both sexes. In these animals, OMC (10^{-7} M) caused a significant decrease in the release of GnRH, correlated with an increase in the GABA hypothalamic release in males, and a decrease of the excitatory amino acids aspartate and glutamate in females. These results suggested that during sexual maturation, OMC would

Table 2
Neuroendocrine changes induced by UV–B filters (4-MBC and OMC), DEHP and NPl in rats of both gender. (↑ increased; ↓ decreased; = not change).

4-MBC	Male	Adult	Prenatal, lactation, and adulthood	0.7 mg/kg/d, sc.	Delayed puberty	Schlumpf et al., 2008
	Male	Adult	Adulthood	2 and 10 mg/kg/d, sc., for 5 days	↓ LH; ↓ FSH ↓ GnRH	Carou et al., 2008
	Male	Adult	Prenatal, lactation, and adulthood	100 mg/kg/d, sc., daily	↓ LH; ↓ FSH ↓ GnRH;↓GABA ↓ Aspartate ↓ Glutamate	Carou et al., 2009b
	Male	Prepubertal	Prenatal	100 mg/kg/d, sc., daily	↓ LH no change Glutamate	Carou et al., 2009a
	Male	Prepubertal	Prenatal	500 mg/kg/d, sc., daily	↓ LH ↓ Glutamate	Carou et al., 2009a
	Male	Peripubertal	Prenatal	100 mg/kg/d, sc., daily	↑ LH no change Aspartate	Carou et al., 2009a
	Male	Peripubertal	Prenatal	500 mg/kg/d, sc., daily	↑ LH ↑ Aspartate	Carou et al., 2009a
	Female	Adult	Prenatal, lactation and adulthood	100 mg/kg/d, sc., daily	↑ LH; ↑ FSH no change GnRH ↑ Aspartate no change Glutamate ↓ GABA	Carou et al., 2009b
OMC	Male	Prepubertal	Pre and postnatal	500, 750 or 1000 mg/kg/d	↓Testosterone	Axelstad et al., 2011
	Male	Adult	Adulthood	In vitro 10^{-7} M	↓GnRH; ↑GABA ↓ Glutamate	Carbone et al., 2010a
	Male	Pre and peripubertal	Pre and peripubertal	In vitro 10^{-7} M	↓GnRH; ↑GABA no change Glutamate no change Aspartate	Szwarcfarb et al., 2008
	Female	Adult	Adulthood	In vitro 10^{-7} M	↓GnRH; =GABA ↓ Glutamate ↓ Aspartate	Carbone et al., 2010a
	Female	Adult	Adulthood	250 mg/20 g food	↓ Testosterone	Seidlová-Wuttke et al., 2006b
	Female	Pre and peripubertal	Pre and peripubertal	In vitro 10^{-7}M	↓GnRH; =GABA ↓ Glutamate no change Aspartate	Szwarcfarb et al., 2008
DEHP	Male	Prepubertal	Prenatal and neonatal	750 mg/kg/d, orally	↓ Testosterone	Parks et al., 2000
	Male	Postnatal day 3	Prenatal and neonatal	750 mg/kg/d, Orally	↓ Testosterone	Gray et al., 2000
	Male	Prepubertal	Prenatal	100 mg/kg/d, orally	↓ Testosterone ↓ LH	Akingbemi et al., 2001
	Male	Prepubertal	Postnatal days 21-48	200 mg/kg/d, orally	↑ LH	Akingbemi et al., 2001
	Male	Young adult	Postnatal days 68-89	200 mg/kg/d, orally	no change LH	Akingbemi et al., 2001
	Male	Adult	Postnatal days 21-120	200 mg/kg/d, orally	↑ LH ↓Testosterone	Akingbemi et al., 2004
	Male	Prepubertal	Prenatal and lactation	30 mg/kg/d, orally	↑ LH; ↑ FSH ↑ Aspartate no change Glutamate ↓ GABA	Carbone et al., 2012
	Male	Peripubertal	Prenatal and lactation	30 mg/kg/d, orally	no change LH; ↓ FSH ↓ Aspartate ↑ GABA	Carbone et al., 2010
	Female	Adult regulary cycling	Adulthood for 12 days	2 g/kg/d, orally,	Anovulatory cycles; ↓ LH ↓ Estradiol	Davis et al., 1994
	Female	Peri pubertal	Postnatal days 20-30	500 mg/kg/d, orally	↓ Progesterone ↓ Estradiol;↑ LH	Svechnikova et al., 2007
	Female	Prepubertal	Prenatal and lactation	30 mg/kg/day, orally	↑ Aspartate no change Glutamate ↑ GABA	Carbone et al., 2012
NP	Male	Young adult	Postnatal days 30-80	250 mg/kg/day, orally for 50 days	↓ Testosterone ↑ LH; ↑ FSH	Han et al., 2004
	Male	Adulthood	Postnatal days 30-80	250 mg/kg/day, orally for 50 days	↓ Testosterone ↑ LH	Gon and Han, 2006
	Male	Neonatal	Prenatal	25, 200, 750 ppm in diet	↓ Testosterone	Laurenzana et al., 2002
	Male	Young adult	Postnatal days 21-70	100 and 200 mg/kg/d, orally	↓ Testosterone ↑LH; ↑FSH; ↑GnRH	Ale et al., 2010
	Male	Prepubertal	Lactation	100 mg/kg/d, orally 200 mg/kg/d, orally	No change LH; ↑GnRH No change LH and GnRH	Samaniego et al., 2012
	Male	Peripubertal	Lactation	100 and 200 mg/kg/d, orally	↑ LH, ↑GnRH	Samaniego et al., 2012

Table 2 (*Continued*)

Male	Young adult	Lactation	100 mg/kg/d, orally 200 mg/kg/d, orally	No change LH; ↑GnRH ↑ LH, ↑GnRH	Samaniego et al., 2012
Male	Prepubertal	Prenatal	100 mg/kg/d, orally 200 mg/kg/d, orally	No change LH; ↑GnRH No change LH and GnRH	Samaniego et al., 2012
Male	Peripubertal	Prenatal	100 mg/kg/d, orally 200 mg/kg/d, orally	↑ LH, =GnRH No change LH and GnRH	Samaniego et al., 2012
Male	Young adult	Prenatal	100 and 200 mg/kg/d, orally	↑ LH, No change GnRH	Samaniego et al., 2012

exert an inhibitory effect on GnRH, which would be related to the action of this ED on the excitatory and inhibitory neurotransmitters in male and female rats (Szwarcfarb et al., 2008) (see Table 2).

3. Endocrine alterations induced by phtalates

Di-(2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate to convey flexibility and transparency to numerous plastic products made of polyvinyl chloride (PVC) (Latini et al., 2003). Humans are daily exposed to this chemical through ingestion, inhalation and by dermal contact (Calafat et al., 2004; Doull et al., 1999; Faouzi et al., 1999; Koch et al., 2006; Latini et al., 2003; Moore et al., 2001). DEHP is not covalently bound to the polymer and therefore, it can leach from plastic products into foods, beverages or directly into body fluids (Lovekamp-Swan and Davis, 2003; Moore et al., 2001). Moreover, also occupational and medical exposure (e.g., tubing, blood bags and dialysis equipment) increase body burden levels, reaching much higher concentrations in this population. Pre and postnatal oral exposure to DEHP may be associated in animals with male reproductive development and function alteration. DEHP in rats is rapidly hydrolyzed in the gut to mono-(2-ethylhexyl) phthalate (MEHP), which pass into breast milk and cross the placental barrier (Latini et al., 2003; Stroheker et al., 2005).

It was shown that the exposure to DEHP during early development can cause birth defects in male reproductive tract. For example, DEHP administered by gavage during gestation and lactation at doses of 405 mg/kg/day (high dose) produced nipple retention, reduced anogenital distance and caused histological changes in the testis, indicating that this chemical could act as an anti-androgen at high dose exposure (Andrade et al., 2006). Also, testicular effects in males rats exposed to DEHP (300 and 750 mg/kg/day by gavage) were observed at different stages of sexual maturation (Borch et al., 2005). In the same way, Parks et al. (2000) reported that DEHP maternal exposure to DEHP (750 mg/kg/day by gavage) induced testicular malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat offspring. Pre and postnatal exposure to DEHP (100 mg/kg/day/by oral gavage) or prepubertal and adult rat exposure (100 and 200 mg/kg/day) was able to induce alterations in Leydig cell development, that may occur through modulation of testosterone- biosynthetic enzyme activity and serum LH levels (Akingbemi et al., 2001). Male offspring exposed to DEHP (0, 20, 100 and 500 mg/kg per day by gavages) in uterus and during lactation, showed reduced absolute and relative weights of androgen-dependent tissue organs (ventral prostate and seminal vesicle) and altered spermatogenic processes, demonstrating the ability of DEHP to disrupt the androgen-regulated development of the male reproductive tract at the highest dose level tested and indicating permanent effects of in uterus and lactational DEHP exposure (Dalsenter et al., 2006). Perinatal exposure to DEHP (750 mg/kg/day from gestational day (GD) 14 to postnatal day (PND) 3) induced a significant incidence of reproductive malformations in male pups such as reduced anogenital distance and testis weights (Gray et al., 2000). These alterations could be directly associated with epidemiological evidence indicating that boys born from women exposed to phthalates during pregnancy have an increased incidence of inborn genital malformations and spermatogenic dysfunction (Hu et al., 2009; Ge et al., 2007). Also, the presence of phthalate monoesters in human breast milk was positively correlated with cryptorchidism and LH increase, and negatively associated with free testosterone in infants three months of age. This fact could indicate that human Leydig cell development and function may also be vulnerable to perinatal exposure to some phthalates (Main et al., 2006).

It has been proposed that DEHP acts in an anti-androgenic manner which appears to result from an androgen receptor independent mechanism of anti-androgenicity. Although DEHP does not bind the androgen receptor but suppresses androgen-stimulated sexual differentiation, they are considered prototype antiandrogen. DEHP could act by interfering the esteroidogenesis (Akingbemi et al., 2001). Chronic postnatal exposure to DEHP (100 mg/kg/day by gavage from weaning to adulthood) was capable of inducing an increase in serum LH and estradiol biosynthesis in Leydig cells, a decrease in testosterone biosynthesis and Leydig cell hyperplasia, suggesting that the decrease in T biosynthesis together with increased estradiol production could be due to LH induction of aromatase activity in Leydig cells (Akingbemi et al., 2004). In uterus exposure to DEHP (750 mg/kg/day) was able to produce a reduction in testosterone production, but neither DEHP nor its metabolite MEHP displayed affinity for the human androgen receptor at concentrations up to 10 μM in vitro, indicating that DEHP could disrupt male rat sexual differentiation by reducing testosterone in the fetal male rat during a critical stage of reproductive tract differentiation (Parks et al., 2000).

Others mechanisms, as reduction in the expression of steroid genesis related factors and in nuclear receptors that regulate cholesterol transport, could explain the suppressive effect of DEHP on testosterone levels. In male fetuses of pregnant rats exposed to DEHP (100 ad 300 mg/kg/day by gavages), Borch et al. (2006) have found a reduction in testicular testosterone production that it was correlated with reduced mRNA expression of the steroidogenesis related factors, the nuclear receptors SF-1 and PPAR gamma, which regulate certain steps in steroid synthesis, and the cryptorchidism-associated Insl-3. Howdeshell et al. (2007) have demonstrated cumulative effects on reproductive tract of male fetuses exposed to phthalates (500 m/kg/day) and alterations in fetal steroid hormones and genes. Wilson et al. (2004) have reported that exposure to phthalates (750 mg/kg/day during gestational day14–18) caused decrease in mRNA expression of key steroidogenic enzymes and also the peptide hormone insulin-like peptide 3 (insl-3) from the fetal Leydig cells.

In adult female rats, DEHP (2 g/kg by gavages during 12 days) produced hypoestrogenic anovulatory cycles and polycystic ovaries (Davis et al., 1994). The reproductive toxicity of DEHP was attributed to suppression of ovarian granulosa cell estradiol production by its metabolite MEHP (mono-(2-ethylhexyl) phthalate), which was also capable of suppressing aromatase transcript levels in cultured rat granulosa cells at 200 μM concentration (Lovekamp and Davis, 2001). Pre and perinatal exposure to DEHP (405 mg/kg/day) has been associated with an increase in the number of ovarian atretic tertiary follicles in adult female offspring

rats (Grande et al., 2007). Also, in vitro assays demonstrated that DEHP (1–100 μg/ml) and the metabolite MEHP (0.1–10 μg/ml) may directly inhibit antral follicle growth in adult mice, via a mechanism that includes reduction in levels of estradiol production and decreased expression of cell cycle regulators (Gupta et al., 2010). It has been reported that on the pituitary–gonadal axis of prepubertal female rats DEHP (500 mg/kg/day by oral gavages for 10 days) exerted dual effects stimulating the hormonal function of the pituitary and, at the same time, inhibiting steroidogenesis by the ovarian granulose cells (Svechnikova et al., 2007).

Few studies of reproductive neuroendocrine effects of phthalates are available in the literature. We have studied the effect of pre and perinatal exposure to DEHP on the neuroendocrine parameters that regulate reproduction in prepubertal male and female rats. DEHP at low doses of 3 and 30 mg/kg/day was administered orally in the drinking water to dam rats since pregnancy onset until the moment of pups sacrifice at 15 days of age. No changes in gonadotropin levels and amino acid neurotransmitters were detected at the low dose in both sexes. However, 30 mg/kg/day of DEHP produced a significant decrease in the hypothalamic inhibitory neurotransmitter GABA and an increase in the stimulatory neurotransmitter aspartate in prepubertal male offspring rats, accompanied by gonadotropin serum levels increase. Therefore, we detected different effects by sex on hypothalamic–pituitary axis of prepubertal offspring rats produced by in uterus and lactational exposure to DEHP. The impact of this chemical at hypothalamic level could have very important consequences on the onset of puberty and on the reproductive function in adult life (Carbone et al., 2012).

In other work (Carbone et al., 2010), we investigated the effect of the pre- and perinatal exposure to DEHP on the hypothalamic-pituitary-gonadal axis in peripubertal male rats. We observed that DEHP (30 mg/kg bw/day) was capable to reduce testis weight and serum FSH, in correlation with a significant increase in the inhibitory GABAergic tone and a reduction in the stimulatory effect of aspartate on gonadotropin levels.

Our results showed that exposition to DEHP has different effect depending of stage of sexual maturation, time exposition and sex. This fact could be related with quantitative and qualitative changes in the regulation of the reproductive axis during sexual maturation, previously described (Moguilevsky and Wutke, 2001; Moguilevsky et al., 1991, 1995; Losada et al., 1993; Szwarfarb et al., 1994).

On these bases, the phthalate DEHP could modify the neuroendocrine regulation of the hypothalamic–pituitary axis in immature rats offspring from dams exposed to DEHP at dose of 30 mg/kg bw/day during gestational and lactational periods. Moreover this effect would be dependent on sex and period of sexual maturation to be evaluated. These findings would indicate a possible action to DEHP at central level, in addition to direct effect on gonads evidenced by the presence of testicular and ovarian histological alterations that were already mentioned (see Table 2).

4. Endocrine alterations induced by nonylphenol

Nonylphenol (NP) is an alkylphenol, industrial intermediary in the production of non ionic detergents, latex paint, adhesives and plastics as the polystyrene, polymer stabilizers to package food, herbicides and pesticides, and also antioxidant agents and lubricant additives (Shaw and McCully, 2002; Inoue et al., 2001; Soto et al., 1991). Some decades ago, the presence of NP in the environment was first reported (Ginger et al., 1984). Sixty percent (60%) of these alkylphenols end finally in environmental waters (Daughton and Ternes, 1999; Heberer et al., 2002; Petrovic et al., 2003; Snyder et al., 2003; Naylor et al., 1992), and also in treated potable water (Servos et al., 2007; Stackelberg et al., 2007; Yu et al., 2007). In humans it can be observed that the most direct contact with NP is through food (Inoue et al., 2001). NP is considered an ED that has chemical and physical properties very similar to natural estrogens and has the possibility to link the estrogenic receptors. NP (50 mg/kg) administered to ovariectomized adult female rats treated with estradiol (15 ng at 12, 13 and 14 days after ovariectomy) was capable to increase the mitotic activity in rat endometrium (Soto et al., 1991). Also in "in vitro" assays this chemical (10^{-6} M) induced cellular proliferation and expression of the progesterone receptor in human breast tumor cells sensitive to estrogens (Soto et al., 1991). In male fish, the exposure to NP (10 μg/l) caused vitellogenin expression, a protein associated with the reproduction in female fish (Schawaiger et al., 2002; Sumpter and Jobling, 1995) as well as inhibition of spermatogenesis (at in vitro concentration of 10 μM) and the appearance of intersexes (Schawaiger et al., 2002). In rodent models exposed to NP (250 mg/kg) it has been described a decrease in the testicular size and sperm production, as well as an increase of the intertubular space and low seminal quality (Jager et al., 1999; Lee et al., 1999). Recently we have demonstrated that exposure to NP (50 and 100 mg/kg by gavages) in early stages of sexual maturation, led to a histological disorganization of the epithelium seminiferus in rat testis and changes in the neuroendocrine regulation of the reproductive system (data unpublished). Coincidentally immature rats exposed neonatally to NP (20.8 mg/kg) showed a reduction in testis, epididymis and seminal vesicles size, as well as an increase of cryptorchidism up to a 60% (Lee, 1998). Similar results were found by Nagao et al. (2001), using NP at dose of 50 mg/kg. The testicular and epididymis atrophy also occur in a dose-dependent manner in adult male rats exposed to NP (50, 100 or 200 mg/kg by gavages) during gestational and lactational periods (Fan et al., 2001). These changes are similar those observed in adult male rats treated neonatally with 0.1, 1 or 10 μg sc. of diethylstilbestrol, an ED with estrogenic action (Atanassova et al., 1999). In addition, Han et al. (2004) have shown a significant decrease in epididymis weight, sperm density and testosterone level, as well as histopathological changes in the seminiferus tubulus of rats treated with NP (250 mg/kg/day by gavage for 50 days).

Decreased testicular and epididymal masses in rats exposed to NP (250 mg/kg/day) in utero until 10 weeks of age were reported by Jager et al. (1999), suggesting an adverse effect of this ED on the fertility potential of male rats after gestational, lactational and direct exposure.

In humans the infertility male factor is commonly observed in the idiopathic oligoasthenotetarospermia (IOAT) syndrome, being possible that estrogenic substances as NP are related with its physiopathology. Multiplication of Sertoli cells, controlled by FSH, occurs during fetal, neonatal and prepubertal life. The inhibition of FSH secretion reduces the multiplication of these cells. It is important to have in mind that in neonates FSH secretion is quite sensitive to the inhibition of exogenous estrogens (Sharpe and Skakkebaek, 1993). Therefore, the NP estrogenic activity could explain this effect. On the other hand, in female offspring's of female rats exposed to NP (12.31 mg/kg/day sc., during pregnancy), an increase of ovary weight has been observed (Kimura et al., 2006) without significant changes in uterine weight. However, when this particular ED at dose of 100 mg/kg/day sc. was directly administered to immature rats, uterine weight, diameter of uterine duct and vaginal luminal epithelial height were significantly increased (Kang et al., 2000).

The effect of NP on the regulating mechanisms of the reproductive system has not been studied so thoroughly as the morphometric parameters. However, it has been described an abrupt decrease in the testosterone level with NP (250 mg/kg/day by gavages) administered during adulthood (Gong and Han, 2006) and also in animals treated with dietary doses of NP (0, 25, 200 and 750 ppm) from fetal development until adulthood (Laurenzana

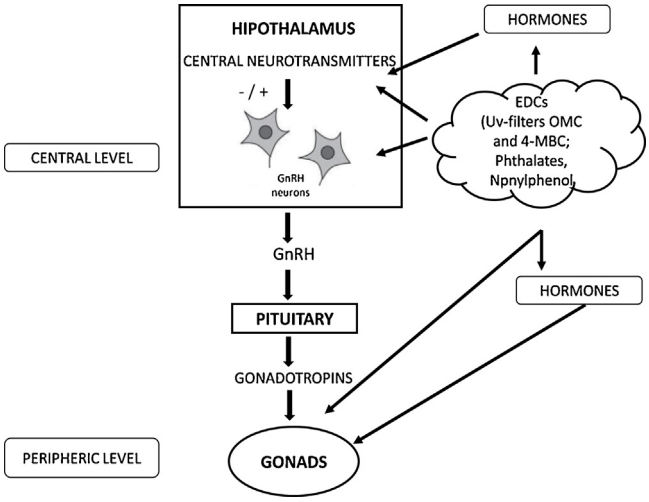

Fig. 1. Schematic description of how hypothalamic neuroendocrine systems are targets of environmental endocrine disruptors. In the brain, hypothalamic neuroendocrine cells are regulated by neurotransmitter systems and by both direct and indirect hormone actions. The hypothalamic gonadotropin – releasing factor (GnRH) is secreted and released from GnRH neurons to the pituitary gland, regulating the secretion of gonadotropins which exert their effect on gonads. Environmental endocrine-disrupting chemicals (EDCs) may mimic or block some of these hormonal effects, disrupting at central or peripheral levels the neuroendocrine processes involved in the reproductive function.

et al., 2002). Recently we have reported that chronic exposure to NP (100 and 200 mg/kg/d by gavages) since weaning to adulthood, in addition to cause an important decrease of plasmatic testosterone, induces a significant increase in the hypothalamic release of GnRH and pituitary gonadotropins (Ale et al., 2010). This exposure to NP would lead to a decrease of the inhibitory control on the neuroendocrine regulation. Similarly, 30 and 70 days old male rats exposed to the same doses of NP during lactation, showed an increase in GnRH and gonadotropins secretion (Samaniego et al., 2012).

In ovariectomized rats treated with NP (10 mg/kg sc.) a significant decrease in plasmatic LH level was observed (Furuta et al., 2006). However, chronic administration of NP (100 and 200 mg/kg/day, from weaning to adulthood) stimulated GnRH and LH release "ex vivo" in non ovariectomized rats in diestrum phase. Also this exposure was associated with an early vaginal opening (Ale et al., 2012).

Administration of NP (50 mg/kg, orally) in the last stage of gestation produced a concentration of NP in the fetal serum within a range from 30 to 40% of the observed in maternal plasma. In these animals, NP was present in the central nervous system (Doerge et al., 2002), suggesting that NP goes through the placenta, and accumulates in the brain. This could cause alterations in the hypothalamic regulation of the excitatory/inhibitory amino acids–GnRH–gonadotropins of the reproductive system as we have already described by others EDs (see Table 2).

5. Conclusion

In conclusion, the exposure to different substances with endocrine disruptive action could produce severe alterations, mainly in the reproductive axis. Recent studies demonstrate that the three reproductive levels are also responsive to environmental EDs. Recently findings in experimental animals have shown new evidences of reproductive disruption associated with neuroendocrine changes induced by UV–B filters, phtalates and nonylphenol during sexual maturation in rats of both gender. The adverse effects of these compounds could be exercised in a sex, age and exposure period dependent manner, on the white organs of the reproductive system and/or at central level, modifying the regulatory neuroendocrine mechanisms (see Fig. 1). One of the neuroendocrine alterations could be the changes in the amino acid neurotransmitters system described by us, as well as the modifications in others neurotransmitters which have not been studied yet. If the exposure to these substances occurs during critical periods of development, such as intrauterine, early neonatal and pubertal stages, when the neuroendocrine mechanisms are very sensitive to changes in the action of estrogenic and androgenic hormones, the impact caused can be even greater and permanent. Given this background, future study should explore other neurotransmitter systems and neuropeptides that could be involved in the reproductive system changes induced by EDs.

Also, considering that neuroendocrine systems do not work in isolation, a possible cross talk with the effects of these EDs on other neuroendocrine axes, which could interfere with the normal functioning of the reproductive axis, should be revised in the future..

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In vitro effect of octyl - methoxycinnamate (OMC) on the release of Gn-RH and amino acid neurotransmitters by hypothalamus of adult rats.

Carbone S[1], Szwarcfarb B, Reynoso R, Ponzo OJ, Cardoso N, Ale E, Moguilevsky JA, Scacchi P.

Author information

Abstract

OMC (octyl-methoxycinnamate), an endocrine disruptor having estrogenic activity, is used in sunscreen creams as UV filter. We studied its "in vitro" effects on the hypothalamic release of Gn-RH as well as on the amino acid neurotransmitter system. OMC significantly decreased Gn-RH release in normal male and female rats as well as in castrated rats with substitutive therapy. No effects were observed in castrated rats without substitutive therapy. In males OMC increases the release of GABA, decreasing the production of glutamate (GLU) while in the female decreases the excitatory amino acid aspartate (ASP) and GLU without modifications in the hypothalamic GABA release. These results suggest that OMC acting as endocrine disruptor could alter the sex hormone-neurotransmitter-Gn-RH axis relationships in adult rats.

Exp Clin Endocrinol Diabetes. 2008 Feb;116(2):94-8. doi: 10.1055/s-2007-1004589.

Octyl-methoxycinnamate (OMC), an ultraviolet (UV) filter, alters LHRH and amino acid neurotransmitters release from hypothalamus of immature rats.

Szwarcfarb B[1], Carbone S, Reynoso R, Bollero G, Ponzo O, Moguilevsky J, Scacchi P.

Author information

Abstract

OMC (octyl-methoxycinnamate), is an endocrine disruptor with estrogenic activity, which is used in sunscreen creams as a UV filter. We studied its " IN VITRO" effects on the hypothalamic release of LHRH as well as on the amino acid neurotransmitter system in immature rats of 15 (prepubertal) and 30 (peripubertal) days of age. OMC decreased the LH-RH release significantly in male and female rats of both age. In male rats OMC increased the release of GABA while in the female ones It diminished the excitatory amino acid aspartate (ASP) and Glutamate (GLU) without modifications in the hypothalamic GABA release. These results suggest that during sexual maturation the inhibitory effect of OMC on LH-RH release appears to be related to its action on the inhibitory and excitatory amino acid neurotransmitters in male and female rats.

Toxicol Appl Pharmacol. 2006 Feb 1;210(3):246-54. Epub 2005 Jun 27.

Comparison of effects of estradiol (E2) with those of octylmethoxycinnamate (OMC) and 4-methylbenzylidene camphor (4MBC)--2 filters of UV light - on several uterine, vaginal and bone parameters.

Seidlová-Wuttke D1, Jarry H, Christoffel J, Rimoldi G, Wuttke W.

Abstract
OMC and 4MBC are 2 absorbers of ultraviolet light which are used in unknown quantities in sunscreens, cosmetics and plastic products to protect against UV light-induced damage of the skin or of fragrances or plastic material. From there, they were shown to reach surface water and/or by direct contamination or ingestion the human. Under various conditions in mice and rats, both substances were shown to be estrogenic. Therefore, we compared in vitro and in vivo the effects of chronic application of these compounds at 2 doses with those of E2, all administered via food. No signs of toxicity were observed under application of 0.6 mg E2, 57.5 or 275 mg of OMC, 57.5 or 250 mg of 4MBC; these amounts were ingested with 21 g of control food, 17.8 g E2 food, 20.6 g or 22.3 g OMC food and 23.7 or 22.8 g 4MBC food. In the uterus, vagina and bone, E2 exerted the expected stimulatory effects which were minimally shared by OMC and 4MBC in the uterus and vagina as assessed by histology and determination of a variety of estrogen-regulated genes such as insulin-like growth factor-1, progesterone receptor and estrogen receptor beta. In the bone, OMC had no effect, while 4MBC shared the antiosteoporotic effects of E2 as measured by quantitative computer tomography in the metaphysis of the tibia. The mechanism of action of 4MBC, however, appears to be different as E2 reduced serum osteocalcin and the C-terminal breakdown products of collagen-1alpha1 which were both increased by 4MBC. Taken together, these data indicate a very weak estrogenic effect of OMC and 4MBC in the uterus and in the vagina but not in the bone where 4MBC exerted antiosteoporotic effects by a different mechanism than E2.

Toxicol Appl Pharmacol. 2006 Jul 1;214(1):1-7. Epub 2005 Dec 20.
Comparison of effects of estradiol with those of octylmethoxycinnamate and 4-methylbenzylidene camphor on fat tissue, lipids and pituitary hormones.

Seidlová-Wuttke D1, Christoffel J, Rimoldi G, Jarry H, Wuttke W.
Author information
Abstract
Octylmethoxycinnamate (OMC) and 4-methylbenzylidene camphor (4MBC) are commercially used absorbers of ultraviolet (UV) light. In rats, they were shown to exert endocrine disrupting including uterotrophic, i.e. estrogenic effects. Estrogens have also metabolic effects, therefore the impact of oral application of the two UV absorbers at 2 doses for 3 months on lipids and hormones were compared with those of estradiol-17beta (E2). E2, OMC and 4MBC reduced weight gain, the size of fat depots and serum leptin, a lipocyte-derived hormone, when compared to the ovariectomized control animals. Serum triglycerides were also reduced by the UV screens but not by E2. On the other hand, E2 and OMC reduced serum cholesterol, low density lipoproteins and high density lipoproteins; this effect was not shared by 4MBC. While E2 inhibited, OMC and 4MBC stimulated serum LH levels. In the uterus, both UV filters had mild stimulatory effects. 4MBC inhibited serum T4 resulting in increased serum TSH levels. It is concluded that OMC and 4MBC have effects on several metabolic parameters such as fat and lipid homeostasis as well as on thyroid hormone production. Many of these effects are not shared by E2. Hence, other than estrogen-receptive mechanisms may be responsible for these effects.

Toxicology. 2004 Dec 1;205(1-2):95-102.
Endocrine active compounds affect thyrotropin and thyroid hormone levels in serum as well as endpoints of thyroid hormone action in liver, heart and kidney.

Schmutzler C1, Hamann I, Hofmann PJ, Kovacs G, Stemmler L, Mentrup B, Schomburg L, Ambrugger P, Grüters A, Seidlova-Wuttke D, Jarry H, Wuttke W, Köhrle J.

Abstract
To assess interference with endocrine regulation of the thyroid axis, rats (female, ovariectomised) were treated for 12 weeks with the suspected endocrine active compounds (EAC) or endocrine disrupters (ED) 4-nonylphenol (NP), octyl-methoxycinnamate (OMC) and 4-methylbenzylidene-camphor (4-MBC) as well as 17beta-estradiol (E2) and 5alpha-androstane-3beta,17beta-diol (Adiol) on the background of a soy-free or soy-containing diet, and endpoints relevant for regulation via the thyroid axis were measured. Thyrotropin (TSH) and thyroid hormone (T4, T3) serum levels were altered, but not in a way consistent with known mechanisms of feedback regulation of the thyroid axis. In the liver, malic enzyme (ME) activity was significantly increased by E2 and Adiol, slightly by OMC and MBC and decreased by soy, whereas type I 5'-deiodinase (5'DI) was decreased by all treatments. This may be due rather to the estrogenic effect of the ED, as there is no obvious correlation with T4 or T3 serum levels. None of the substances inhibited thyroid peroxidase (TPO) in vitro, except for NP. In general, several endocrine active compounds disrupt the endocrine feedback regulation of the thyroid axis. However, there was no uniform, obvious pattern in the effects of those ED tested, but each compound elicited its own spectrum of alterations, arguing for multiple targets of interference with the complex network of thyroid hormone action and metabolism.

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Octyl methoxycinnamate: Two generation reproduction toxicity in Wistar rats by dietary administration

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Abstract

Wistar rats continuously received octyl methoxycinnamate (OMC) in the diet through two successive generations at nominal doses of 0, 150, 450 or 1000 mg/kg bw/day. OMC had no adverse effects on estrous cycles, mating behavior, conception, parturition, lactation and weaning, sperm and follicle parameters, macropathology and histopathology of the sexual organs. 1000 mg/kg bw/day reduced parental food consumption and body weight (−14% to −16% in males, −4% to −5% females), increased liver weight, produced hepatic cytoplasmic eosinophilia and erosion/ulceration of glandular stomach mucosa. and led to a slightly decreased implantation rate in the top dose F0 and F1 dams. The high dose F1 and F2 pups had reduced lactation weight gain and organ weights and delayed sexual maturation landmarks. There was no evidence of a selective influence of the test compound on pups' sexual landmarks. The NOAEL (no observed adverse effect level) is 450 mg/kg bw/day for fertility and reproductive performance, for systemic parental and developmental toxicity.

Keywords: CAS 5466-77-3; Octyl methoxycinnamate; OMC; Two-generation toxicity study; Rat; Fertility; Development; Reproduction

1. Introduction

Octyl methoxycinnamate (=2-ethylhexyl-4-methoxycinnamate = *p*-methoxycinnamic acid 2-ethylhexyl ester = octinoxate = OMC; $C_{18}H_{26}O_3$, 290 g/mol; CAS# 5466-77-3, EINECS/ELINCS# 226-775-7) is an ultraviolet absorber marketed under the trade name Uvinul® MC 80 N. In addition to its use as a light stabilizer, OMC is an FDA Category 1 sunscreen, approved worldwide at concentrations up to 10%, and is the most frequently used sunscreening agent. At room temperature it is a colorless or slightly yellow liquid, freely soluble in organic solvents and oils, but insoluble in water (estimated log P_{ow} = 5.80).

In animal studies, OMC is acutely non-toxic by oral administration (rat oral LD50 > 5 g/kg), does not irritate the skin and mucous membranes, has no sensitizing effect (SCC, 1996) and is not mutagenic in the Ames test (Zeiger et al., 1985; BASF AG, unpublished data).

OMC has been reported to display no androgenic or antiandrogenic activity at androgen receptors in the human breast carcinoma cell line, MDA-kb2 at any tested concentration (1 nM–10 μM) (Ma et al., 2003). It has been reported, however, by Schlumpf et al., 2001, to stimulate in vitro proliferation of MCF-7 cells

Abbreviations: NOAEL, no observed adverse effect level; OMC, octyl methoxycinnamate; bw, body weight; ppm, parts per million of test substance in diet, by weight.

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(human estrogen-sensitive breast cancer cells) with an EC50 of 2.37 μM (although secretion of the estrogen-regulated protein pS2 in these cells was not significantly increased), and to have estrogenic activity in the uterotrophic assay in immature Long-Evans rats, with an ED50 of 935 mg/kg bodyweight (bw)/day and a NOEL of 522 mg/kg bw/day. However, no estrogenic activity of OMC has been found in the uterotrophic assay using immature Wistar rats at doses up to 1000 mg/kg bw/day (SCCNFP, 2001; BASF AG, unpublished data). In addition, OMC does not bind in vitro to estrogen receptors from the porcine uterus or to recombinant ERα and ERβ proteins and there are no indications of an antiandrogenic activity in the Hershberger assay in castrated male rats (BASF AG, unpublished data). These somewhat conflicting data on the potential estrogenic activity of OMC in screening systems suggest that at the most the compound has a very weak estrogenic potential at high dosages only and that the toxicological relevance for the reproductive cycle is therefore questionable.

There are no data on the potential effects of OMC on fertility and reproductive performance after continuous multigeneration exposure throughout development from gametogenesis to weaning and sexual maturity. This study was conducted to address this need, i.e. to determine the potential hazard of OMC concerning fertility and reproduction.

2. Materials and methods

2.1. Study design

This two-generation reproduction toxicity study was designed in accordance with OECD test guideline 416 (OECD, 2001), and was conducted in accordance with Good Laboratory Practice guidelines and applicable animal welfare legislation (OECD, 1981; German Chemicals Act, 1994).

2.2. Test animals

Male and female Wistar rats (CrlGlxBrlHan:WI), 27–29 days old on arrival, were supplied by Charles River, Germany. Males and females were from different litters, to exclude the possibility of sibling matings. All animals were free from clinical signs of disease, and the females were nulliparous and non-pregnant. These animals were the F0 generation parental animals. All other animals on study (F1 and F2 pups, F1 parental rats and F2 young adults) were bred from these animals.

Parental adults were uniquely identified with an ear tattoo identification number, and were housed individually (except during mating) in stainless steel wire-mesh cages (DK III, Becker & Co., Castrop-Rauxel, Germany) or, during cohabitation for mating and in females from day 18 of pregnancy until day 14 of lactation, in Makrolon type M III cages containing nesting material (certified cellulose wadding, supplied by Ssniff Spezialdiaeten GmbH, Soest, Germany). All live pups were identified by skin tattoo on day 1 post partum and with picric acid marking of the fur between day 10 and 15 post partum. The animal quarters were air-conditioned (20–24 °C, 30–70% relative humidity) with a 12 h light/12 h dark cycle; walls and floors were washed weekly. Certified feed (ground Kliba maintenance diet rat/mouse meal, Provimi Kliba SA, Kaiseraugst, Switzerland) was available *ad libitum* throughout the study, as was tap water (human drinking quality) in drinking bottles.

2.3. Treatment

After acclimatization to the housing facility for one week, groups of 25 male and 25 female rats were allocated randomly, stratified by weight, to one of four treatment groups, and received OMC (Uvinul MC 80 N, batch UV2-01.019, purity 99.9% by HPLC-UV) admixed to dry ground diet to produce nominal target doses of 0 (control), 150, 450 or 1000 mg/kg bw/day. Doses were based on a preceding 1-generation range finding study in rats dosed at 0, 1500, 4500 or 10,000 ppm in the diet (fixed concentrations) resulting in test substance uptakes of 180, 525 and 1190 mg/kg bw/day. In that study, 10,000 ppm reduced final male bodyweight by 10% (relative to controls), decreased female plasma urea, creatinine, total protein and albumin, increased cholesterol, and reduced ovary weight by 20%; pup weaning body weights were reduced 37% at 10,000 ppm and 10% at 4500 ppm; treatment had no effects on clinical observations, food consumption, fertility, reproductive performance, offspring viability and lactation indices, or parental and offspring gross necropsy findings (BASF, unpublished data). In the present study, dietary test substance concentrations were adjusted weekly in both sexes during the premating period, based on actual food consumption and body weight, and also weekly in males during the gestation, lactation and post-weaning periods. During mating in both sexes and during gestation and post-weaning periods in females, dietary test substance concentrations were kept the same as in the last week of the premating period. During lactation, dietary test substance concentrations in females were set to 50% of those used during the last week of the premating period, to compensate for the increased maternal food intake during this period. Post-weaning F1 and F2 pups were dosed on the basis of historical body weight and food consumption data for rats of similar ages. To achieve the final doses of 0, 150, 450 and 1000 mg/kg bw/day, the nominal dietary concentrations used ranged between 674 and 16,090 ppm; actual concentrations were confirmed by UV-HPLC to be 94.0–105.6% of nominal.

F0 parental animals were exposed to test substance throughout a premating period of at least 73 days (i.e. for more than one complete cycle of spermatogenesis in males, for several complete cycles of oogenesis in females), through cohabitation for mating (maximum 21 days), and then throughout gestation (about 21 days), parturition and lactation up to weaning of the F1 offspring (about 21 days), ending about 16 h before necropsy. From the F1 pups, 25 males and 25 females per dose group were selected as F1 parental animals, and dietary dosing continued for at least 74 days prior to cohabitation for mating, and then during mating, gestation and lactation up to weaning of the F2 offspring, ending about 16 h before necropsy. Dosing continued in F2 offspring until necropsy.

Parental animals were cohabitated overnight for mating (one male and one non-sibling same-dose female per cage) daily until positive mating (sperm in vaginal smear = day 0 post-coitum) or for a maximum of 2 weeks.

2.4. Observations

Parental animal health status and clinical signs were checked daily. Food and water consumption and body weights were measured weekly throughout the study, and in mated females during gestation on days 0, 7, 14 and 20 post-coitum, and in those with litters during lactation on days 1, 4, 7, 14 and (body weights only) 21 post partum. Food consumption was not measured from day 14 to 21 post partum since by this time pups also consume considerable amounts of solid food, and so maternal food consumption alone cannot be quantified during this time. Estrous cycle length and cytological normality were evaluated daily from vaginal smears for all F0 and F1 female parental rats for a minimum of 3 weeks prior to mating and throughout the mating period until positive mating.

All offspring (F1 and F2) were examined as soon as possible on the day of birth (day 0 post partum) to determine the number of liveborn and stillborn per litter; they were sexed based on anus to genital tubercle distance, and subsequently by anogenital distance and mammary line appearance, and at necropsy. Pup health status and clinical signs were checked at least once daily throughout lactation.

Litters (both F1 and F2) were randomly culled on day 4 post partum to 4 male and 4 female pups where possible. Pups were weighed on the day after birth (day 1 post partum) and on days 4, 7, 14 and 21 post partum. To quantify sexual maturation, female vaginal opening was assessed daily from day 27 post partum and male preputial separation daily from day 40 post partum in F1 offspring selected as F1 parental animals and in all surviving F2 offspring.

2.5. Pathology

F0 and F1 parental animals, as well as offspring not selected for mating, were necropsied at or after litter weaning on day 21 post partum. Parental animals were killed by decapitation under CO_2 anesthesia. Whole body, liver, kidneys, epididymides and testes weights, and major organ macropathology were recorded in all animals. Implantation sites were counted after uterine staining for about 5 min in 10% ammonium sulfide solution (Salewski, 1964). Histopathology of vagina, cervix uteri, uterus, ovaries, oviducts, left testis and epididymis (caput, corpus and cauda), seminal vesicles, coagulating gland, prostate gland, adrenal glands, brain, pituitary gland, spleen and kidneys was recorded in all control and high dose (1000 mg/kg bw/day) F0 and F1 parental animals, and in all low and mid-dose animals with suspected impaired fertility. Liver and spleen (males only) were examined in all groups. For gross lesions detected macroscopically and liver, histopathology was recorded in all animals from all groups. Testes, epididymides and ovaries were fixed in Bouin's solution, the other organs in formaldehyde solution. Standard stain was hematoxylin and eosin; to investigate observed brown pigmentation in male spleens, these were treated with Perls stain (for iron). Differential ovarian follicle counts were performed in control and high dose (1000 mg/kg bw/day) F1 females, follicles were quantified in serial sections of both ovaries as primordial (types 1, 2, 3a and b) or growing (types 4, 5a and b) according to Plowchalk et al. (1993). Sperm motility was microscopically quantified immediately after necropsy and organ weight determination in the right testis and cauda epididymis from all males (in randomized order) according to Slott et al. (1991). Mean number of homogenization-resistant testicular spermatids and caudal epididymal sperm, and percentages of morphologically abnormal sperm were quantified based on Feuston et al. (1989) in all control and high dose group males.

Pups culled on day 4 post partum and superfluous F1 and F2 pups at weaning were killed by CO_2 inhalation. Post-weaning F2 pups were sacrificed by cervical dislocation. All these pups, including stillborn and subsequent mortalities, were examined externally, eviscerated and their organs assessed macroscopically. At scheduled necropsy in F1 and F2 pups, brain, spleen and thymus were weighed in 1 pup/sex and litter; relative organ weights were calculated relative to in-life body weight on day 21 post partum.

2.6. Reproductive parameters

Reproductive performance of F0 and F1 parental animals was summarized by the following indices: male mating index (%): (number of males with confirmed

mating = vaginal sperm or pregnancy in cohabited female × 100)/number of males placed with females; male fertility index (%): (number of males proving their fertility = parturition or presence of embryos or fetuses in utero in cohabited female × 100)/number of males placed with females; female mating index (%): (number of females mated = vaginal sperm or pregnancy × 100)/number of females placed with males); female fertility index (%): (number of females pregnant = embryos or fetuses in utero or giving birth × 100)/number of females placed with males); gestation index (%): (number of females with liveborn pups × 100)/number of females pregnant; livebirth index (%): (number of liveborn pups × 100)/number of liveborn + stillborn pups; post-implantation loss (%): (number of implantations−number of pups delivered) × 100/number of implantations; viability index (%): (number of pups alive on day 4 post partum (preculling) × 100)/number of liveborn pups; lactation index (%): (number of pups alive on day 21 post partum × 100)/number of pups alive on day 4 post partum (postculling).

2.7. Statistical analysis

The experimental unit of analysis was the parental animal or litter, except for livebirth, viability and lacatation indices, which were analysed by dose group. Dunnett's test (Dunnett, 1955; Dunnett, 1964) was used for simultaneous comparison of all dose groups with the control group in food and water consumption (g/parental animal), body weights and body weight change (parental animals and litters), estrus cycle length, number of mating days to successful mating, duration of gestation, number of pups delivered per litter, and time to sexual maturation (days to vaginal opening or preputial separation). Kruskal–Wallis (2-sided) tests followed if significant by pairwise Wilcoxon tests (Hettmansperger, 1984; Nijenhuis and Wilf, 1978; Siegel, 1956). Wilcoxon tests were also used to assess group differences in terminal parental body weights and parental and pup absolute and relative organ weights, proportion of pups per litter with necropsy observations, differential follicle counts, total spermatids/g testis or cauda epididymides, and % sperm motility, for which variable Bonferoni–Holm adjustment (Holm, 1979) was applied. Fisher's Exact test of equal proportions (Siegel, 1956), one-sided, was used for pairwise comparison of each dose group with the control for male and female mating and fertility indices, gestation index, females with liveborn, stillborn and with all stillborn pups, livebirth index, pups stillborn, pups dead, pups cannibalized, pups sacrificed moribund, viability and lactation indices, number of litters containing pups with necropsy findings, sexual maturation data (vaginal opening or preputial separation), and males with >4% abnormal sperm.

3. Results

3.1. F0 parental observations

Calculated test substance intake in the 150, 450 and 1000 mg/kg bw/day groups for the premating phase was 153, 460 and 1015 mg/kg bw/day body weight/day in males (mean of weeks 0–17) and 156, 468 and 1039 mg/kg bw/day for females (mean of weeks 0–10). For females test substance intake was 152, 451 and 1025 mg/kg bw/day during gestation (mean of days 0–20), and 137, 413 and 867 mg/kg bw/day during lactation (mean of days 1–14).

Except for two 1000 mg/kg bw/day males with urine-smeared fur (week 7–17), there were no treatment-related clinical signs. One non-pregnant 150 mg/kg bw/day female was found dead in week 19 (the last week of treatment) with severe chronic progressive glomerulonephropathy; this death was not considered treatment-related.

Food consumption was reduced by up to 10% compared to controls in 1000 mg/kg bw/day males, starting in premating week 5. Treatment had less consistent effects on female food consumption, which was reduced at 1000 mg/kg bw/day during lactation days 4–14, and at 450 mg/kg bw/day during premating weeks 2–9 and gestation days 0–14).

Body weight was reduced at 1000 mg/kg bw/day in males, starting in premating week 3–4; mean terminal body weight was significantly lower than controls (−16%). Effects in females were not so marked or consistent; at 1000 mg/kg bw/day, body weights were reduced during gestation days 7–20 and throughout lactation; lactation (days 0–20), but mean terminal body weight was not significantly lower than controls (−5%).

At necropsy, significant effects on organ weights (defined as statistical significance in both absolute and relative weights, and consistent with a dose–response relationship) were seen only in females at 1000 mg/kg bw/day, with increased liver weight (117% of control mean absolute weight and 123% of control mean relative weight) and reduced ovary weight (83% of control mean absolute weight and 86% of control mean relative weight). Histopathology revealed minimal or slight hepatic cytoplasmic eosinophilia in most 1000 mg/kg bw/day animals (males 0/0/0/22, females 0/0/0/23, in the 0, 150, 450 and 1000 mg/kg bw/day groups, respectively). There was no histopathological correlate for the reduced ovary weights. As summarized in Table 3, erosion/ulceration of the glandular stomach mucosa was noted in a single 1000 mg/kg bw/day male and few dosed females (0/1/3/4 in the 0, 150, 450 and 1000 mg/kg bw/day groups, respectively). Treatment had no adverse effects on spermatid and sperm number, morphology or motility (Table 7).

3.2. F0 reproduction/F1 pup data

As shown in Table 1, treatment had no effect on estrus cycle length, time to positive mating, duration of gestation, post-implantation losses, offspring sex and survival at and after birth, and the associated mating, fertility, livebirth, viability and lactation indices. At 1000 mg/kg bw/day, there was a significantly reduced mean number of implantation sites per dam and correspondingly reduced litter size, reduced pup weight gain from day 4 to 21 post partum, and delayed vaginal opening and preputial separation. Vaginal opening was also delayed at 450 mg/kg bw/day. From these findings only reduced pup body weight gain was considered to be related to the test substance. The average number of implants per dam and the litter size of the high dose group (10.0 and 9.2) were just marginally below the historical control range, whereas the number of implants per dam in the control group (12.0) was unusually high and above the historical range of the test facility (10.2–11.5). The statistically significantly lower litter size is a direct consequence of this phenomenon and is therefore not considered to be an independent toxicologically relevant adverse effect of the test compound. Furthermore, in the preceding 1-generation range finding study in rats, even at 10,000 ppm in the diet (about 1190 mg/kg bw/day) no test substance-related effects on implantation and litter size were recorded (BASF AG, unpublished data).

Vaginal patency was within the historical control range (30.8–33.8 days) in all groups, the control value (31.3 days) being close to the lower limit and the high dose value (33.8 days) being close to the upper limit of the historical range. Thus, the statistically significant difference between control and high dose group is likely to be the caused by an unusually low control value rather than indicating a particular effect of the test compound on sexual maturation. Therefore, this finding is considered to be an incidental event. Preputial separation (45.4 days) in high dose males slightly exceeded the historical range of the test facility (42.5–45.0 days). However, the average body weights of these males were significantly below the concurrent control, which presumably contributed to this slight delay of sexual maturation. Thus, this apparent slight delay is probably the result of a general retardation of the development of the male F1 pups rather than a specific effect of the test compound.

As shown in Table 2, the litter incidence of pups at weaning with dilated renal pelvis was statistically significantly increased at 450 and 1000 mg/kg bw/day (2.4% and 2.9%, respectively). Dilated renal pelvis is a physiological stage of development of this organ and is frequently observed (historical control range for affected pups per litter 0–3.5%). Thus, an association of the slightly increased incidence of this finding to the test substance is not assumed. All other pup necropsy observations were not related to dose. Changes of high dose

Table 1
F0 reproductive data/F1 progeny

Dose group (mg/kg bw/day)	0	150	450	1000
F0 mean ± SD estrus cycle length (days)	4.1 ± 0.7	4.3 ± 1.4	4.7 ± 3.1	4.2 ± 1.3
F0 male mating index (%)	100	100	100	100
F0 male fertility index (%)	96	88	92	100
F0 female mating index (%)	100	100	100	100
F0 female fertility index (%)	96	88	92	100
Mean ± SD days to positive mating	2.6 ± 1.2	2.5 ± 0.9	2.4 ± 1.0	2.5 ± 0.9
Mean ± SD duration of gestation (days)	22.1 ± 0.6	21.7 ± 0.6	22.0 ± 0.7	22.0 ± 0.5
F0 gestation index (%)	100	100	96	100
Number of F1 litters	24	22	22	25
Mean implantation sites per dam	12.0 ± 2.3	12.3 ± 1.0	11.3 ± 2.4	10.0 ± 2.0**
Mean ± SD % post-implantation loss	8.1 ± 11.2	3.6 ± 4.7	11.8 ± 22.6	7.6 ± 8.8
Mean ± SD F1 pups delivered per litter	11.0 ± 2.5	11.9 ± 0.9	10.9 ± 2.1	9.2 ± 2.0**
F1 livebirth index (%)	99	99	99	99
Sex ratio of live F1 newborns (% male)	46	49	53	44
Number of F1 pups stillborn	3	3	2	3
Number of F1 pups died preweaning	3	2	5	6
Number of F1 pups cannibalized	1	0	0	5
F1 viability index (survival day 0–4 post partum) (%)	98	100	98	96
F1 lactation index (survival day 4–21 post partum) (%)	100	99	99	99
F1 pup weight, mean ± SD per litter, day 1 post partum (g)	6.3 ± 0.6	6.1 ± 0.5	6.4 ± 0.7	6.2 ± 0.7
F1 pup weight gain, mean ± SD per litter, day 1–4 post partum, precull (g)	3.1 ± 0.7	2.9 ± 0.6	3.0 ± 0.6	2.7 ± 0.8
F1 pup weight gain, mean ± SD per litter, day 4–21 post partum, postcull (g)	37.1 ± 2.7	36.7 ± 2.6	36.8 ± 2.8	31.6 ± 4.2**
F1 mean ± SD age at vaginal opening (days)	31.3 ± 1.6	32.5 ± 1.3	33.6 ± 2.1**	33.8 ± 2.2**
F1 mean ± SD weight at vaginal opening (g)	90.8 ± 10.6	95.0 ± 10.1	98.7 ± 10.1*	91.9 ± 8.2
F2 mean ± SD age at preputial separation (days)	43.6 ± 1.2	43.7 ± 1.4	43.4 ± 1.3	45.4 ± 1.5**
F1 mean ± SD weight at preputial separation (g)	172.5 ± 10.3	175.7 ± 15.3	173.9 ± 10.4	168.7 ± 15.8

* $p \leqslant 0.05$; ** $p \leqslant 0.01$ versus control.

Table 2
F1 pup necropsy observations

Number of affected pups/litters				
Dose group (mg/kg bw/day)	0	150	450	1000
Number of pups/litters evaluated	213/24	207/22	189/22	171/25
Cardiomegaly (globular shaped heart) (%)	0.0	0.0	0.8	0.0
Dilated renal pelvis (%)	0.0	0.5	2.4*	2.9*
Hemorrhagic thymus (%)	0.0	0.0	2.1*	1.0
Malpositioned carotid branch (abnormal course of carotids) (%)	0.6	0.0	0.0	0.0
Misshapen spleen (%)	0.0	0.5	0.0	0.0
Small testis (%)	0.0	0.0	0.5	0.0
Partly cannibalized (%)	0.0	0.0	0.6	0.0
Incisors sloped (%)	1.0	0.5	0.0	0.0
Post-mortem autolysis (%)	1.0	0.0	0.6	2.3
Number with any finding (%)	2.7	1.5	6.9*	6.2

Terminology according to Wise et al. (1997).
* $p \leqslant 0.05$ versus control.

Table 3
F0 parental pathology

Sex	Male				Female			
Number of animals with finding (most relevant observations; n-examined = 25 per dose and sex)								
Dose group (mg/kg bw/day)	0	150	450	1000	0	150	450	1000
Glandular stomach-erosion/ulcer	0	0	0	1	0	1	3	4
Liver-minimal or slight cytoplasmic eosinophilia	0	0	0	22	0	0	0	23
Pituitary-cyst, pars intermedia	0	0	0	3	1	0	0	0
Spleen-marked hemosiderin (Perls stain)	7	7	5	18	0	0	0	0

pup brain, spleen and/or thymus weights were secondary to the observed body weight decrements.

3.3. F1 parental observations

Calculated test substance intake in the 150, 450 and 1000 mg/kg bw/day groups was 154, 461 and 1028 mg/kg bw/day in males (mean of weeks 0–15). In females, it was 158, 474 and 1057 mg/kg bw/day during the premating period (mean of weeks 0–10); 149, 443 and 976 mg/kg bw/day during gestation (mean of days 0–20), and 133, 396 and 873 mg/kg bw/day during lactation (mean of days 1–14).

Except for four males and one female at 1000 mg/kg bw/day with urine-smeared fur starting in week 7–15, there were no treatment-related clinical signs. One 450 mg/kg bw/day female was sacrificed moribund on the first day of the premating period (i.e. just after selection as a parental animal), with severe dilation of the cecum and moderate dilation of the jejunum (both with liquid contents), but no other notable pathology; this animal was not replaced. One 450 mg/kg bw/day female was found dead, unable to deliver on day 23 of gestation, with no notable pathology findings. Neither of these deaths was considered treatment-related.

Food consumption was reduced at 1000 mg/kg bw/day, in males during the first 7 weeks of the premating period, and in females during gestation and lactation, by about 10% compared to controls.

Mean body weight at 1000 mg/kg bw/day was reduced by approximately 10% in both males and females throughout the premating, gestation and lacatation periods. At necropsy, mean terminal body weights were significantly lower than controls in both males (−14%) and females (−4%). Significant effects on organ weights, defined as statistical significance in both absolute and relative weights, and consistent with a dose-response relationship, were seen only in females: at 450 and 1000 mg/kg bw/day, absolute liver weight was significantly increased compared to controls (111% and 116%, respectively) as well as relative organ weight (110% and 121%, respectively). Histopathology revealed minimal or slight hepatic cytoplasmic eosinophilia at 1000 mg/kg bw/day in all males and half of the females (males 0/0/0/25, females 0/0/0/12, in the 0, 150, 450 and 1000 mg/kg bw/day groups, respectively). Absolute and relative ovary weight was reduced only at 1000 mg/kg bw/day (82% and 86% of control, respectively). There was no histopathological correlate for the reduced ovary weights.

As summarized in Table 6, erosion/ulceration of the glandular stomach mucosa was noted in 1000 mg/kg bw/day males (group incidences 0/0/0/4 in males and 3/3/2/4 in females). One 1000 mg/kg bw/day male had unilateral reduced testis size and diffuse tubular

Table 4
F1 reproductive data/F2 progeny

Dose group (mg/kg bw/day)	0	150	450	1000
F1 mean ± SD estrus cycle length (days)	4.1 ± 0.5	4.2 ± 0.5	3.9 ± 0.3	4.2 ± 0.6
F1 male mating index (%)	100	100	100	100
F1 male fertility index (%)	96	96	96	100
F1 female mating index (%)	100	100	100	100
F1 female fertility index (%)	96	96	96	100
Mean ± SD days to positive mating, F1	3.0 ± 1.4	3.0 ± 1.2	3.1 ± 1.4	2.6 ± 0.7
Mean ± SD duration of gestation, F1 (days)	22.0 ± 0.4	22.3 ± 0.5	22.3 ± 0.5	22.3 ± 0.5
F1 gestation index (%)	100	100	96	100
Number of F2 litters	24	24	22	25
Mean ± SD implantation sites per dam	12.4 ± 2.3	11.5 ± 2.4	10.7 ± 2.8*	10.3 ± 1.8**
Mean ± SD % post-implantation loss	8.8 ± 9.4	13.5 ± 19.2	8.3 ± 12.5	5.7 ± 8.4
Mean ± SD F2 pups delivered per litter	11.4 ± 2.4	10.2 ± 3.2	9.8 ± 2.9	9.7 ± 1.8
F2 livebirth index (%)	99	97	96	97
Sex ratio of live F2 newborns (% male)	47	54	46	48
Number of F2 pups stillborn	2	7	9*	8*
Number of F2 pups died preweaning	4	2	4	6
Number of F2 pups cannibalized	5	6	0	12*
F2 viability index (survival day 0–4 post partum) (%)	97	97	98	92
F2 lactation index (survival day 4–21 post partum) (%)	99	100	100	100
F2 pup weight, mean ± SD per litter, day 1 post partum (g)	5.9 ± 0.6	6.3 ± 0.8	6.6 ± 0.9**	5.9 ± 0.7
F2 pup weight gain, mean ± SD per litter, day 1–4 post partum, precull (g)	2.8 ± 0.5	3.2 ± 0.7	3.5 ± 0.8**	2.8 ± 0.6
F2 pup weight gain, mean ± SD per litter, day 4–21 post partum, postcull (g)	36.5 ± 3.1	36.5 ± 4.1	38.0 ± 4.7	29.3 ± 4.0**
F2 mean ± SD age at vaginal opening (days)	32.9 ± 2.7	33.5 ± 3.4	33.8 ± 3.7	35.5 ± 3.5**
F2 mean ± SD weight at vaginal opening (g)	93.5 ± 10.9	96.8 ± 13.4	100.2 ± 12.6	95.3 ± 13.4
F2 mean ± SD age at preputial separation (days)	42.8 ± 1.5	42.8 ± 1.3	42.9 ± 1.3	45.1 ± 1.9**
F2 mean ± SD weight at preputial separation (g)	165.5 ± 8.9	165.8 ± 13.5	170.0 ± 9.3	153.9 ± 12.8**

* $p \leqslant 0.05$; ** $p \leqslant 0.01$ versus control.

Table 5
F2 pup gross pathology (day 0–21 p.p.)

Number of affected pups/litters				
Dose group (mg/kg bw/day)	0	150	450	1000
Number of pups/litters evaluated	215/24	190/23	165/22	181/25
Conjoined twins (%)	0.0	0.4	0.0	0.0
Dilated renal pelvis (%)	0.8	0.0	0.0	1.7
Empty stomach (%)	0.0	0.0	0.6	0.0
Hemorrhagic thymus (%)	0.0	0.0	0.6	1.8
Hydronephrosis (%)	0.0	0.0	0.0	0.5
Hydroureter (%)	0.0	0.0	0.0	0.5
Incisors sloped (%)	0.0	0.0	0.9	0.0
Post-mortem autolysis (%)	4.5	3.9	4.1	5.8
Situs inversus (%)	0.5	0.0	0.0	0.0
Small testis (%)	0.4	0.4	0.0	0.8
Number with any finding (%)	6.3	4.7	6.2	10.1*

Terminology according to Wise et al. (1997).
* $p \leqslant 0.05$ versus control.

degeneration (seminiferous tubuli contained only Sertoli cells), and corresponding left epididymal reduced size and aspermia, but the contralateral (right) testis and epididymis were grossly normal with normal sperm counts, and the animal mated successfully. This isolated finding has been observed in historical controls; it was considered spontaneous and unrelated to treatment. Minimal or slight focal testicular tubular degeneration was observed in one control and two high dose males, but sperm parameters were normal and all three animals mated successfully; this finding was also considered unrelated to treatment.

Treatment had no adverse effects on spermatid and sperm number, morphology or motility, or on differential follicle counts (Table 7). (The significant difference in epididymal spermatids between 1000 mg/kg bw/day and control groups is attributable to anomalously high control values, as noted in Table 7.)

3.4. F1 reproduction/F2 pup data

Calculated test substance intake of the F2 offspring in the 150, 450 and 1000 mg/kg bw/day groups was 175, 523 and 1172 mg/kg bw/day in males (mean of weeks 0–3 post-weaning) and 173, 519 and 1077 mg/kg bw/day in females (mean of weeks 0–2 post-weaning).

As shown in Table 4, treatment had no significant effect on estrus cycle length, time to positive mating, duration of gestation, post-implantation losses, offspring sex and survival after birth, and the associated mating, fertility, livebirth, viability and lactation indices.

The mean number of implantation sites was statistically significantly reduced at 450 mg/kg bw/day and 1000 mg/kg bw/day (10.7 and 10.3, respectively) compared to controls (12.4). However, the average number of implants per dam in the control group was unusually high and considerably above the historical range of the test facility (10.2–11.5), whereas the number of implants

Table 6
F1 parental pathology

Sex	Male				Female			
Number of animals with finding (most relevant observations; n-examined = 25 per dose and sex)								
Dose group (mg/kg bw/day)	0	150	450	1000	0	150	450	1000
Glandular stomach-erosion/ulcer	0	0	0	4	3	3	2	4
Liver-minimal or slight cytoplasmic eosinophilia	0	0	0	25	0	0	0	12
Pituitary-cyst, pars intermedia	2	0	0	5	0	0	0	3
Spleen-marked hemosiderin (Perls stain)	3	3	4	6	0	0	0	0

Table 7
Parental sperm analyses and follicle counts (mean ± SD)

Dose group (mg/kg bw/day)	0	1000
Number evaluated per sex	25	25
F0 sperm analysis		
Total spermatids/g testis	125 ± 16.2	125 ± 27.7
Total spermatids/g cauda epididymis	677 ± 133.7	641 ± 141.2
% abnormal sperm	1.4 ± 0.9	2.0 ± 1.4
% motility (mean ± SD)	88 ± 11.0	86 ± 11.2
F1 sperm analysis		
Total spermatids/g testis	119 ± 11.5	128 ± 14.0
Total spermatids/g cauda epididymis	763 ± 108.9	700 ± 135.7*
% abnormal sperm	1.3 ± 1.1	1.5 ± 1.5
% motility (mean ± SD)	89 ± 7.7	87 ± 10.5
F1 differential follicle counts		
Primordial follicles (mean)	224	243
Growing follicles (mean)	42	43

* $p \leqslant 0.05$ versus control, due to anomalously high control value, which exceeded historical control range (in 19 control groups from 10 previous studies of this type, total spermatids/g cauda epididymis means ranged from 517 to 727; mean of historical means = 625).

per dam in the high dose group was well within the historical range. These findings were therefore considered to be incidental and not associated to the test substance.

The incidence of stillborn pups was statistically significantly higher than controls in the 450 and 1000 mg/kg bw/day groups (4.2% and 3.3%, respectively). However, this increase was not related to dose and the rates of stillborn pups were well within the historical range of the test facility (0–4.3%), and was therefore considered not test substance-related.

The viability index as indicator for pup mortality (pups died and cannibalized) between days 0 and 4 p.p. was statistically significantly reduced in the 1000 mg/kg bw/day group. Loss of one single complete litter was mainly responsible for the reduced viability index in this dose group as all of the delivered pups died or were cannibalized between day 1 and 4 p.p. This is considered to be an incidental event and not due to the test compound.

At 1000 mg/kg bw/day, pup weight gain was reduced from day 4 to 21 post partum and in the selected offspring also during entire rearing; subsequently vaginal opening and preputial separation were slightly delayed (Table 4). F2 gross pathology did not reveal any other treatment related findings (Table 5). Changes of high dose pup brain, spleen and/or thymus weights were secondary to the observed body weight decrements.

4. Discussion

In the present two generation reproduction toxicity study, OMC had no adverse effect on estrus cycle, sperm number, morphology and motility, differential follicle counts, mating, fertility, gestation and parturition. Parental food consumption and body weight were reduced at 1000 mg/kg bw/day (necropsy bodyweight −14% to −16% in males, −4% to −5% females). General systemic effects were evident at 1000 mg/kg bw/day in terms of increased liver weight and hepatic cytoplasmic eosinophilia due to hepatic enzyme induction and are considered to be an adaptive rather than a frank toxicological effect. An apparent increase in the incidence of erosion/ulceration of the glandular stomach mucosa was noted in F0 females (0/1/3/4) as well as in F1 males (0/0/0/4) and females (3/3/2/4). From the F1 females it can be seen that this finding is also observed in control animals. However, an incidence of 4 affected animals was not observed in this study and also not in our historical control data. Therefore, it is concluded that the increased occurrence of erosion/ulceration of the glandular stomach mucosa at 1000 mg/kg bw/day may have been related to treatment. An effect of OMC on liver was previously reported in a 13-week repeat-dose toxicity study, in which females at the high dose (1000 mg/kg bw/day) had increased glutamate dehydrogenase activity which reversed during the recovery period (SCC, 1996), but there are no previous reports of an OMC effect on glandular stomach mucosa.

There was a statistically significantly reduction of the number of implantation sites at 1000 mg/kg bw/day in both parental generations, and also at 450 mg/kg bw/day in F1 parents, compared to controls. It should be noted that the number of implantation sites in the F0 and F1 female control animals was particularly high, in fact exceeded the historical range recorded to date in the test facility. The number of implantation sites in the F0 females at 1000 mg/kg bw/day (10.0 ± 2.0) was in fact very close to the historical control range (10.2–11.5). In the F1 generation females the number of

implantation sites at 450 mg/kg bw/day (10.7 ± 2.8) and 1000 mg/kg bw/day (10.3 ± 1.8) was fully within the historical range. The fact that subsequent follicle counts were normal in all F1 parents indicates that if the reduction in implantation rate was truly related to treatment, it was not related to egg maturation.

Moreover, in both generations, post-implantation loss was normal for all groups, again indicating the absence of a treatment related effect on this parameter. Since a marginally lower implantation rate was noted in both parental generations, it is possible that this is a secondary, albeit a small effect, at the high dose. Reductions in the implantation rate are not unusual in animals which are showing toxicity, as is the case in the high dose females here, which had reduced food consumption and body weight, liver effects and stomach erosion. A small pre-implantation loss in a multigeneration study such as this, at a dose where the parents show signs of toxicity, is not unusual and is most likely secondary to the maternal toxicity. Therefore, we conclude that the slight reduction in the number of implantation sites in the high dose F0 and F1 females was unlikely to be directly related to treatment.

In both generations, pup weight gain was reduced at 1000 mg/kg bw/day from day 4 to 21 of lactation, resulting in pup body weights that were 13–16% below controls at weaning. At 1000 mg/kg bw/day, significantly lower body weights were also noted post-weaning in the reared offspring of both generations. In weeks 2 and 3 after weaning (the time when sexual maturation was recorded), the average body weights ranged 6–12% below concurrent controls. Sexual maturation (vaginal opening and preputial separation) was slightly delayed, i.e. occurred a few days later than in control animals, in male and female offspring of both F1 and F2 generations at 1000 mg/kg bw/day. The conclusion that this is a test substance-specific effect could be made if time to sexual maturation were the only relevant parameter. However, if body weight rather than calendar age is the essential component driving sexual maturation, then a different conclusion would need to be drawn. In this study the body weights of the 1000 mg/kg bw/day offspring (both sexes) on the day of sexual maturity were quite comparable to the concurrent control values at the time when they reached sexual maturation (i.e. 2–3 days earlier). It is not unlikely that delayed body weight development would result in a delay in sexual maturation. To better determine the relationship between body weight development and the time to reach sexual maturity, we recommend that a daily determination of body weight during recording of vaginal patency (from day 27 p.p. onwards) and preputial separation (from day 40 p.p. onwards) should be performed. Such data may help to assess more correctly if a primary (selective) or secondary test substance-related effect is implicated.

As noted in the Introduction, OMC has no androgenic or antiandrogenic activity, and no or, at high dosages only, very weak estrogenic potential. The results of the present study indicate that OMC has no estrogenic potential in vivo in parental animals and their offspring when continuously treated over two generations during premating, gestation, lactation and sexual maturation.

In conclusion, the no observed adverse effect level (NOAEL) of OMC by continuous dietary administration in this study is 450 mg/kg bw/day for fertility and reproduction parameters, for systemic parental and developmental toxicity. This is based on reduced body weights, increased liver weight and hepatic cytoplasmic eosinophilia in the parents, a secondary reduction in implantation rate, and reduced body weights and delayed sexual maturation of the pups at 1000 mg/kg bw/day.

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4MBC and OMC, components of UV-sunscreens, exert organ specific alterations on type I 5'-Deiodinase activity and expression in female rats

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Endocrine disrupting compounds (EDC) of natural or synthetic origin were originally found to impair development and endocrine regulation of the reproductive system. Here, we assessed whether they also exert effects on the thyroid hormone axis. Female ovariectomized rats were treated for 12 weeks with two substances used as UV absorbers in sunscreens: Octyl-methoxycinnamate (OMC; 66 and 310mg/day) and 4-methylbenzylidene-camphor (4-MBC; 0.4 and 1.74mg/day) alone or in combination with soy-containing food. These substances are known to be resorbed by the skin, circulating in the blood (Janjua et al. 2004J Invest Dermatol 123:57–61) and suspected to have endocrine disrupting effects. The soy isoflavone genistein has estrogenic and anti-estrogenic effects and is known to inhibit thyroid hormone biosynthesis. As type I 5'-deiodinase (5'DI) is a well known T3-regulated endpoint, 5'DI activity was measured by deiodination of 125I--reverse T3, and 5'DI gene expression was determined by real time RT-PCR. Treatment with OMC caused a significant reduction of 5'DI activity from a basal level of 6.6 to 3.0 and 3.1 pmol iodide released x mg–1 x min–1 in the liver in the absence and presence of soy in the diet, respectively, while 4MBC did not show any effects. A similar decrease was also observed for 5'DI mRNA levels. In the kidney OMC as well as 4MBC led to a marked decline of 5'DI activity from 8.4 to 3.0 pmol iodide released x mg–1 x min–1, independent of the diet. Additionally, higher 5'DI activities were found in the kidney of animals consuming soy containing diet, while no such effect was obtained in liver. This data indicates that 4MBC and OMC affect thyroid hormone metabolism via tissue-specific inhibition of 5'DI activity and gene expression. Thus, 4MBC and OMC do not only interfere with steroid-hormone dependent regulation, but may also disturb endocrine regulation via the thyroid hormone axis.

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Aggregate consumer exposure to UV filter ethylhexyl methoxycinnamate via personal care products

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ABSTRACT

Ultraviolet (UV) filters are substances designed to protect our skin from UV-induced damage and can be found in many categories of personal care products (PCPs). The potential endocrine-disrupting effects attributed to UV filter ethylhexyl methoxycinnamate (EHMC) are being debated. We evaluated the aggregate exposure of the Swiss–German population (N = 1196; ages ≤1–97 years) to EHMC via the use of PCPs; thus we provide the first comprehensive information about the current EHMC exposure sources and aggregate exposure levels. In our probabilistic modeling method performed at an individual level, PCP use data obtained by a postal questionnaire were linked to concentration data on EHMC gained from chemical analyses of PCPs used by the questionnaire respondents. The modeled median and 99.9th percentile of the internal aggregate exposure for the general population were 0.012 and 0.873 mg day^{-1} kg^{-1} and 0.008 and 0.122 mg day^{-1} kg^{-1} for the summer/autumn and winter/spring period, respectively. The major contributors to internal aggregate exposure were sunscreen products in summer/autumn (females: 64%; males: 85%; children aged ≤12 years 93%). In winter/spring, lip care dominated for females (30%) and sunscreen for males (38%) and children aged ≤12 years (50%). Overall, the internal aggregate exposure estimates for the studied population are shown to be below the Derived No Effect Level (DNEL) for EHMC i.e., the level of exposure above which humans should not be exposed; however, when an intense short-term exposure via sunscreen is accounted for during a sunbathing day, at the high-end percentiles (99.9th) the predicted aggregate exposure exceeds the DNEL for thyroid-disrupting effects such as for children aged ≤4 years, who might be particularly susceptible to endocrine disrupting events. It is nevertheless critical to acknowledge that quantitative data on transdermal penetration of EHMC from PCPs are currently insufficient. Since long-term effects of endocrine disruptors are not known, future studies are warranted to provide accurate quantitative data on transdermal penetration of EHMC and to determine its metabolic fate in humans.

1. Introduction

Ultraviolet (UV) filters are a class of chemicals that are increasingly added into personal care products (PCPs) (Maier et al., 2005; Séhédic et al., 2009) to protect human skin from the harmful effects of UV radiation such as immediate skin damage (sunburn), premature skin aging, and skin cancer. PCPs may also contain UV filters as UV absorbers to prevent light-induced product degradation. The worldwide UV filter production volume was estimated at about 10,000 metric tons per year (Gago-Ferrero et al., 2012). It is therefore not surprising that several UV filters are high production volume chemicals. One such chemical is ethylhexyl methoxycinnamate (EHMC) (Bachelot et al., 2012); its molecules are lipophilic (log K_{ow} 6.0; Balmer et al., 2005) and can accumulate in biota (Bachelot et al., 2012; Fent et al., 2010), with potentially adverse effects. Potential health risks to aquatic life, wildlife, and human populations associated with the possible endocrine-disrupting effects of EHMC have received wide attention not only within the scientific community, but also from the general public through media channels and non-governmental organizations (e.g., Bund für Umwelt und Naturschutz Deutschland, English: Friends of the Earth Germany, 2013; Environmental Working Group, 2014). Multiple hormonal activities of EHMC have been reported both in vitro and in vivo (Axelstad et al., 2011; Christen et al., 2011; Gomez et al., 2005; Inui et al., 2003; Klammer et al., 2007; Ozáez et al., 2013; Schlumpf et al., 2001, 2004; Seidlová-Wuttke et al., 2006), but few studies have investigated EHMC levels in human matrices (blood, urine: Janjua et al., 2004; breast milk: Hany and Nagel, 1997; Schlumpf et al., 2010), and the metabolic fate of EHMC in the human body remains unknown.

Abbreviations: PCP(s), personal care product(s); UV, ultraviolet; EHMC, ethylhexyl methoxycinnamate; SPF, sun protection factor; MCS, Monte Carlo Simulation; RCR, Risk characterization ratio; DNEL, Derived No Effect Level; US, the United States.

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Available data on human exposure levels to EHMC via the use of PCPs are very limited (Andersen et al., 2012). Since EHMC is contained in multiple categories of PCPs that can be concurrently used by the same consumer, aggregate exposure estimates are highly needed for risk assessment. The major difficulty in modeling the aggregate consumer exposure to EHMC, particularly in European populations, is the lack of publicly available national-level data on use patterns for PCPs, including co-use data (i.e., which PCP categories are concurrently used by consumers), and exact EHMC concentrations in PCPs that are kept confidential in Europe.

EHMC is frequently contained in PCPs sold on the Swiss market (Manová et al., 2013b). Not surprisingly, therefore, it has been found in the Swiss environment in both abiotic (Fent et al., 2010; Plagellat et al., 2006; Poiger et al., 2004) and biotic matrices (Balmer et al., 2005; Fent et al., 2010), including breast milk (Schlumpf et al., 2010). The objective of the present study was to provide the very first comprehensive information about the current EHMC aggregate exposure levels through multiple PCP categories. Our novel probabilistic modeling method is performed at an individual level for the German-speaking Swiss consumers and is well suited for ingredients not present in all PCPs of a given product category, such as EHMC, to avoid unreasonable overestimation of exposure. Furthermore, in comparison to the conventional probabilistic modeling approaches, our approach takes into account correlations between frequencies of use of the different PCP categories used by the same consumer. Our work also explores the contribution of different PCP categories to aggregate exposure, as filling this major data gap is necessary to enable concerned consumers to minimize their exposure to EHMC via the use of PCPs.

2. Materials and methods

Despite its widespread use in PCPs, not all products in a given PCP category contain EHMC. To avoid overestimation of exposure, we assessed the aggregate exposure probabilistically at an individual level. For this purpose, two data sets were previously collected. As a first component of this work, we conducted a postal questionnaire survey to determine the use patterns of PCPs (Manová et al., 2013a). As a second component, we sampled 116 PCPs selected on the basis of the questionnaire and measured their EHMC concentrations (Manová et al., 2013b). For each respondent, we then linked the self-reported data on PCP use patterns with EHMC concentrations measured in the self-reported PCPs. Seven widely used leave-on PCP categories that are often used simultaneously were considered (face cream, aftershave lotion/balm, hand cream, makeup foundation, lip care, lipstick, sunscreen) for their high likelihood of containing UV filters.

2.1. Study design and data collection

First, we established a list of PCP categories that often contain UV filters. Based on our market analysis, eight target PCP categories have initially been chosen: face cream, body lotion, aftershave lotion/balm (hereafter referred to as aftershave), hand cream, makeup foundation, lip care, lipstick, and sunscreen. Other PCP categories (e.g., rinse-off products) may also contain EHMC as a UV absorber to prevent photodegradation of the product, but UV absorbers are usually only needed in very low concentrations. Usage data for the selected leave-on PCP categories were collected through postal questionnaires between January and March 2011. The study was conducted in the German-speaking part of Switzerland. Throughout this work, we use the term “children” for participants aged ≤12 years, “adolescents” for participants aged between 13 and 17 years, and “adults” for those ≥18 years old. Two questionnaire versions were designed; one for adults, and one for children and adolescents. The questionnaire for children and adolescents was mailed to 1000 eligible families recruited using a commercially available address database. The adult questionnaire was sent to 2500 household addresses randomly selected from the Swiss telephone directory. The overall response rate to the questionnaires was 48.8% for children and adolescents and 36.8% for adults. After exclusion of ineligible, incomplete, and contradictory responses, the final dataset included 1196 respondents (age range of ≤1–97 years). Detailed information about the questionnaire survey and the population characteristics have been published previously (Manová et al., 2013a). The present study was conducted as part of a larger research project focused on several organic UV filters, therefore ingredient lists of the frequently used PCPs identified by the questionnaire were inspected for the presence of all organic UV filters, not only EHMC. Body lotion was subsequently excluded from further consideration due to the absence of organic UV filters in the ingredient lists of body lotions used by the questionnaire respondents.

A sample of 116 UV filter-containing PCPs used by the respondents was selected for analysis. PCP samples were collected locally from several Swiss retailers (Migros, Coop, Denner, Lidl, ALDI SUISSE), local pharmacies and perfumeries in Zürich and Basel, Switzerland, and a Swiss internet retail store. Concentrations of 22 organic UV filters were measured in the selected PCPs. EHMC was detected in 59 products (51% of all PCPs tested). Details of the chemical methods and UV filter concentrations in the PCPs analyzed were described by Manová et al. (2013b).

2.2. Model background

The Ford model given in Eq. (1) (Ford, 1998; Wormuth et al., 2005) considers dermal application of liquid and viscous products and is thus well suited for characterizing EHMC exposure via the investigated PCP categories. For each individual in our study population, the Ford model was first used to derive the daily external exposure to EHMC, $E_{EHMC(external)}$ [mg day^{-1} kg^{-1}], from their self-reported PCP application frequency, f_{event} [day^{-1}], the retention factor of the considered PCP category (i.e., a measure of the fraction of EHMC-containing PCP that remains in contact with a human body over a longer period of time), a_{ret} [−], the amount of the EHMC-containing PCP applied per application, q_{PCP} [mg], and the weight fraction, w_{PCP} [−], of EHMC in the PCP applied by the respondent. Finally, the exposure values were adjusted for the respondent's self-reported body weight, m_{bw} [kg]. The daily internal exposure, $E_{EHMC(internal)}$ [mg day^{-1} kg^{-1}], was estimated by introducing the absorption factor, *abs* [−], representing the fraction of the external aggregate dose of unmetabolized EHMC that is transferred into the systemic circulation.

$$E_{EHMC\ (internal)} = f_{event} \cdot a_{ret} \cdot q_{PCP} \cdot w_{PCP} \cdot m_{bw}{}^{-1} \cdot abs \quad (1)$$

If a respondent was exposed to EHMC via more than one PCP category, then the aggregate exposure was defined as the sum of EHMC exposures across the different PCP categories. In accordance with our individual-oriented approach, each questionnaire respondent was taken as a starting point and the daily EHMC internal aggregate exposure from the different PCP categories was aggregated at an individual basis, while maintaining the information about each respondent's self-reported PCP co-use patterns, as given in Eq. (2), where n represents the number of PCP categories used by the respondent.

$$E_{EHMC,agg(internal)} = \sum_{i=1}^{n} E_{EHMC,PCP_i(internal)} \quad (2)$$

We computed the aggregate exposure separately for summer/autumn (May to October) and winter/spring (November to April), as the frequency of sunscreen use and the extent of application to different areas of the body both depend on the outdoor activities associated with the respective season. However, we have also derived the acute internal aggregate exposure to EHMC to account for the very intense exposure to EHMC related to sunscreen whole-body use on a sunbathing day

(e.g., during holiday periods, summer weekends). We did not have data for PCP co-use for specific days of the year. Therefore, the daily internal aggregate exposure to EHMC resulting from the regular use of the remaining PCPs throughout the year was added to the acute sunscreen exposure estimate to derive a sunbathing day internal aggregate exposure $E_{\text{EHMC-SUNBATHING, agg (internal)}}$ [mg day^{-1} kg^{-1}].

2.2.1. Model inputs: sources and uncertainty

The aggregate exposure distributions for EHMC were generated probabilistically using Monte Carlo Simulations (MCSs) at an individual level to integrate parameter uncertainty. MCSs were performed (50,000 iterations for each PCP category) using Scientific Python (SciPy; version 0.13.1), including both consumers and non-consumers (for more details see Supplementary Section 1.1). Data sources for model input parameters and their defined probability distributions (assumed to be independent of each other) are detailed below and summarized in Table 1.

Data on PCP co-use and PCP application frequency, f_{event}, were obtained from the questionnaire (Manová et al., 2013a) for each respondent. If participants did not indicate the f_{event} and the names of the individual PCPs they used, we assumed that they did not use that PCP category. However, if they did not indicate the f_{event}, but filled out the names of the individual PCPs, the f_{event} record was substituted in every MCS run by the median based on f_{event} values indicated by other respondents (for percentage of missing answers replaced by medians see Supplementary Table S1). Since PCP application frequencies differ with age, we used different median values for children under the age of 13 and adolescents/adults (aged 13 years and older). The range of application events f_{event} in the answers given by the respondents was modeled by assigning uniform distributions in the respective range. For instance, the answer '2–3 times per week' was represented by a uniform distribution with minimum of 2/7 and maximum of 3/7 times per day. For sunscreen, we asked respondents to indicate for each season the number of days on which they used sunscreen and the number of sunscreen applications per day. We then determined the average number of whole-body applications of sunscreen per day for the summer/autumn months and head-only applications for the winter/spring months. Since an open-ended question format was employed to derive the individual frequencies of sunscreen use, quantitative data were not available to estimate the magnitude of the error within respondents' answers. To address the uncertainty in the exposure analysis, we assumed an uncertainty range expressed in percentage points, namely ±20% of the value determined from the questionnaire thus resulting in a triangular distribution. In the sunbathing scenario, we replaced the average application frequency for the summer/autumn period per day with the exact number of whole-body sunscreen applications on a single sunbathing day as reported by each respondent in the questionnaire (see Supplementary Section 1.2 for more details).

The retention factor, a_{ret}, is used in the Ford model to make a distinction between rinse-off and leave-on PCPs. All the PCP categories considered in this study belong to leave-on PCP categories that are intended to stay on the skin for prolonged periods of time, thereby increasing the chance of systemic absorption of the constituent ingredients. As proposed by the Scientific Committee on Consumer Safety (SCCS) (SCCS, 2012) for such PCPs, the retention factor of 1 was used in the exposure analysis (treated as a fixed-point value in the MCS).

The amount of PCP used per application, q_{PCP}, was taken from a recent study conducted in the Netherlands (Biesterbos et al., 2013). Biesterbos et al. (2013) asked respondents to estimate PCP use amounts based on photographs showing different amounts of the PCP used, the number of PCP layers applied on the lips, etc. This information was converted into numerical ranges. The most frequently applied amount range for each PCP category was chosen to represent the uniform distributions for q_{PCP} for adults (Supplementary Table S2). As Biesterbos et al. (2013) only collected the q_{PCP} data for adults, for children and adolescents, the q_{PCP} ranges were extrapolated from the adult q_{PCP} distributions by applying a correction factor accounting for their smaller body surface area (see Supplementary Section 1.3).

The weight fraction, w_{PCP}, of EHMC in the applied PCP was determined based on the names of the specific PCPs reported by respondents. We used open-ended questions to gather information on specific PCPs used by the respondents in order to obtain as much details about the PCPs as possible and hence deduce whether they contain EHMC. However, the gathered dataset was extremely complex as the degree of detail given by respondents varied widely, making the identification of the individual products and consequently whether they contain EHMC very challenging. For respondents who reported more than one PCP name in each PCP category, exposure was calculated based on the EHMC presence in the first PCP indicated. If the selected PCP category was not used or the PCP was fully identified and did not contain EHMC, exposure from this product was considered to be zero. When a respondent reported a full PCP name and EHMC concentrations were measured for this particular PCP, exposure was directly estimated using the exact EHMC concentrations as fixed-point values. If the exact PCP name was missing, however, the PCP brand name was available; we first determined whether the particular brand uses EHMC in their products. If EHMC was not used, exposure was considered to be zero. To address the uncertainty associated with EHMC concentration in cases when (a) the exact/full PCP name was available (including brand name), but not the exact concentration, (b) only the brand name was available and the brand used EHMC in their PCPs, and (c) the brand name was not available (i.e., there was still a chance that the use of this PCP contributed to the aggregate exposure), a uniform distribution was defined for the range between zero and the highest measured value for the given PCP category, as our chemical analyses have shown that EHMC concentrations in PCPs used by the respondents are markedly lower than the maximum authorized concentration of 10% (Manová et al., 2013b). We did not include the uncertainty associated with measurement accuracy, as it is negligible in comparison to the other uncertainties. The step-by-step procedure for selecting the EHMC concentration inputs as described above is illustrated in Fig. 1.

For each respondent, his or her self-reported body weight, m_{bw}, was obtained from the questionnaire (age- and gender-specific distributions

Table 1
Parameters and input probability distributions types.

Parameter	Symbol	Unit	Distribution type	Reference
PCP application frequency	f_{event}	day^{-1}	Uniform/Triangular	Manová et al. (2013a)
Retention factor	a_{ret}	–	Fixed value	SCCS (2012)
Amount of PCP applied per application	q_{PCP}	mg	Uniform	Biesterbos et al. (2013)
Weight fraction of EHMC in PCP	w_{PCP}	–	Uniform/Fixed value	Manová et al. (2013b)
Body weight	m_{bw}	kg	Triangular	Manová et al. (2013a)
Absorption factor	*abs*	–	Uniform	Benech-Kieffer et al. (2000), Chatelain et al. (2003), Durand et al. (2009), Jiménez et al. (2004), Potard et al. (1999), Potard et al. (2000), Treffel and Gabard (1996)

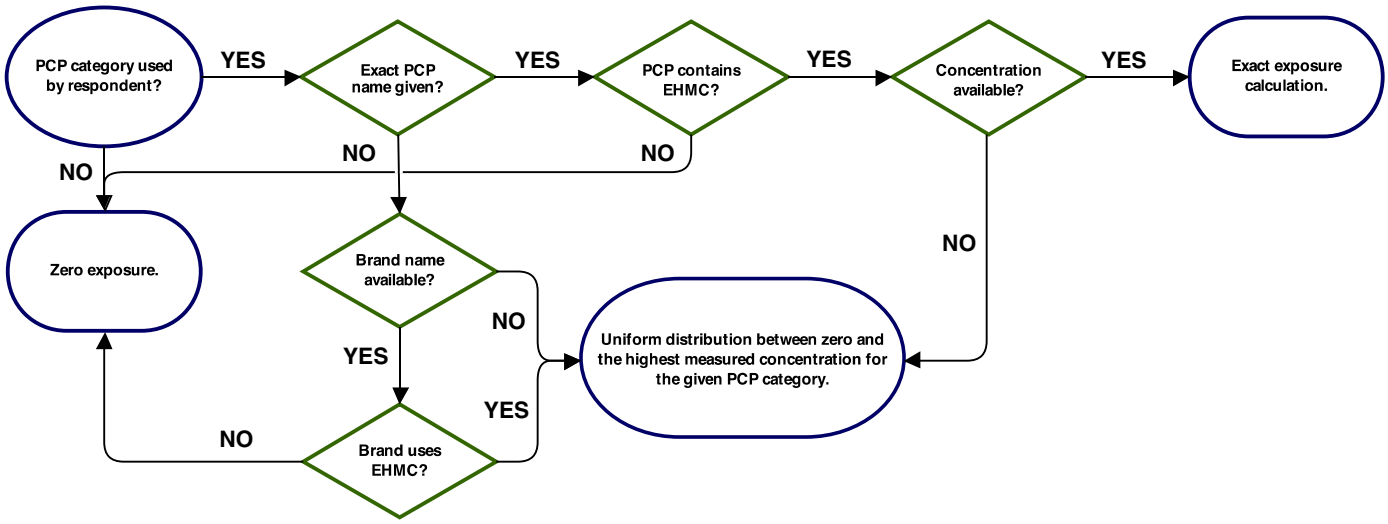

Fig. 1. Method for selecting the EHMC concentration inputs.

from Manová et al., 2013a). However, to account for the fact that self-reported body weight values may be biased, with a more pronounced tendency for weight underestimation (Danubio et al., 2008; Elgar et al., 2005; Paccaud et al., 2001; Spencer et al., 2001), a triangular distribution was defined for each respondent, in which the lower bound deviates by −2 kg and the upper bound by +3 kg, from the single self-reported m_{bw} value.

To estimate the internal aggregate exposure, accurate quantitative data on dermal absorption are required given the evidence that EHMC is systematically absorbed through the skin from EHMC containing PCPs (Janjua et al., 2004). In general, current understanding of the systemic absorption of chemicals via dermal exposure is derived from in vitro and in vivo experiments. Due to ethical and economic reasons, and shorter experimental time, in vitro tests are more commonly used, in which the diffusion of chemicals into and across the skin layers (epidermis, dermis) into a receptor fluid, imitating the blood supply, is measured. The sum of the EHMC amount present in the dermis and in the receptor fluid, thus considered to have penetrated through the skin, is hereafter referred to as "transdermal penetration." It represents the bioavailable amount of EHMC. We considered only studies using formulations that are similar to the finished PCPs used by consumers; therefore experiments with EHMC concentrations above the maximum authorized concentration of 10% applicable to Switzerland (VKos, 2012) and the European Union (EU, 2009) were excluded. Overall, seven in vitro studies investigating the transdermal penetration of EHMC were assessed. Human skin is the best choice for in vitro experiments; however, experiments conducted with pig skin, which is a good surrogate for human skin (Barbero and Frasch, 2009), were also considered. In vitro studies using static Franz-type diffusion cells have shown large differences in transdermal penetration, ranging from 0.004 to 5.7% (Benech-Kieffer et al., 2000; Chatelain et al., 2003; Durand et al., 2009; Jiménez et al., 2004; Potard et al., 1999; Potard et al., 2000; Treffel and Gabard, 1996). Studies reviewed applied a dose of EHMC-containing formulation between 2 and 8 mg cm^{-2}. The minimum dose used (2 mg cm^{-2}) is in accordance with the general recommendations for sunscreen use and corresponds to the amount used for the determination of the sun protection factor (SPF). However, it has been shown that consumers on average apply 0.5 mg cm^{-2} of sunscreen (Isedeh et al., 2013) and even less of other PCP categories. Application amounts exceeding 2 mg cm^{-2} per application may be unsuitable to mimic the real-life habits of consumers, and in turn poorly represent their systemic exposure to EHMC, as the transdermal penetration of the applied dose may decrease with increasing dose (Buist et al., 2009). On the other hand, consumers may apply products several times per day. PCP re-application has not been considered in the reviewed studies. Furthermore, only a single concentration of EHMC has been examined in most studies, despite that EHMC concentrations in PCPs have been shown to vary widely (Manová et al., 2013b). The experimental duration varied from 6 to 24 h. As mentioned above, EHMC is a lipophilic molecule (log K_{ow} 6.0) and therefore it may be accumulating and perhaps metabolizing in the skin. It has been shown that the penetration of EHMC into the blood continues from the site of application after 6 and even 16 h (Durand et al., 2009; Jiménez et al., 2004). Regular sampling over the whole exposure period is desirable so that an absorption profile over time can be constructed. From the reviewed studies, only two included more than two sampling time-points during the exposure period investigated. Different product formulations have been tested including water-in-oil, oil-in-water, and silicone-in-water emulsions, petroleum jelly and emulsion gel, and the vehicle used has been shown to influence the transdermal penetration of EHMC (Durand et al., 2009; Treffel and Gabard, 1996). Encapsulation of EHMC may also influence its transdermal penetration (e.g., nanoencapsulation; Jiménez et al., 2004). In summary, currently available data on transdermal penetration of EHMC are very difficult to assess due to methodological differences between the studies. For the purpose of this study, the absorption factor, *abs*, is described by a uniform distribution in which the lowest and highest values reported for EHMC transdermal penetration i.e., 0.004 and 5.7%, represent the lower and upper bounds used in the exposure analysis. In vitro studies discussed above have been summarized in the Supplementary Table S4. Exposure to EHMC via the use of lip care products and lipsticks can occur by absorption through the skin as well as by oral intake. In this study, we assume a high potential for oral intake (dermal uptake was not considered for these two PCP categories); thus 80 and 100% are considered as lower and upper bounds for ingestion.

2.3. Human health risk characterization

For the purpose of human health risk characterization, the risk characterization ratio (RCR) Eq. (3) (EU, 2006) was computed for EHMC by comparing the daily internal aggregate exposure, $E_{EHMC,\ agg\ (internal)}$ [mg day^{-1} kg^{-1}], to a Derived No Effect Level (DNEL) [mg day^{-1} kg^{-1}], defined as the level of a substance above which a human should not be exposed.

$$RCR = \frac{E_{EHMC,agg(internal)}}{DNEL}. \quad (3)$$

For cases where RCR < 1, no risk reduction measures are needed. The same approach to evaluate EHMC risks to human health was employed by the Danish Environmental Protection Agency (EPA) (Andersen et al., 2012). Based on oral gavage studies in rats, the DNEL values derived by the Danish EPA for EHMC are 1.67 mg day^{-1} kg^{-1} and 1.00 mg day^{-1} kg^{-1} for estrogenic effects ($DNEL_E$) and for thyroid-disrupting effects ($DNEL_T$), respectively.

2.4. Comparison to probabilistic modeling results without data on specific PCPs

The weight fraction of EHMC in a product was determined on the basis of information provided by each respondent about the names of the specific PCPs they used to avoid exposure overestimation. However, such detailed data on the use of specific PCPs at an individual level as generated within this study is often not available. Therefore, exposure assessments include assumptions regarding the market fraction or assume that the market fraction is one. To assess the effect of including detailed high-quality data about PCPs as used in our method (hereafter referred to as Method 1), for comparison, an additional MCS was performed (hereafter referred to as Method 2) that models the exposure to EHMC assuming that data on the use of specific PCPs are unavailable i.e., the weight fraction of EHMC is always a range between zero and the highest measured value for the given PCP category.

3. Results

3.1. Modeled aggregate exposure levels using Method 1

The estimated median, 95th, 99th, and 99.9th percentile of the distribution of internal aggregate exposure to EHMC via the investigated PCP categories by the general population were 0.012, 0.156, 0.508, and 0.873 mg day^{-1} kg^{-1}, respectively, for the summer/autumn period. The corresponding values for the winter/spring season were markedly lower, namely, 0.008, 0.048, 0.080, and 0.122 mg day^{-1} kg^{-1} (Table 2). The median estimated internal aggregate exposure levels are higher for female than for male respondents; however, at the upper end percentiles the exposure levels are similar for both genders and even higher for males in some cases (e.g., 99.9th percentile for both seasons).

The median estimated internal aggregate exposure is highest for children aged 5–8 years (Table 2), followed by children aged 9–12 years and adults aged 18–42 years. The aggregate exposure levels for children at the 99.9th percentile are markedly higher than for adolescents and adults. At the 99.9th percentile the exposure levels of the least exposed consumers aged ≥66 are in fact seven times lower than those of the highest exposed consumers i.e., the youngest consumers aged ≤4 years. In the winter/spring months, the median estimated internal aggregate exposure is highest for adults aged 18–42 years. At the 99.9th percentile, however, the estimated aggregate exposure for children aged ≤4 years is noticeably higher than for all other age groups. Since specific data on transdermal penetration of EHMC for humans are currently insufficient, yet critical to the calculation of internal exposure, we included the external aggregate exposure data for EHMC for use with more accurate absorption data hopefully available in the future (Supplementary Table S5).

The internal aggregate exposure levels for the whole modeled population are below the threshold values (DNELs) for endocrine disrupting effects; hence RCR is less than 1. However, to take into account an intense short-term exposure to EHMC related to whole-body sunscreen use on specific days, we calculated the internal aggregate exposure on a sunbathing day for each respondent. Table 3 shows that for the 99.9th percentile of the male respondents ($E_{EHMC\text{-}SUNBATHING,\ agg\ (internal)}$ 1.09 mg day^{-1} kg^{-1}) and the youngest children aged ≤4 years ($E_{EHMC\text{-}SUNBATHING,\ agg\ (internal)}$ 1.24 mg day^{-1} kg^{-1}), the $DNEL_T$ (1.00 mg day^{-1} kg^{-1}) is exceeded, which means that 0.1% of these subpopulations experience a level of exposure where RCR is larger than 1 and risk cannot be excluded. Moreover, as shown in Table 2, for the youngest children aged ≤4 years the internal aggregate exposure during the summer/autumn season is closely approaching 1. External aggregate EHMC exposure data for a sunbathing day can be found in the Supplementary Table S6.

3.2. PCP categories: contribution to aggregate exposure

To identify the key contributors to EHMC aggregate exposure, the relative contribution (%) of each PCP category was computed on the basis of aggregate population exposure by season and separately for males and females (see Fig. 2) and children.

For both genders, sunscreen was by far the major source of EHMC exposure for the summer/autumn months, with contributions of 85% and 64% and to aggregate exposure for males and females, respectively, followed face cream for males (6%) and lip care for females (13%). While sunscreen (head only application) remains the main exposure source of EHMC (38%) for males during the winter/spring months, lip care is the main source of EHMC (30%) for females. For children, the highest contribution to aggregate exposure comes from sunscreen in both seasons (summer/autumn 93%; winter/spring 50%). In the summer/autumn months, the contribution of the remaining PCP categories is very low, whereas during the winter/spring months, lip care and face cream do contribute a greater proportion of aggregate exposure, with 20 and 17%, respectively. Contributions (%) for all PCP categories for children as well as data used for Fig. 2 are available in the Supplementary Table S7.

Table 2
EHMC internal aggregate exposure levels in mg day^{-1} kg^{-1} for each season.

		Summer/Autumn (S)	Winter/Spring (W)						
Age group (years)	n	P50	P50	P95 (S/W)		P99 (S/W)		P99.9 (S/W)	
≤4	75	0.001	0.001	0.334	0.043	0.852	0.128	0.992	0.135
5–8	103	0.028	0.008	0.618	0.042	0.768	0.094	0.879	0.101
9–12	103	0.018	0.005	0.305	0.033	0.523	0.057	0.584	0.062
13–17	116	0.015	0.009	0.168	0.055	0.245	0.086	0.287	0.107
18–42	207	0.017	0.011	0.079	0.048	0.159	0.057	0.215	0.078
18–42 (female only)	131	0.020	0.014	0.098	0.065	0.182	0.076	0.227	0.079
43–52	203	0.012	0.008	0.099	0.061	0.175	0.090	0.233	0.104
53–65	210	0.012	0.009	0.069	0.045	0.110	0.060	0.148	0.070
≥66	179	0.008	0.008	0.062	0.039	0.078	0.067	0.140	0.080
All ages	1196	0.012	0.008	0.156	0.048	0.508	0.080	0.873	0.122
Female (all ages)	653	0.019	0.014	0.156	0.064	0.517	0.089	0.822	0.111
Male (all ages)	543	0.003	0.004	0.160	0.028	0.461	0.062	0.841	0.130

Table 3
EHMC internal aggregate exposure levels in mg day^{-1} kg^{-1} on a sunbathing day.

Age group (years)	P50	P95	P99	P99.9
≤4	0.001	0.583	0.938	1.237
5–8	0.142	0.527	0.602	0.891
9–12	0.010	0.518	0.687	0.781
13–17	0.067	0.407	0.538	0.744
18–42	0.027	0.394	0.479	0.543
18–42 (female only)	0.034	0.418	0.476	0.554
43–52	0.104	0.367	0.437	0.562
53–65	0.021	0.345	0.566	0.688
≥66	0.018	0.251	0.357	0.442
All ages	0.025	0.434	0.583	0.904
Female (all ages)	0.037	0.449	0.594	0.802
Male (all ages)	0.008	0.422	0.556	1.088

3.3. Modeled aggregate exposure levels: comparison of Method 1 and Method 2

In summer/autumn and on a sunbathing day, the aggregate exposure estimates for the whole population can be up to about nine times higher (sunbathing day P50) when conservatively modeled without the data on EHMC occurrence/concentrations in the specific PCPs (Table 4). In the winter/spring months, the aggregate exposure estimates are also higher, but slightly less. One has to bear in mind that in Method 2 we still used a uniform distribution for the transdermal penetration of EHMC. For a more conservative exposure assessment, the highest value available for the transdermal penetration might be used.

Table 4
Method 1 and Method 2: comparison of EHMC internal aggregate exposure levels in mg day^{-1} kg^{-1} (all ages and both genders).

	Method 1			Method 2		
	Summer/ Autumn	Winter/ Spring	Sunbathing day	Summer/ Autumn	Winter/ Spring	Sunbathing day
P50	0.012	0.008	0.025	0.040	0.022	0.224
P95	0.156	0.048	0.434	0.312	0.067	0.537
P99	0.508	0.080	0.583	0.729	0.103	0.783
P99.9	0.873	0.122	0.904	1.442	0.125	0.981

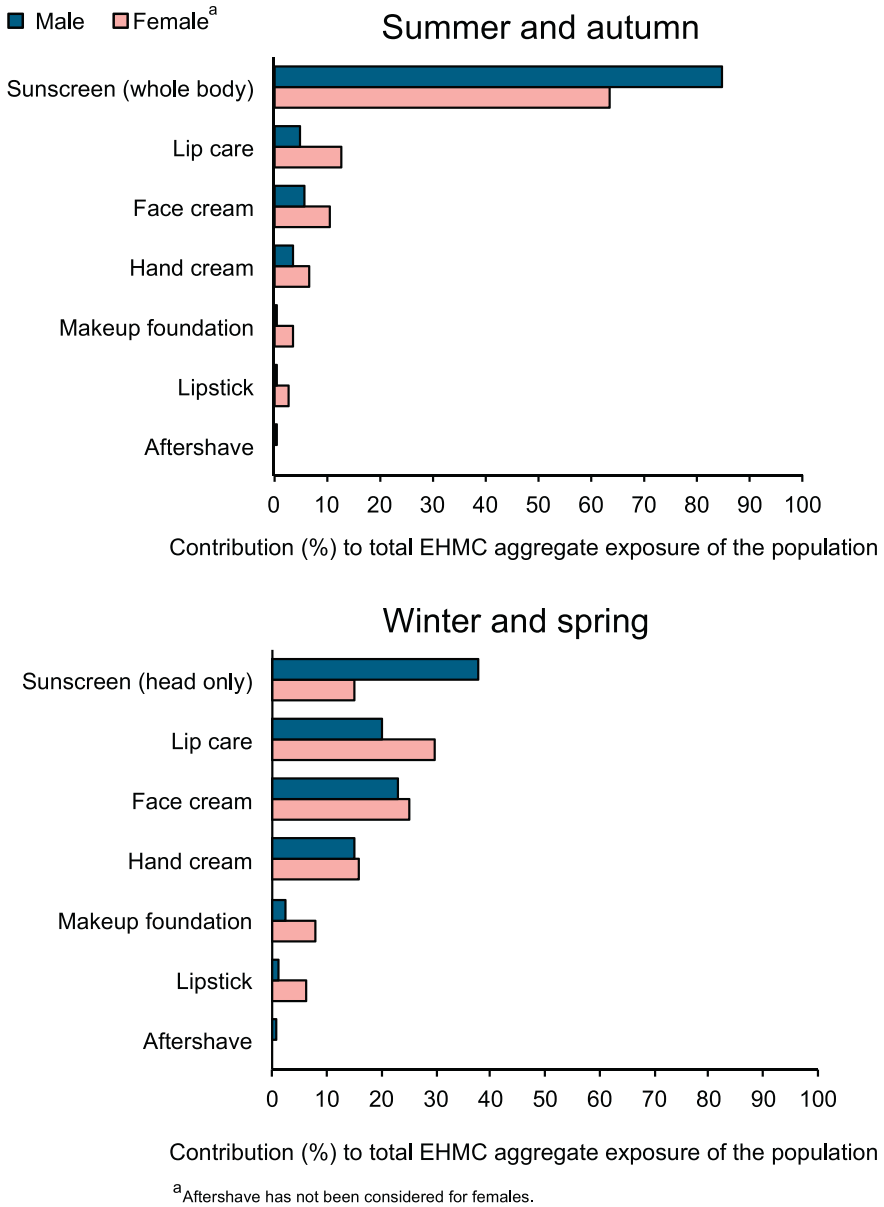

Fig. 2. Contributions (%) of the investigated PCP categories to EHMC internal aggregate exposure stratified by season and gender.

4. Discussion

The approach of using individual-level data for exposure estimation has also recently been used to calculate paraben exposure via PCP use in children aged ≤3 years old (Gosens et al., 2013), however, while assuming that all PCPs used by the target population contained parabens. This approach may be valid for substances widely used like the parabens, but for substances like EHMC, which are only present in some PCPs of a given category, identification of PCP users, who are using products without EHMC, is crucial. We have therefore refined the exposure estimation procedure by including detailed information on EHMC occurrence/concentration in the specific PCPs used by each questionnaire respondent. Our results show that excluding this information leads to considerable overestimation of exposure. Hence, the modeling method used should be chosen based on the extent of use of an ingredient in PCPs. However, this might be challenging, as data on the use of specific PCPs at an individual level are rarely available. Cowan-Ellsberry and Robison (2009) suggested that this information can be replaced by combining the extent of use of an ingredient in PCPs with the market share of the different PCP categories. There are companies offering information about market share of different PCP categories, brands, and ingredients; however, the usually very high costs of these data hinder their use in research. Additionally, because these data are not primarily collected for use in exposure assessments, they may not be fully suited for consumer exposure modeling.

Overall, children were the highest exposed individuals in our study population. Moreover, in an exposure scenario representing a sunbathing day the 99.9th percentile dose for consumers aged ≤4 years was above the $DNEL_T$, thus the risk of adverse health effects from endocrine disruption cannot be excluded. The main reasons for the high aggregate exposure levels in children were their larger ratio of surface area to body weight and the very high prevalence and frequency of sunscreen use (Manová et al., 2013a). The specific sunscreens that parents used on their children were products often explicitly labelled for use by infants and children and several of these contained EHMC. In general, children have immature organ systems and higher metabolic rates, which make them more susceptible to the effects of chemical exposures in comparison to adults. Although changes caused by early-life chemical exposures may not be immediately apparent, they may lead to late-life effects (Grandjean et al., 2008). Therefore, special caution should be taken to minimize children's exposure to any potentially harmful chemicals. On the other hand, since children's skin is also still in development, it is thought to be unusually vulnerable to UV radiation and its long-term harmful effects (Balk, 2011), thus their sun protection should be guaranteed. Sunscreen use is an important sun protective measure. However, EHMC-containing sunscreens may not offer the desired maximum level of sun protection, as EHMC photoisomerizes upon exposure to UV radiation to its *cis*-isomer that has lower absorption capacity (Pattanaargson et al., 2004). Furthermore, it can react with other UV filters to form photo-adducts (Sayre et al., 2005). As a result, the product claim may not be fulfilled (Gonzalez et al., 2007). Consequently, the use of EHMC-containing sunscreens is not advisable in children.

The Danish EPA performed a deterministic risk assessment (Andersen et al., 2012) to evaluate the human health risks from exposure to EHMC via sunscreen and face cream. The assessment focused on women of childbearing age in order to account for exposure of the fetus in sensitive stages of development. Even more than for the childhood exposure, the window of exposure plays a crucial role for fetal exposure to endocrine disruptors (WHO, 2012). Thus, short-term, but intense, EHMC exposure during pregnancy warrants special attention. The medium and maximum daily exposures to EHMC resulting from concurrent use of face cream and sunscreen on a sunbathing day were calculated by the Danish EPA to be 0.32 and 1.25 mg day^{-1} kg^{-1}, respectively. Therefore, in the maximum exposure scenario the RCR_T exceeded 1, and the outcome of the risk assessment resulted in a recommendation to limit the exposure to EHMC (e.g., by avoiding using EHMC-containing sunscreens). Comparing the results with our probabilistic calculations for women of childbearing age (18–42 years old; Table 3), the median aggregate exposure estimates in the Danish study, despite only including co-use of two PCP categories, are about ten times higher (0.03 mg day^{-1} kg^{-1}) and the maximum daily exposure compared to our 99.9th percentile are more than twice as high (0.55 mg day^{-1} kg^{-1}). The deterministically and probabilistically determined exposure values are difficult to compare, as they are based on different concepts (for more details see Supplementary Section 1.1). Nevertheless, as mentioned above, in the exposure scenario representing a sunbathing day, at the 99.9th percentile, the aggregate dose for the general male population and consumers aged ≤4 years, the RCR_T was above 1. Thus, both exposure modeling approaches suggest that some PCP users may be at risk due to short-term, but intense, sunscreen use when sunbathing.

Sunscreen represents by far the single largest source of EHMC exposure for both genders in the summer/autumn months. This finding may be surprising considering that the measured EHMC concentrations in sunscreens were, on average, lower than in the other PCP categories considered (Manová et al., 2013b). However, sunscreens have a very high prevalence of use amongst the respondents for both genders (Manová et al., 2013a) and are applied to large areas of the body, whereas the other PCP categories are applied to a significantly smaller surface area (i.e., face, lips, hands) and have much lower use prevalence (e.g., makeup foundation, aftershave). For males, sunscreen is also the major source of exposure in the winter/spring months. This can be explained by lower use prevalence and frequency of the remaining PCP categories amongst males as well as lower frequency of occurrence of EHMC in the specific products used by male respondents. Skin care PCPs that claim to reduce the risk of sun-induced premature skin aging are widely available for women; however, men's grooming, in general, is becoming more popular. According to MINTEL (2013), beauty and personal care launches that target men increased by 70% between 2007 and 2012 worldwide. Men nowadays also want to diminish the signs of skin aging and, consequently, men's skin care PCPs with UV protection are now quickly appearing on the market. In future studies, it will therefore be of interest to compare temporal trends of EHMC exposure, including the contribution of the specific PCP categories to aggregate exposure. Also, in light of the fact that EHMC occurrence in sunscreens in Switzerland has significantly declined in recent years (Manová et al., 2013b).

The aggregate exposure estimates presented in this study have some limitations. First, considerable uncertainties are associated with the available experimental data on transdermal penetration of EHMC. Second, respondents were asked retrospective questions between January and March. Therefore, we assume some level of memory recall bias, especially, in the case of the sunscreen frequency of use during the past summer/autumn (for details see Manová et al., 2013a). Third, Biesterbos et al. (2013) asked respondents to estimate the q_{PCP} visually (using photographs) or via questions describing the product amounts. Other studies are available that used the more accurate daily diary methodology combined with products weighing to determine the q_{PCP} (Loretz et al., 2005, 2006). Nevertheless, these studies only include females and were conducted in the United States (US), where the consumer habits and product container types are different than in Europe. Fourth, we focused on leave-on PCP categories left on the skin for prolonged periods, which increase the likelihood of transdermal penetration of EHMC. However, there are also rinse-off PCP categories that were not accounted for in the exposure analysis (e.g., shampoos), but still may contain low levels of EHMC. This could lead to some underestimation of the actual aggregate exposure. Fifth, a large number of products included in this study are also available elsewhere in Europe. Nevertheless, our results should be generalized with caution due to

geographical variation in the availability of PCP brands, especially the local private label brands. In countries outside Europe, diverging legislation will impact EHMC occurrence and/or concentrations in PCPs. Additionally, PCP use patterns in other countries can differ. Sixth, PCPs sold on the Swiss and EU markets must display a list of ingredients in decreasing order of concentration if >1% or a leaflet may be enclosed with the PCP or available at the point of sale (EU, 2009; VKos, 2012). However, no freely accessible database exists that contains ingredient information for all PCPs on the market. Therefore, PCP ingredient lists had to be collected for all products used by the questionnaire respondents and sometimes the product name had to be reconstructed, which is subjective, labour-intensive, and requires a very thorough knowledge of the PCPs on the market, making the approach time-consuming and costly. A publicly available international database is, thus, highly needed to provide updated reliable data on occurrence and safety of ingredients in PCPs. Such a database could also be used to determine trends over time in PCP ingredient occurrence and to identify emerging substances of concern. Furthermore, consumers nowadays demand PCPs that meet high safety and quality standards. To obtain information on ingredient safety and environmental impacts, concerned consumers nowadays have to rely on the Internet and other media, which may provide conflicting, incomplete or even false information. Consumers often lack the knowledge to evaluate the reliability and relevance of the retrieved data. Misinterpretation and misuse of scientific studies can easily occur and, in turn, influence consumers' purchase decisions. It is therefore in the manufacturers' best interest to offer objective PCP ingredient information to the public via a reliable, trusted Internet source.

5. Conclusions

In summary, our approach of using aggregate exposure modeling performed probabilistically at an individual level, while linking self-reported PCP use patterns and concentrations for the self-reported PCPs can be used to provide informative aggregate estimates of EHMC exposure and can also be applied to other ingredients present in multiple PCP categories. Our results indicate that despite the current limitations it is a highly suitable method for ingredients that are not present in all the PCPs of a certain category, as it avoids unreasonable overestimation of exposure. However, in order to routinely assess aggregate exposure at individual level in this manner, more efforts are needed to improve and accelerate the data collection and analysis methodologies, to enable more refined exposure assessments in future. In this respect, a free online international database containing reliable ingredient information for individual PCPs would benefit both consumers and scientific risk assessment.

Our results should be seen as a first step in informing scientists as well as consumers about the current EHMC exposure sources and aggregate exposure levels in Europe. Overall, for the Swiss–German population the aggregate exposure levels were below the DNELs for both estrogenic and thyroid-disrupting effects for both summer/autumn and winter/spring seasons. However, during an intense short-term exposure, such as during a sunbathing day, at the 99.9th percentile the RCR exceeded 1 for thyroid-disrupting effects for children aged ≤4 years, who might be particularly susceptible to endocrine disrupting effects, and therefore, we cannot exclude the risk of adverse health effects. Moreover, one has to keep in mind that there are significant data gaps in our knowledge of transdermal penetration and metabolism of this UV filter. To conclusively confirm or disprove the recommendation that e.g., children ≤4 years old should not be exposed to EHMC, transdermal penetration of EHMC needs to be thoroughly evaluated by a standardized and validated method, and the metabolic fate and pharmacokinetics of EHMC in humans will need to be determined.

Conflict of interest

The authors declare they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2014.09.008.

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 7:55 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 6 of 9
Attachments: 63 WHO and EDC.pdf.zip

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

World Health Organization

UNEP
United Nations Environment Programme

State of the Science of Endocrine Disrupting Chemicals - 2012

Edited by
Åke Bergman, Jerrold J. Heindel, Susan Jobling,
Karen A. Kidd and R. Thomas Zoeller

IOMC INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

State of the Science of

Endocrine Disrupting Chemicals - 2012

An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization.

Edited by
Åke Bergman, Jerrold J. Heindel, Susan Jobling,
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World Health Organization

UNEP STOCKHOLM • NAIROBI • RIO • JOHANNESBURG

1972-2012:
Serving People and the Planet

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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Preface

The *State of the Science of Endocrine Disrupting Chemicals—2012*, is an update of the scientific knowledge, including main conclusions and key concerns, on endocrine disruptors as part of the ongoing collaboration between the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) to address concerns about the potential adverse health effects of chemicals on humans and wildlife.

We live in a world in which man-made chemicals have become a part of everyday life. It is clear that some of these chemical pollutants can affect the endocrine (hormonal) system, and certain of these endocrine disruptors may also interfere with the developmental processes of humans and wildlife species. Following international recommendations in 1997 by the Intergovernmental Forum on Chemical Safety and the Environment Leaders of the Eight regarding the issue of endocrine disrupting chemicals (EDCs), WHO, through the International Programme on Chemical Safety (IPCS), a joint programme of WHO, UNEP and the International Labour Organization, developed in 2002 a report entitled *Global Assessment of the State-of-the-Science of Endocrine Disruptors*.

The Strategic Approach to International Chemicals Management (SAICM) was established by the International Conference on Chemicals Management (ICCM) in February 2006, with the overall objective to achieve the sound management of chemicals throughout their life cycle so that, by 2020, chemicals are used and produced in ways that minimize significant adverse effects on human health and the environment.

SAICM recognizes that risk reduction measures need to be improved to prevent the adverse effects of chemicals on the health of children, pregnant women, fertile populations, the elderly, the poor, workers and other vulnerable groups and susceptible environments. It states that one measure to safeguard the health of women and children is the minimization of chemical exposures before conception and through gestation, infancy, childhood and adolescence.

SAICM also specifies that groups of chemicals that might be prioritized for assessment and related studies, such as for the development and use of safe and effective alternatives, include chemicals that adversely affect, inter alia, the reproductive, endocrine, immune or nervous systems. A resolution to include EDCs as an emerging issue under SAICM was adopted in September 2012 by ICCM at its third session.

EDCs represent a challenge, as their effects depend on both the level and timing of exposure, being especially critical when exposure occurs during development. They have diverse applications, such as pesticides, flame retardants in different products, plastic additives and cosmetics, which may result in residues or contaminants in food and other products. Therefore, EDCs may be released from the products that contain them.

The protection of the most vulnerable populations from environmental threats is a key component of the Millennium Development Goals. As the challenge in meeting the existing goals increases, with work under way in developing countries to overcome traditional environmental threats while dealing with poverty, malnutrition and infectious disease, emerging issues should be prevented from becoming future traditional environmental threats. Endocrine disruption is a challenge that must continue to be addressed in ways that take into account advances in our knowledge.

UNEP and WHO, in collaboration with a working group of international experts, are taking a step forward by developing these documents on endocrine disruptors, including scientific information on their impacts on human and wildlife health and key concerns for decision-makers and others concerned. The well-being of future human and wildlife generations depends on safe environments.

UNEP and WHO convened, in December 2009, a meeting of the planning group for the development of an update to the 2002 IPCS "Global Assessment of the State-of-the-Science of Endocrine Disruptors". This was followed by teleconferences and a planning meeting

in Geneva in June 2010. These meetings allowed for defining the scope, the outline, the development process and suggestions of main authors that would be integrated in the working group. Authors were identified because of previous peer-reviewed publications and according to their area of expertise. The following experts provided guidance and expertise for the planning stages:

- Åke Bergman, Stockholm University, Sweden
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- Niels Erik Skakkebaek, University of Copenhagen, Denmark
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The working group consequently met in Stockholm in November 2010, in Copenhagen in May 2011 and in Geneva in December 2011, as well as through teleconferences, to develop and revise various drafts of the documents. Professor Åke Bergman led the working group and facilitated the development of the chapters with the main authors in coordination with UNEP and WHO.

The following international scientific experts were part of the working group that developed the documents:

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The development of these documents would not have been made possible without the significant contributions of the planning and working groups and the valuable leadership of Professor Åke Bergman, as well as of the lead authors of the main chapters Professor Susan Jobling, Dr. Jerrold J. Heindel, Professor Karen A. Kidd and Professor R. Thomas Zoeller. UNEP and WHO are very grateful for their extensive support and for the hard work of all.

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The working group members, scientific experts and contributors of text served as individual scientists and not as representatives of any organization, government or industry. All individuals who participated in the preparation of these documents served in their personal capacity and were required to sign a Declaration of Interest statement informing the Responsible Officer if, at any time, there was a conflict of interest perceived in their work. Such a procedure was followed, and no conflicts of interest were identified.

The development and publication of the two documents were supported by funds provided to UNEP by the Norwegian government, the Swedish Environment Ministry, the Swedish Research Council (FORMAS) and the Swedish Environmental Protection Agency. Further support was provided to WHO by the United States National Institute of Environmental Health Sciences (NIEHS) through cooperative agreement 1 U01 ES02617. The contents of the documents are solely the responsibility of the contributors and do not necessarily represent the official views of the NIEHS.

Executive summary

Introduction

In 2002, the International Programme on Chemical Safety (IPCS), a joint programme of the World Health Organization (WHO), the United Nations Environment Programme (UNEP) and the International Labour Organization, published a document entitled *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002). This work concluded that scientific knowledge at that time provided evidence that certain effects observed in wildlife can be attributed to chemicals that function as endocrine disrupting chemicals (EDCs); that the evidence of a causal link was weak in most cases and that most effects had been observed in areas where chemical contamination was high; and that experimental data supported this conclusion. The document further concluded that there was only weak evidence for endocrine-related effects in humans. Uncertainties regarding global endocrine disrupting effects were put forward; simultaneously, concern was expressed that endocrine disruption may affect developmental processes if exposure occurs during early life stages. Almost no data regarding endocrine-related effects were available for chemicals other than those defined as persistent organic pollutants (POPs) according to the Stockholm Convention on Persistent Organic Pollutants: polychlorinated biphenyls (PCBs), dioxins and dichlorodiphenyltrichloroethane (DDT). Even for these chemicals, the data gaps were obvious for parts of the world other than western Europe, North America and Japan. The IPCS (2002) document finally concluded that there was a need for broad, collaborative and international research initiatives and presented a list of research needs.

Since the start of this century, intensive scientific work has improved our understanding of the impacts of EDCs on human and wildlife health. Scientific reviews published by, for example, the Endocrine Society (Diamanti-Kandarakis et al., 2009), the European Commission (Kortenkamp et al., 2011) and the European Environment Agency (2012) show the scientific complexity of this issue. These documents implicate EDCs as a concern to public and wildlife health. In addition, the European Society for Paediatric Endocrinology and the Pediatric Endocrine Society have put forward a consensus statement calling for action regarding endocrine disruptors and their effects (Skakkebaek et al., 2011).

Now, in 2012, the United Nations Environment Programme (UNEP) and WHO present an update of the IPCS (2002) document, entitled *State of the Science of Endocrine Disrupting Chemicals—2012*. This document provides the global status of scientific knowledge on exposure to and effects of EDCs. It explains, in the first chapter, what endocrine disruption is all about, and then it discusses in detail, in 12 sections in the second chapter, endocrine disrupting effects in humans and wildlife. The work is based on the fact that endocrine systems are very similar across vertebrate species and that endocrine effects manifest themselves independently of species. The effects are endocrine system related and not necessarily species dependent. Effects shown in wildlife or experimental animals may also occur in humans if they are exposed to EDCs at a vulnerable time and at concentrations leading to alterations of endocrine regulation. Of special concern are effects on early development of both humans and wildlife, as these effects are often irreversible and may not become evident until later in life. The third and final chapter of this document discusses exposure of humans and wildlife to EDCs and potential EDCs.

Key concerns

- **Human and wildlife health depends on the ability to reproduce and develop normally. This is not possible without a healthy endocrine system.**
- **Three strands of evidence fuel concerns over endocrine disruptors:**
  - the high incidence and the increasing trends of many endocrine-related disorders in humans;
  - observations of endocrine-related effects in wildlife populations;

- the identification of chemicals with endocrine disrupting properties linked to disease outcomes in laboratory studies.

- **Many endocrine-related diseases and disorders are on the rise.**
  - Large proportions (up to 40%) of young men in some countries have low semen quality, which reduces their ability to father children.
  - The incidence of genital malformations, such as non-descending testes (cryptorchidisms) and penile malformations (hypospadias), in baby boys has increased over time or levelled off at unfavourably high rates.
  - The incidence of adverse pregnancy outcomes, such as preterm birth and low birth weight, has increased in many countries.
  - Neurobehavioural disorders associated with thyroid disruption affect a high proportion of children in some countries and have increased over past decades.
  - Global rates of endocrine-related cancers (breast, endometrial, ovarian, prostate, testicular and thyroid) have been increasing over the past 40–50 years.
  - There is a trend towards earlier onset of breast development in young girls in all countries where this has been studied. This is a risk factor for breast cancer.
  - The prevalence of obesity and type 2 diabetes has dramatically increased worldwide over the last 40 years. WHO estimates that 1.5 billion adults worldwide are overweight or obese and that the number with type 2 diabetes increased from 153 million to 347 million between 1980 and 2008.

- **Close to 800 chemicals are known or suspected to be capable of interfering with hormone receptors, hormone synthesis or hormone conversion. However, only a small fraction of these chemicals have been investigated in tests capable of identifying overt endocrine effects in intact organisms.**
  - The vast majority of chemicals in current commercial use have not been tested at all.
  - This lack of data introduces significant uncertainties about the true extent of risks from chemicals that potentially could disrupt the endocrine system.

- **Human and wildlife populations all over the world are exposed to EDCs.**
  - There is global transport of many known and potential EDCs through natural processes as well as through commerce, leading to worldwide exposure.
  - Unlike 10 years ago, we now know that humans and wildlife are exposed to far more EDCs than just those that are POPs.
  - Levels of some newer POPs in humans and wildlife are still increasing, and there is also exposure to less persistent and less bioaccumulative, but ubiquitous, chemicals.
  - New sources of human exposure to EDCs and potential EDCs, in addition to food and drinking-water, have been identified.
  - Children can have higher exposures to chemicals compared with adults—for example, through their hand-to-mouth activity and higher metabolic rate.

- **The speed with which the increases in disease incidence have occurred in recent decades rules out genetic factors as the sole plausible explanation. Environmental and other non-genetic factors, including nutrition, age of mother, viral diseases and chemical exposures, are also at play, but are difficult to identify. Despite these difficulties, some associations have become apparent:**
  - Non-descended testes in young boys are linked with exposure to diethylstilbestrol (DES) and polybrominated diphenyl ethers (PBDEs) and with occupational pesticide exposure during pregnancy. Recent evidence also shows links with the pain-killer paracetamol. However, there is little to suggest that PCBs or dichlorodiphenyldichloroethylene (DDE) and DDT are associated with cryptorchidism.
  - High exposures to polychlorinated dioxins and certain PCBs (in women who lack some detoxifying enzymes) are risk factors in breast cancer. Although exposure to natural and synthetic estrogens is associated with breast cancer, similar evidence linking estrogenic environmental chemicals with the disease is not available.
  - Prostate cancer risks are related to occupational exposures to pesticides (of an unidentified nature), to some PCBs and to arsenic. Cadmium exposure has been linked with prostate cancer in some, but not all, epidemiological studies, although the associations are weak.
  - Developmental neurotoxicity with negative impacts on brain development is linked with PCBs. Attention deficit/hyperactivity disorder (ADHD) is over-represented in populations with elevated exposure to organophosphate pesticides. Other chemicals have not been investigated.
  - An excess risk of thyroid cancer was observed among pesticide applicators and their wives, al-

though the nature of the pesticides involved was not defined.

- **Significant knowledge gaps exist as to associations between exposures to EDCs and other endocrine diseases, as follows:**
  - There is very little epidemiological evidence to link EDC exposure with adverse pregnancy outcomes, early onset of breast development, obesity or diabetes.
  - There is almost no information about associations between EDC exposure and endometrial or ovarian cancer.
  - High accidental exposures to PCBs during fetal development or to dioxins in childhood increase the risk of reduced semen quality in adulthood. With the exception of these studies, there are no data sets that include information about fetal EDC exposures and adult measures of semen quality.
  - No studies exist that explore the potential link between fetal exposure to EDCs and the risk of testicular cancer occurring 20–40 years later.
- **Numerous laboratory studies support the idea that chemical exposures contribute to endocrine disorders in humans and wildlife. The most sensitive window of exposure to EDCs is during critical periods of development, such as during fetal development and puberty.**
  - Developmental exposures can cause changes that, while not evident as birth defects, can induce permanent changes that lead to increased incidence of diseases throughout life.
  - These insights from endocrine disruptor research in animals have an impact on current practice in toxicological testing and screening. Instead of solely studying effects of exposures in adulthood, the effects of exposures during sensitive windows in fetal development, perinatal life, childhood and puberty require careful scrutiny.
- **Worldwide, there has been a failure to adequately address the underlying environmental causes of trends in endocrine diseases and disorders.**
  - Health-care systems do not have mechanisms in place to address the contribution of environmental risk factors to endocrine disorders. The benefits that can be reaped by adopting primary preventive measures for dealing with these diseases and disorders have remained largely unrealized.
- **Wildlife populations have been affected by endocrine disruption, with negative impacts on growth and reproduction. These effects are widespread and have been due primarily to POPs. Bans of these chemicals have reduced exposure and led to recovery of some populations.**
  - It is therefore plausible that additional EDCs, which have been increasing in the environment and are of recent concern, are contributing to current population declines in wildlife species. Wildlife populations that are also challenged by other environmental stressors are particularly vulnerable to EDC exposures.
- **Internationally agreed and validated test methods for the identification of endocrine disruptors capture only a limited range of the known spectrum of endocrine disrupting effects. This increases the likelihood that harmful effects in humans and wildlife are being overlooked.**
  - For many endocrine disrupting effects, agreed and validated test methods do not exist, although scientific tools and laboratory methods are available.
  - For a large range of human health effects, such as female reproductive disorders and hormonal cancers, there are no viable laboratory models. This seriously hampers progress in understanding the full scale of risks.
- **Disease risk due to EDCs may be significantly underestimated.**
  - A focus on linking one EDC to one disease severely underestimates the disease risk from mixtures of EDCs. We know that humans and wildlife are simultaneously exposed to many EDCs; thus, the measurement of the linkage between exposure to mixtures of EDCs and disease or dysfunction is more physiologically relevant. In addition, it is likely that exposure to a single EDC may cause disease syndromes or multiple diseases, an area that has not been adequately studied.
- **An important focus should be on reducing exposures by a variety of mechanisms. Government actions to reduce exposures, while limited, have proven to be effective in specific cases (e.g. bans and restrictions on lead, chlorpyrifos, tributyltin, PCBs and some other POPs). This has contributed to decreases in the frequency of disorders in humans and wildlife.**
- **Despite substantial advances in our understanding of EDCs, uncertainties and knowledge gaps still exist that are too important to ignore. These knowledge gaps hamper progress towards better protection of the public and wildlife. An integrated, coordinated international effort is needed to define the role of EDCs in current declines in human and wildlife health and in wildlife populations.**

General aspects on endocrine disruption (chapter 1)

The present document uses the same definitions of EDCs and potential EDCs that were developed in IPCS (2002): "An *endocrine disruptor* is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"; and "A *potential endocrine disruptor* is an exogenous substance or mixture that possesses properties that might be expressed to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations".

In addition to the key concerns presented above, the most relevant main messages from chapter 1 are presented below:

- What is endocrine disruption all about? Some endocrine disruptors can act directly on hormone receptors as hormone mimics or antagonists. Others can act directly on any number of proteins that control the delivery of a hormone to its normal target cell or tissue.
- The affinity of an endocrine disruptor to a hormone receptor is not equivalent to its potency. Chemical potency on a hormone system is dependent upon many factors.
- Endocrine disruptors produce non-linear dose–response curves both in vitro and in vivo, by a variety of mechanisms.
- Environmental chemicals can exert endocrine disrupting activity on more than just estrogen, androgen and thyroid hormone action. Some are known to interact with multiple hormone receptors simultaneously.
- Sensitivity to endocrine disruption is highest during tissue development; developmental effects will occur at lower doses than are required for effects in adults.
- Testing for endocrine disruption must encompass the developmental period and include lifelong follow-up to assess latent effects.
- Endocrine disruption represents a special form of toxicity, and this must be taken into consideration when interpreting the results of studies on EDCs or when designing studies to clarify the effects of EDCs and quantifying the risks to human and wildlife health.

Over the last 10 years, it has been established that endocrine disruptors can work together to produce additive effects, even when combined at low doses that individually do not produce observable effects.

Evidence for endocrine disruption in humans and wildlife (chapter 2)

Over the last decade, scientific understanding of the relationship between exposure to endocrine disruptors and health has advanced rapidly. There is a growing concern that maternal, fetal and childhood exposure to EDCs could play a larger role in the causation of many endocrine diseases and disorders than previously believed. This is supported by studies of wildlife populations and of laboratory animals showing associations between exposure to EDCs and adverse health effects and by the fact that the increased incidence and prevalence of several endocrine disorders cannot be explained by genetic factors alone. Epidemiological studies to date have explored quite narrow hypotheses about a few priority pollutants, without taking account of combined exposures to a broader range of pollutants. The main messages for each endocrine disease or disorder described in chapter 2 are presented below, focusing on advances in knowledge and understanding since publication of the IPCS (2002) report.

Female reproductive health

- Increased understanding of endocrine pathways governing female reproductive processes suggests that a role for EDCs in the multicausality of female reproductive dysfunction is biologically plausible.
- There is limited and conflicting experimental and epidemiological evidence to support a role for EDCs in advancing puberty and breast development and in causing fibroids (phthalates) and endometriosis (PCBs, phthalates and dioxins) and almost no evidence for causation of polycystic ovary syndrome or infertility; however, few studies have examined chemical causation of these diseases directly, and very few chemicals have been investigated.
- Historically high incidences of fibroids have also occurred in seal populations in the Baltic Sea and have been associated with exposure to contaminants (particularly PCBs and organochlorine pesticides). Recovery of these populations is now occurring, following a decline in the environmental concentrations of these chemicals. More evidence now exists that reduced reproductive success in female birds, fish and gastropods is related to exposure to PCBs, organochlorine pesticides, tributyltin and dioxins. As exposure to these EDCs decreased, adverse reproductive effects in wild populations also decreased.
- There is more evidence from laboratory studies now than in 2002 that chemical exposures can interfere with endocrine signalling of pubertal timing, fecundity and fertility and with menopause.

- There are many gaps in our knowledge of endocrine disruption of the female reproductive system. Many of the mechanisms are poorly understood, and the number of chemicals that have been investigated is limited.
- There are many gaps in the available chemical test methods for screening chemicals for endocrine disrupting effects on female reproduction. Regulatory tests for many wildlife taxa are currently not developed, and the endocrine end-points measured in mammalian assays are sometimes not adequate to detect possible roles of EDCs in inducing many of the female reproductive disorders and diseases described here.

Male reproductive health

- In comparison with 2002, the incidence of testicular cancer has further increased in the European countries in which it has been carefully studied.
- Although geographical differences exist, semen quality has declined in some countries; 20–40% of young men in the general population of Denmark, Finland, Germany, Norway and Sweden have sperm counts in the subfertile range.
- Decreases in semen quality reported in Scandinavian studies parallel increases in the incidence of both genital abnormalities in babies and testis germ cell cancer in men in the same areas over the last 60 years. The occurrence of cryptorchidism at birth is associated with a 5-fold increased risk of testicular cancer and with impaired semen quality and subfecundity.
- Several epidemiological studies show weak associations between cryptorchidism in sons and exposure of their mothers to DES, paracetamol, mixtures of PBDEs or unknown pesticides during pesticide application. No associations have been found with individual pesticides, underlining the importance of including mixtures assessment in epidemiological and laboratory investigations. Studies have not identified associations with PCBs or with DDT/DDE.
- High accidental exposures to PCBs during fetal development or to dioxins in childhood increase the risk of reduced semen quality in adulthood. With the exception of these studies, there are no data sets that include information about fetal EDC exposures and adult measures of semen quality. No studies have been performed to explore the potential link between fetal EDCs and the risk of testicular cancer occurring 20–40 years later.
- Limited evidence suggests a slightly increased risk of hypospadias or of reduced semen quality associated with exposure to mixtures of endocrine disrupting pesticides. Limited evidence also suggests links between maternal phthalate exposure and reduced anogenital distance (a proxy for reduced semen quality) in baby boys. For most chemicals, potential associations between fetal exposure and childhood or adult male reproductive health have not been studied.
- An animal model for aspects of testicular dysgenesis syndrome has been established in the rat and shows an interrelationship between testicular dysgenesis and exposure to some EDCs during the fetal male programming window. There is now a mechanism demonstrated in the rat by which irreversible disorders of the male reproductive tract can be caused.
- Exposures to several anti-androgenic pesticides have been shown to induce cryptorchidism, hypospadias and reduced semen quality in rodent experiments and are also often linked to shortened anogenital distance.
- Not all effects seen in the rat appear across species, and vice versa. Recent data show that effects of phthalates in the rat are not seen in the mouse or in human testis studied in culture. For bisphenol A (BPA), the human testis model is more sensitive to toxic effects than the rat model.
- With the exception of testicular germ cell cancers, which are logistically difficult to detect, symptoms of androgen deficiency and estrogen exposure occur in a variety of wildlife species in both urban and rural environments and have been linked to exposure to chemicals in a limited number of species in some areas.
- The feminizing effects of estrogenic chemicals from sewage effluents on male fish were first reported in the 1990s and have now been seen in many countries and in several species of fish, indicating that this is a widespread phenomenon. Feminized (intersex) male fish have reduced sperm production and reduced reproductive success.
- The suite of effects seen in wildlife can be reproduced in laboratory studies in which experimental animals are exposed to EDCs.

Sex ratio

- EDC-related sex ratio imbalances, resulting in fewer male offspring in humans, do exist (e.g. in relation to dioxin and 1,2-dibromo-3-chloropropane), although the underlying mechanisms are unknown. The effects of dioxin on sex ratio are now corroborated by results obtained in the mouse model.
- EDC-related sex ratio imbalances have been seen in wild fish and molluscs, and the effects of EDCs on sex ratios in some of these species are also supported by laboratory evidence.

Thyroid-related disorders

- Compared with 2002, increased but still limited evidence exists showing associations between thyroid-related disorders and chemical exposures. There is, however, very little direct evidence that effects on thyroid hormone action mediate these associations. There is currently no direct approach to test this hypothesis on human populations.
- Some epidemiological studies report associations between chemical exposures (PCBs, PBDEs, phthalates, BPA and perfluorinated chemicals) and thyroid function, including in pregnant women, but few of these report associations with thyroid measures in the cord blood of their offspring or with abnormal function in these offspring.
- Laboratory experiments with rodents show that there are many chemicals that can interfere with thyroid function. For example, exposure to PCBs clearly reduces serum thyroid hormone levels in rodents.
- Similarly, there are chemicals that can interfere directly with thyroid hormone action in a manner that will not be captured by measuring serum hormone levels only.
- The variability of effects seen is interpreted by some to indicate that there is no convincing evidence that chemicals can interfere with thyroid hormone action in humans.
- Evidence of relationships between exposure to chemicals and thyroid hormone disruption in wildlife species has increased in the last decade, especially in relation to exposure to the flame retardant PBDEs and PCBs, but other chemicals are inadequately studied.
- The strength of evidence supporting a role for EDCs in disrupting thyroid function in wildlife adds credence to the hypothesis that this could occur in humans.
- Thyroid disruption is acknowledged to be poorly addressed by the chemical tests currently listed in the Organisation for Economic Co-operation and Development conceptual framework. Genetic lines of mice are now widely available that could help clarify the mechanisms by which chemical exposures can interfere with thyroid hormone action.

Neurodevelopmental disorders in children and wildlife

- There are some strong data sets (e.g. for PCBs, lead and methylmercury) showing that environmentally relevant developmental exposures to these EDCs and potential EDCs have caused cognitive and behavioural deficits in humans.
- Sufficient data indicate that in utero exposure to EDCs also affects cognition in animal studies, and limited data indicate that sexually dimorphic behaviours are also affected.
- Studies of exposed wildlife provide important information on exposure levels, early and subclinical effects and the clinical neurotoxicity of EDCs, because the mechanisms, underlying effects and outcomes of exposures are often similar to those in humans. Data showing effects on growth, development and behaviour in wildlife exist for some PCBs and mercury, but are sparse or non-existent for other EDCs.
- Since 2002, increased evidence supports the involvement of thyroid hormone mechanisms in neurodevelopmental disorders in humans and wildlife and the sensitivity of embryonic and postnatal development to EDCs when compared with adulthood.
- Severe thyroid hormone deficiency causes severe brain damage. Moderate (25%) or even transient insufficiency of thyroxine during pregnancy is also associated with reduced intelligence quotient, ADHD and even autism in children.
- Chemical testing strategies do not routinely require evaluation of the ability of a chemical to produce developmental neurotoxic effects in a pre-market setting.

Hormone-related cancers

- The increase in incidence of endocrine-related cancers in humans cannot be explained by genetic factors; environmental factors, including chemical exposures, are involved, but very few of these factors have been pinpointed.
- For breast, endometrial, ovarian and prostate cancers, the role of endogenous and therapeutic estrogens is well documented; this makes it biologically plausible that xenoestrogens might also contribute to risks. However, chemicals shown to be associated with breast (dioxins, PCBs and solvents) or prostate (unspecified agricultural pesticides, PCBs, cadmium and arsenic) cancer either do not have strong estrogenic potential or are unspecified. The possibilities of involvement of EDCs in ovarian and endometrial cancers have received little attention.
- For thyroid cancer, there are indications of weak associations with pesticides and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, but there is no evidence that hormonal mechanisms are involved.
- Models of hormonal cancers are not available for regulatory testing. This makes the identification

of hormonal carcinogens very difficult and forces researchers to rely on epidemiological studies. However, epidemiological studies cannot easily pinpoint specific chemicals and can identify carcinogenic risks only after the disease has occurred.

- Similar types of cancers of the endocrine organs, particularly reproductive organs, are also found in wildlife species (several species of marine mammals and invertebrates) and in domestic pets. In wildlife, endocrine tumours tend to be more common in animals living in polluted regions than in those inhabiting more pristine environments.

Adrenal disorders in humans and wildlife

- Experimental data and data from exposed wildlife populations suggest that both the hypothalamic–pituitary–adrenal (HPA) axis and the adrenal gland are targets for endocrine disruption caused by pollutants at environmentally relevant exposure concentrations; for example, adrenocortical hyperplasia is found in Baltic Sea seals exposed to a mixture of DDT and PCBs and their methyl sulfone metabolites. Despite this fact, and compared with other endocrine axes, the HPA axis has so far gained relatively little attention in endocrine disruptor research.
- Developing organs are particularly sensitive to alterations in hormone levels, and exposure to chemicals during critical windows of development may cause irreversible effects on the adrenal glands that may not be expressed until adulthood. Recent experimental data suggest that environmentally relevant exposures to pollutants (PCBs) affect development of the fetal adrenal cortex and the function of the HPA axis and induce delayed effects in the response to stress in animal models.
- For the great majority of chemicals, there is no evidence for effects of exposures on adrenal function, nor have there been any in vivo studies to test for this. A variety of chemicals and mixtures have, however, been shown to cause effects in vitro (in the H295R cell line).

Bone disorders

- Limited studies indicate that accidental poisoning of humans with hexachlorobenzene, PCBs and DDT caused bone disorders, and a plausible, although not proven, endocrine mechanism for these effects has been proposed.
- Epidemiological studies on humans also show a relationship between exposure to endocrine disrupting POPs and decreased bone mineral density or increased risk of bone fractures.

Metabolic disorders

- Obesity, diabetes and metabolic syndrome are due to disruption of the energy storage–energy balance endocrine system and thus are potentially sensitive to EDCs.
- Exposures of animal models to a variety of chemicals during early development have been shown to result in weight gain, revealing the possibility of an origin for obesity early in development. Because they are disrupting many components of the endocrine system involved in controlling weight gain (adipose tissue, brain, skeletal muscle, liver, pancreas and gastrointestinal tract), these chemicals constitute a new class of endocrine disruptors called “obesogens”.
- Obesity is also correlated with type 2 diabetes, and chemicals that have been shown to cause obesity in animal models also result in altered glucose tolerance and reduced insulin resistance.
- There are no compelling animal data linking chemical exposures with type 1 diabetes, although some chemicals can affect the function of insulin-producing beta cells in the pancreas, including BPA, PCBs, dioxins, arsenic and phthalates. Many of these chemicals are also immunotoxic in animal models, and so it is plausible that they could act via both immune and endocrine mechanisms to cause type 1 diabetes.
- Limited epidemiological data exist to support the notion that EDC exposure during pregnancy can affect weight gain in infants and children. Limited epidemiological data show that adult exposures to some EDCs (mainly POPs, arsenic, BPA) are associated with type 2 diabetes, but there are no data for type 1 diabetes, there is insufficient evidence of endocrine mechanisms and there is insufficient study of this area in general.

Immune function and diseases in humans and wildlife

- It is clear from both laboratory data and human and wildlife samples that EDCs can play a role in the development of immune-related disorders and are at least partially responsible for their rise in recent years.
- Since 2002, molecular mechanisms connecting a variety of nuclear receptors to NF-κB (one of the master regulators of inflammation and immunity) have been elucidated, and developmental immunotoxicity studies link compounds such as DES and the phytoestrogen genistein to postnatal immune disorders. Estrogen exposure has been shown to cause prostate inflammation, and BPA caused allergic sensitization, antibody production and type 2 helper T cell immune responses.

- Systemic inflammation, immune dysfunction and immune cancers such as lymphoma and leukaemia in humans have been associated with EDC exposures. These chemicals may exert their effects through nuclear receptor signalling pathways that have well-established ties with the immune system through cross-talk with inflammatory pathways.
- There are good epidemiological data associating exposure to polycyclic aromatic hydrocarbons, PCBs and other persistent POPs with autoimmune thyroid disease, exposure to phthalates and dioxins with endometriosis and allergies, and exposure to phthalates with asthma and other airway disorders. Endocrine mechanisms are not, however, clear.
- Together, these new insights stress a critical need to better understand how EDCs affect normal immune function and immune disorders and how windows of exposure may affect disease incidence (particularly for childhood respiratory diseases).

Population declines

- Wildlife species and populations continue to decline worldwide. This is due to a number of factors, including overexploitation, loss of habitat, climate change and chemical contamination.
- Given our understanding of EDCs and their effects on the reproductive system, it is extremely likely that declines in the numbers of some wildlife populations (raptors, seals and snails) have occurred because of the effects of chemicals (DDT, PCBs and tributyltin, respectively) on these species. The evidence for EDCs as a cause of these population declines has increased now relative to 2002, due to recoveries of these populations following restrictions on the use of these chemicals.
- EDCs in modern commerce with mechanisms of action similar to those of the endocrine disrupting POPs are suspected to also be a factor contributing to declines seen in wildlife species today. Demonstrating a clear link between endocrine effects in individuals and population declines or other effects will always be challenging, because of the difficulty in isolating effects of chemicals from the effects of other stressors and ecological factors. An endocrine mechanism for current wildlife declines is probable, but not proven.
- In spite of concerns about rising human populations on a global scale, numerous industrialized countries have fertility rates well below replacement levels. It has generally been accepted that socioeconomic factors play a role in these changes. It is plausible that widespread poor semen quality and subfertility levels also contribute to this trend; however, this has not been explored systematically.

Human and wildlife exposures to EDCs (chapter 3)

There is far more knowledge on exposure to EDCs and potential EDCs today compared with 10 years ago. This applies to the diversity of chemicals being implicated as EDCs and to the exposure routes and levels in humans and wildlife. As examples, brominated flame retardants were mentioned only briefly and perfluorinated compounds not at all when the IPCS document on EDCs was prepared 10 years ago (IPCS, 2002). In addition to these, there are now many more EDCs being found in both humans and wildlife. The most relevant main messages regarding exposure to EDCs follow:

- Unlike 10 years ago, it is now better understood that humans and wildlife are exposed to far more EDCs than just POPs. However, only a fraction of the potential EDCs in the environment are currently known.
- EDCs are chemically diverse, are primarily man-made chemicals and are used in a wide range of materials and goods. EDCs are present in food, nature (wildlife) and human beings. They can also be formed as breakdown products from other anthropogenic chemicals in the environment and in humans, wildlife and plants.
- Humans and wildlife are exposed to multiple EDCs at the same time, and there is justifiable concern that different EDCs can act together and result in an increased risk of adverse effects on human and wildlife health.
- Right now, only a narrow spectrum of chemicals and a few classes of EDCs are measured, making up the "tip of the iceberg". More comprehensive assessments of human and wildlife exposures to diverse mixtures of EDCs are needed. It should be a global priority to develop the capacities to measure any potential EDCs. Ideally, an "exposome", or a highly detailed map of environmental exposures that might occur throughout a lifetime, should be developed.
- Exposures to EDCs occur during vulnerable periods of human and wildlife development—from fertilization through fetal development and through nursing of young offspring—which raises particular concern.
- New sources of exposure to EDCs, in addition to food, have been identified and include indoor environments and electronics recycling and dumpsites (the latter being issues of particular concern for developing countries and countries with economics in transition). Children can have higher exposures due to their hand-to-mouth activities and higher metabolic rate.

- Not all sources of exposure to EDCs are known because of a lack of chemical constituent declarations for materials and goods.
- Spatial and temporal monitoring is critical for understanding trends and levels of exposure. This monitoring should include tissues from both humans and wildlife (representing a range of species) as well as water or other environmental compartments to capture the less persistent EDCs.
- Levels in humans and wildlife are related to how much a chemical is used. Bans on several POPs have led to declines in environmental levels and human body burdens. In contrast, there are increasing levels of some newer EDCs, such as perfluorinated alkyl compounds and replacements for banned brominated flame retardants.
- There is global transport of EDCs through natural processes (ocean and air currents) as well as through commerce, leading to worldwide exposure of humans and wildlife to EDCs.

Concluding remarks

EDCs have the capacity to interfere with tissue and organ development and function, and therefore they may alter susceptibility to different types of diseases throughout life. This is a global threat that needs to be resolved.

Progress

We are beginning to understand the importance of certain events during development and throughout the lifespan that interact with genetic background to increase susceptibility to a variety of diseases. It is clear that a large number of all non-communicable diseases have their origin during development. It is also clear that one of the important risk factors for disease is exposure to EDCs during development. Exposure to EDCs during development can, as demonstrated in animal models and in an increasing number of human studies, result in increased susceptibility to, and incidence of, a variety of diseases. These include some of the major human diseases that are increasing in incidence and prevalence around the world. The incidence of these diseases and dysfunctions is increased at current levels of exposure to EDCs in normal populations. It is also clear from human studies that we are exposed to perhaps hundreds of environmental chemicals at any one time. It is now virtually impossible to identify an unexposed population around the globe. There is an increasing burden of disease across the globe in which EDCs are likely playing an important role, and future generations may also be affected.

There have been clear benefits for human and wildlife health from the declining use of these chemicals. Government actions to reduce exposures, while limited, have proven to be effective in specific cases (e.g. bans and restrictions on lead, chlorpyrifos, tributyltin, PCBs and some other POPs). This has contributed to decreases in the frequency of disorders in humans and wildlife.

The advances in our understanding of EDCs have been based mainly on information derived from studies in developed regions. There is still a major lack of data from large parts of the world, in particular from Africa, Asia and Central and South America.

Future needs

Better information on how and when EDCs act is needed to reduce exposures during development and prevent disease from occurring. A clear example of the success of primary prevention through exposure control is lead. We have identified the following needs to take advantage of current knowledge to improve human and wildlife health by prevention of environmentally induced diseases.

A. Strengthening knowledge of EDCs: It is critical to move beyond the piecemeal, one chemical at a time, one disease at a time, one dose approach currently used by scientists studying animal models, humans or wildlife. Understanding the effects of the mixtures of chemicals to which humans and wildlife are exposed is increasingly important. Assessment of EDC action by scientists needs to take into account the characteristics of the endocrine system that are being disrupted (e.g. low-dose effects and non-monotonic dose–response curves, tissue specificity and windows of exposure across the lifespan). Interdisciplinary efforts that combine knowledge from wildlife, experimental animal and human studies are needed to provide a more holistic approach for identifying the chemicals that are responsible for the increased incidence of endocrine-related disease and dysfunction. The known EDCs may not be representative of the full range of relevant molecular structures and properties due to a far too narrow focus on halogenated chemicals for many exposure assessments and testing for endocrine disrupting effects. Thus, research is needed to identify other possible EDCs. Endocrine disruption is no longer limited to estrogenic, androgenic and thyroid pathways. Chemicals also interfere with metabolism, fat storage, bone development and the immune system, and this suggests that all endocrine systems can and will be affected by EDCs. Together, these new insights stress a critical need to acquire a better understanding of the endocrine system to determine how EDCs affect normal endocrine function, how windows of exposure may affect disease incidence (particularly for childhood respiratory diseases) and how these effects may be passed on to generations to come.

Furthermore, new approaches are needed to examine the effects of mixtures of endocrine disruptors on disease susceptibility and etiology, as examination of one

endocrine disruptor at a time is likely to underestimate the combined risk from simultaneous exposure to multiple endocrine disruptors. Assessment of human health effects due to EDCs needs to include the effects of exposure to chemical mixtures on a single disease as well as the effects of exposure to a single chemical on multiple diseases. Since human studies, while important, cannot show cause and effect, it is critical to develop cause and effect data in animals to support the studies on humans.

B. Improved testing for EDCs: Validated screening and testing systems have been developed by a number of governments, and it requires considerable time and effort to ensure that these systems function properly. These systems include both in vitro and in vivo end-points and various species, including fish, amphibians and mammals. New approaches are also being explored whereby large batteries of high-throughput in vitro tests are being investigated for their ability to predict toxicity, the results of which may be used in hazard identification and potentially risk assessment. These new approaches are important as one considers the number of chemicals for which there is no information, and these high-throughput assays may provide important, albeit incomplete, information. An additional challenge to moving forward is that EDC research over the past decade has revealed the complex interactions of some chemicals with endocrine systems, which may escape detection in current validated test systems. Finally, it will be important to develop weight-of-evidence approaches that allow effective consideration of research from all levels—from in vitro mechanistic data to human epidemiological data.

C. Reducing exposures and thereby vulnerability to disease: It is imperative that we know the nature of EDCs to which humans and wildlife are exposed, together with information about their concentrations in blood, placenta, amniotic fluid and other tissues, across lifespans, sexes, ethnicities (or species of wildlife) and regions. Many information gaps currently exist with regard to what is found in human and wildlife tissues, more so for developing countries and countries with economies in transition and for chemicals that are less bioaccumulative in the body. Long-term records to help us understand changes in exposures exist only for POPs and only for a few countries.

In addition, there is a need to continue expanding the list of chemicals currently examined to include those contained in materials and goods as well as chemical by-products; it is impossible to assess exposure without knowing the chemicals to target. The comprehensive measurement of all exposure events during a lifetime is needed, as opposed to biomonitoring at specific time points, and this requires longitudinal sampling, particularly during critical life stages, such as fetal development, early childhood and the reproductive years. Wildlife and humans are exposed to a wide variety of EDCs that differ greatly in their physical and chemical properties. Further, these compounds are generally present at trace concentrations and in complex matrices, requiring highly selective and sensitive analytical methods for their measurement. The wide range of different compound classes requires a variety of analytical approaches and techniques, making it challenging to understand all of the different chemicals in the environment and in human and wildlife tissues. There is a growing need to develop new analytical techniques and approaches to prioritize the assessment of EDCs. There is global transport of EDCs through natural processes (ocean and air currents) as well as commerce, leading to worldwide exposures. New sources of exposure to EDCs, in addition to food, have been identified and include indoor environments and electronics recycling and dumpsites (of particular concern in developing countries and countries with economies in transition). The sources and routes of exposure to EDCs need to be further investigated.

D. Identifying endocrine active chemicals: Identifying chemicals with endocrine disrupting potential among all of the chemicals used and released worldwide is a major challenge, and it is likely that we are currently assessing only the "tip of the iceberg". It is possible to trace high production volume chemicals, but that is not the case for the numerous additives and process chemicals. Adding greatly to the complexity, and to the number of chemicals in our environment, are the unknown or unintended by-products that are formed during chemical manufacturing, during combustion processes and via environmental transformations. While the active ingredients in pharmaceuticals and pesticides have to be documented on the final product, this is not the case for chemicals in articles, materials and goods. Personal hygiene products and cosmetics require declarations of the ingredients, and the number of chemicals applied in this sphere of uses counts in the thousands. Many sources of EDCs are not known because of a lack of chemical constituent declarations in products, materials and goods. We need to know where the exposures are coming from.

E. Creating enabling environments for scientific advances, innovation and disease prevention: Exposure to EDCs and their effects on human and wildlife health are a global problem that will require global solutions. More programmes are needed that foster collaboration and data sharing among scientists and between governmental agencies and countries. To protect human health from the combined effects of EDC exposures, poor nutrition and poor living conditions, there is a need to develop programmes and collaborations among developed and developing countries and those in economic

transition. There is also a need to stimulate new adaptive approaches that break down institutional and traditional scientific barriers and stimulate interdisciplinary and multidisciplinary team science.

F. Methods for evaluating evidence: There is currently no widely agreed system for evaluating the strength of evidence of associations between exposures to chemicals (including EDCs) and adverse health outcomes. A transparent methodology is also missing. The need for developing better approaches for evaluating the strength of evidence, together with improved methods of risk assessment, is widely recognized. Methods for synthesizing the science into evidence-based decisions have been developed and validated in clinical arenas. However, due to differences between environmental and clinical health sciences, the evidence base and decision context of these methods are not applicable to exposures to environmental contaminants, including EDCs. To meet this challenge, it will be necessary to exploit new methodological approaches. It is essential to evaluate associations between EDC exposures and health outcomes by further developing methods for which proof of concept is currently under development.

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Chapter 1

What is endocrine disruption all about?

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1.0 Introduction

We live in a world in which man-made chemicals are part of everyday life. Some of these chemical pollutants can affect the endocrine system, and as such can interfere with hormonally-controlled processes of humans and wildlife. In response to this recognition, the joint International Programme on Chemical Safety (IPCS) of WHO, UNEP and ILO (International Labour Organisation) developed in 2002 a "*Global Assessment of the State of the Science of Endocrine Disruptors*". In the intervening decade, a great deal of research has provided new information about the mechanisms by which environmental chemicals can interfere with hormone actions, the degree to which our environment is contaminated with such chemicals, and the relationship between chemical exposures and health outcomes in humans and in wildlife. The goal of this chapter is to provide an introduction to the concept of endocrine disruption before delving into the details of human and wildlife health effects in Chapter 2 and the exposure science in Chapter 3.

1.1 Overview of human and wildlife health

Chronic (non-infectious) diseases are the principal causes of sickness and death around the world (WHO, 2011; Hanson & Gluckman, 2011). In the pediatric population, this includes – but is not limited to – asthma, birth defects, neurodevelopmental disorders, cancer, diabetes and obesity (Bloom, Cohen & Freeman, 2009); in adults, this includes – but is not limited to – cardiovascular diseases (CVDs), cancer, diabetes and obesity, allergic and autoimmune diseases (Pleis, Ward & Lucas, 2010). Many of these diseases and disorders are increasing, some globally (WHO, 2011; Woodruff et al. 2004; reviewed in Chapter 2 of this document) (**Table 1.1**). Important examples are the increases in the global rates of obesity, elevated blood pressure, diabetes and metabolic syndrome. Taken together, chronic illness represents a significant burden on the world's populations (WHO, 2011).

As developed more fully in Chapter 2, the World Health Organization in 2008 estimated that 1.5 billion adults, aged 20 and older, were overweight and nearly 500 million were considered obese. In some developed countries like the USA, the prevalence reaches approximately 27% of adults and 17% of children and adolescents. Developing countries like Kuwait also have a very high prevalence and it is common to find obesity and malnutrition side by side in low- and middle-income countries. In the USA, the complications of obesity are now more financially costly than any other preventable cause of death with expenditures estimated to be 17% of all USA medical costs each year (Cawley & Meyerhoefer, 2012). Further, at age 12, obese children who remain overweight will have direct medical expenses throughout life associated with their excess weight that is estimated at US$ 6.24 billion (Trasande &Liu, 2011). Obesity is also a significant risk factor for other diseases and other disorders; worldwide estimates of billons of humans suffer from diseases associated with obesity such as glucose intolerance, insulin resistance, and raised blood pressure.

The number of diabetics in the world is expected to increase from 194 million in 2003 to 330 million in 2030 with three of four affected individuals living in developing countries. The global health expenditure on diabetes alone is expected to rise to US$ 490 billion in 2030–12% of all per capita health-care expenditures (Zhang et al., 2010). The burden of premature death from diabetes in developing countries is similar to that of HIV/AIDS, yet the problem is largely unrecognised in these areas.

Worldwide, an estimated 17 million people die of CVDs every year (mostly from heart attacks and strokes). Once associated with industrialized countries, CVDs are now emerging or rapidly increasing in some developing countries.

Alongside CVDs, adult cancers are also an increasing cause of mortality throughout the world and are exceeded only by CVDs in developed countries. As with CVDs also cancer frequency increases are strongly influenced by ageing. However, endocrine related cancers may not fully follow the same pattern. Breast cancer is the second most common cancer in the world and the most common among women. Other reproductive endocrine cancers such as prostate and cervical cancers are amongst the top ten most common cancers globally, together with colorectal, stomach, liver, oesophageal, head, neck and bladder cancers. The rates of breast, pancreatic, endometrial, prostate and kidney cancers are up to five times higher in industrialized countries than developing countries, whereas the rates of stomach cancer show decreasing trends with increasing economic development.

Limited data suggest difficulties among women to conceive and maintain pregnancy in the last two decades, (Swan et al., 1999; Chandra, 1998). Female reproductive disorders such as polycystic ovarian syndrome (PCOS), uterine fibroids and endometriosis are leading causes of sub fecundity and infertility, affecting 3 to 15%, 25-50%, and 10.35%, respectively, of women of reproductive age (Chapter 2.2). Large proportions (up to 40%) of young men in some countries have low semen quality which reduces their ability to father children (Chapter 2.3).

Table 1.1. Trends in childhood diseases and disorders. National statistics provide information on these trends.

Outcome	Years Available	Data Source	Data Description	Notes
Asthma	1980-2000	Centers for Disease Control and Prevention, National Center for Health Statistics, National Health Interview Survey	Data for ages 0-17. The NHIS is a continuing nationwide sample survey of the civilian non-institutionalized population collected by personal household interviews. In 2000, 32 374 people 18 years or older and 13 376 children aged 0-17 were interviewed. Data are based on parental response to whether child has had asthma in last 12 months (see text).	
ADHD	1997-2000	Centers for Disease Control and Prevention, National Center for Health Statistics, National Health Interview Survey	See NHIS description above. Data for ages 15-17. Terminology for this condition has evolved. The American Psychiatric Association adopted the name "attention deficit disorder" in early 1980s and revised it to "attention-deficit/hyperactivity disorder" in 1987. The NHIS of 1997-2000 used here to represent prevalence of ADHD used the term "attention deficit disorder". Data are based on parental response to the question, "Has a doctor or health professional ever told you that (child's name) had attention deficit disorder?"	Data for 1997-2000 are combined because of small response in single years. Data for children aged 5-17 are used because of difficulty in diagnosing ADHD in younger children
Mental retardation	1997-2000	Centers for Disease Control and Prevention, National Center for Health Statistics, National Health Interview Survey	See NHIS description above. Data for ages 0-17. Data are based on parental response to the to the question "Has a doctor or health professional ever told you that (child's name) had mental retardation?"	Most common definitions emphasize sub average intellectual functioning before 18 years of age, usually defined as IQ <70, and impairments in life skills. Different severity categories, ranging from mild retardation to severe retardation, are defined by IQ scores.
Childhood cancer	1974-1998	National Cancer Institute; Surveillance, Epidemiology and End Results Program (incidence); Centers for Disease Control and Prevention, National Center for Health Statistics, National Vital Statistics system (mortality).		

There are also trends in pediatric health (Woodruff et al. 2004). In the United States, United Kingdom and Scandinavia, the preterm birth rate has increased by more than 30% since 1981. This is of concern because these infants experience increased rates of morbidity, including respiratory and neurological conditions, and mortality during the perinatal period. They are also more likely to suffer from CVDs and obesity, lung disease, and type 2 diabetes in adulthood (Chapter 2.2). In addition, birth defects are the leading cause of infant death and certain birth defects, such as those of the male reproductive organs are rising in many countries (Caione, 2009). Neurobehavioral disorders, including dyslexia, mental retardation, attention deficit hyperactivity disorder, and autism affect nearly 20% of children in those countries where it has been evaluated; autism spectrum disorders now occur at a rate that approaches 1% (Chapter 2.6). Thyroid diseases and disorders also represent a particularly high and increasing disease burden in children and adolescents in several countries in which they have been studied (Chapter 2.5). The prevalence of paediatric asthma has more than doubled over the past 20 years, and is now the leading cause of hospitalizations and school absenteeism (Landrigan & Goldman, 2011). The incidences of paediatric leukemia and brain cancer have also risen (Woodruff et al., 2004), as well as the incidence of testicular cancer (increases of up to 400%), the most common cancer in young men in many industrialized countries (Chapter 2.3). Aside from these disease trends, there is a secular trend toward premature puberty among American and European girls which is concerning because it can lead to reduced adult height, increased risk of breast cancer and polycystic ovarian syndrome, and a greater likelihood of engaging in risky behaviours (i.e. smoking, unprotected sex, alcohol and drugs; Chapter 2.2).

These public health statistics have important parallels in some wildlife populations. For example, testicular non descent was observed in 68% of males in a population of black deer in Alaska; similar trends were also observed in Montana. There is recent evidence that animals living near humans have trended toward increasing body weight (Klimentidis et al., 2011). All of these diseases have both a genetic and an environmental component and because the increase in incidence and prevalence cannot be due solely to genetics, it is important to focus on understanding the contributions made by the environment, often easier to study in wildlife than in

human populations. For example, as early as 1915, lead-related neurological disorders were observed in horses and cattle living near industrial facilities; adverse neurological effects of mercury were seen in the local wildlife species in Minimata Bay (Japan) before they were seen in the human residents there and in those living in the Great Lakes basin (Chapter 2.6). Differences in mammary cancer prevalence between carnivores and herbivores and between captive and wild carnivores are striking and support the hypothesis that diet is a major risk factor for these cancers. In the St Lawrence estuary, both the beluga whale and human populations were affected by higher rates of cancer than populations in other parts of Quebec and Canada, and some of these cancers were epidemiologically related to exposure to chemical contaminants, also observed in the beluga whale. In another example, a study of more than 8 000 dogs showed that canine bladder cancer mimicked the distribution of bladder cancer among their human owners (Chapter 2.7.4).

More recent evidence of environmentally caused human diseases and disorders may exist in wildlife species. For example, an inverse association between mercury exposure and DNA methylation in the brain stem of Greenland polar bears may be related to a similar association between mercury exposure and neurological deficits in Inuit children (Chapter 2.6). Genital malformations, lowered semen quality and altered sex hormone levels seen in male fish in urban areas and amphibians in agricultural areas appear to mirror those observed in human populations in similar environments and may reflect common causes (Chapter 2.3). The apparent similarities between diseases and disorders reported in humans and in various wildlife populations are not surprising given that there is often considerable overlap between their environments and food chains as well as in their physiology.

Prüss-Üstün and Corvalán have estimated that as much as 24% of human diseases and disorders globally are due at least in part to environmental factors (Prüss-Üstün & Corvalán, 2006). This provides both a challenge to identify and address, but also a tremendous opportunity to improve human and wildlife health by improving elements of the environment that impact public and wildlife health (Landrigan & Goldman, 2011). The recognition of these challenges and opportunities, along with the fact that many of the most prevalent diseases are associated with the endocrine system, has led to a focus on chemical exposures and specifically endocrine disruptors; a subclass of chemicals that act by disrupting the normal functioning of the endocrine system.

Attention has focused increasingly over the past 20 years on the hypothesis that environmental chemicals may cause human and wildlife diseases by interfering with normal hormone action. In 2002, UNEP, in collaboration with WHO, brought together a group of scientists knowledgeable about research on endocrine disruptors to produce the IPCS Global Assessment of the State of the Science of Endocrine Disruptors document (IPCS, 2002). Since that time, a great deal of scientific work has improved our understanding of each of these issues, and these will be highlighted below. Research published over the past 10 years has confirmed the scientific complexity of endocrine disruption. As an index of the scientific complexity of this issue, the Endocrine Society published a scientific review in 2009 that cited nearly 500 scientific articles focused on various aspects of endocrine disruption (Diamanti-Kandarakis et al., 2009).

The goal of the current document is to update the 2002 IPCS document, providing a current state-of-the science of endocrine disruptors as it relates to human and wildlife population health. It is essential to frame the issue of EDCs within the context of normal endocrine function; therefore in Chapter 1, we begin with a discussion of hormones and human and wildlife health. In Chapter 2, we provide a detailed scientific review of the literature concerning human and wildlife endocrine disorders and diseases plausibly impacted by endocrine disruptors. And in Chapter 3, we review the literature documenting the exposure of humans and wildlife to environmental chemicals with endocrine disrupting properties. The following review of the state of the science of endocrine disruption was developed by a large number of experts who have contributed significantly to the primary literature, and who have an international reputation for their work.

1.2 What are hormones?

To understand endocrine disruption, we must understand the basic features of the endocrine system; a series of ductless glands that secrete hormones directly into the blood to regulate various body functions. The traditional definition of a hormone is a molecule produced by an endocrine gland that travels through the blood to produce effects on distant cells and tissues (Melmed & Williams, 2011). Traditional concepts of endocrine glands include the pituitary gland at the base of the brain, the thyroid gland in the neck, the adrenal glands in the abdomen next to the kidneys, the gonads and certain parts of the pancreas. In addition to these specialized endocrine glands, many other organs that are part of other body systems, such as the heart, body fat, muscle, liver, intestines, and kidneys – have secondary endocrine functions and also secrete hormones (**Figure 1.1**).

Hormone effects are mediated by specific proteins called receptors (defined in section 1.2.2). Without receptors, hormones cannot exert their hormonal effects. Steroid hormones tend to be carried through the blood by specific “carrier” proteins, and are able to passively enter cells and interact with receptors inside the cells. In contrast, protein and amine hormones cannot passively enter cells, so specific mechanisms must be in place to allow these hormones to affect their target organs and cells and this usually involves interactions with cell membrane associated receptors on the outside of the cell. Thyroid hormones are unique in that they act on receptors inside cells, but require specific transport proteins to gain access to the inside of a cell, unlike steroid hormones. We are learning that steroid hormones can also act through cell membrane receptors and this is likely to be important to fully understand their effects and to understand the ways in which exogenous chemicals can interfere with their actions.

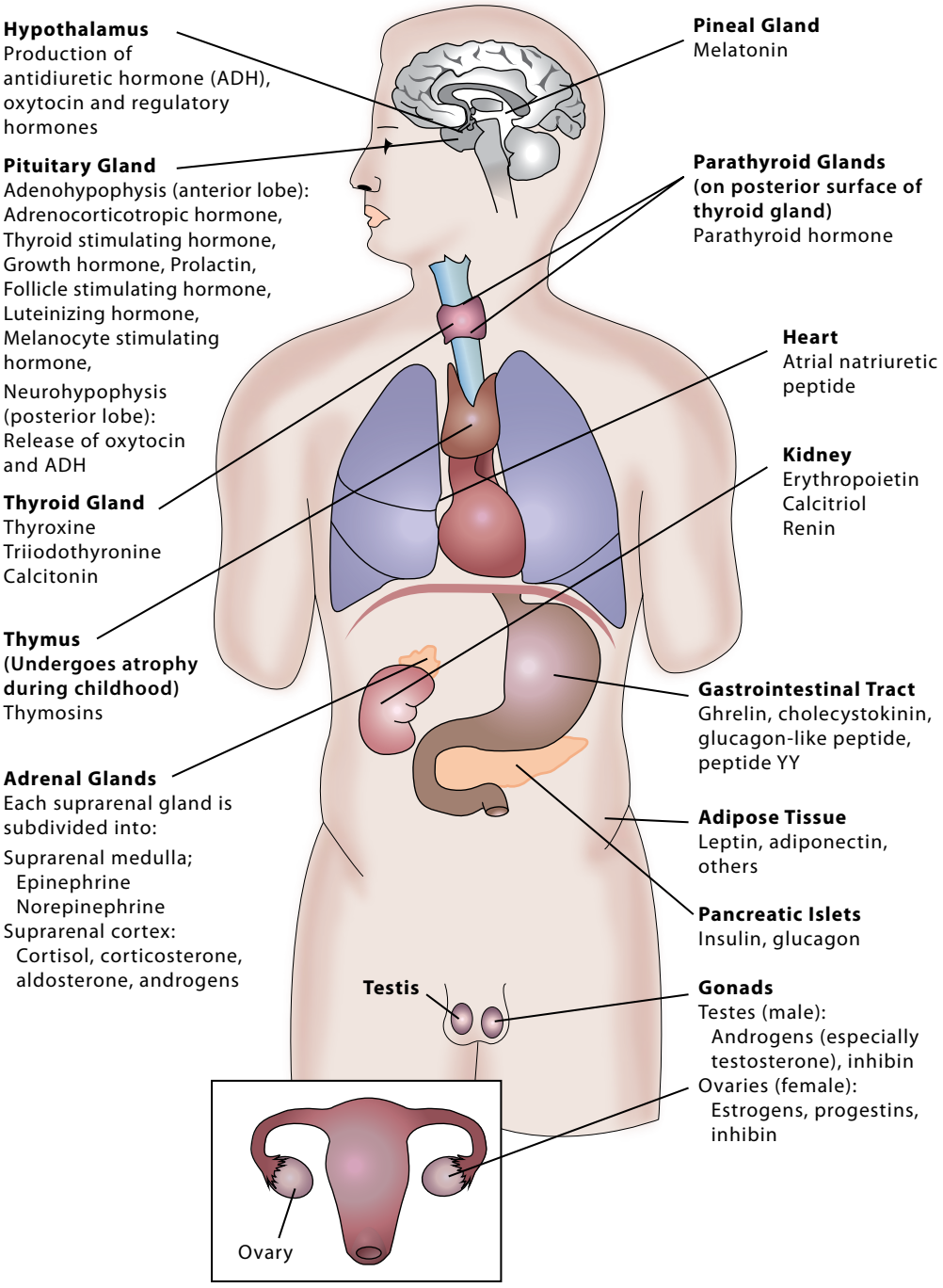

Figure 1.1. What are hormones? Hormones are molecules produced by specialized cells in a large variety of glands and tissues. These molecules travel through the blood to produce effects at sometimes distant target tissues.

1.2.1 Hormones control major physiological processes

Hormones are important to both vertebrates and invertebrates. They are essential for controlling a large number of processes in the body from early processes such as cell differentiation during embryonic development and organ formation, to the control of tissue and organ function in adulthood (Melmed & Williams, 2011). A well-known example is that of insulin, a small protein hormone produced by specialized cells in the pancreas called “beta cells”. These cells are stimulated to secrete insulin into the blood by the direct action of the sugar, glucose. As blood levels of glucose rise during and after a meal, it enters the beta cells through a specific protein transporter on the cell membrane and is converted inside the cell to the high-energy compound ATP. This process directly causes changes inside the beta cells, resulting in the secretion of insulin that was already produced and stored in anticipation of these events. Insulin then travels through the blood to many different tissues and cells, causing glucose to be taken up into those tissues via specific membrane receptors linked to transport systems.

As a result, blood glucose levels fall, which then shuts off insulin secretion from beta cells. In this way, insulin is important not simply to maintain glucose levels within a fairly narrow range in the blood, but it is important for tissues to be able to take up and use glucose for energy. In addition, insulin reaches the brain, where it has important effects on appetite. Because insulin rises during and after a meal, this hormone plays a role in regulating the feeling of hunger. This is typical of hormones –

Table 1.2. Basic overview of the endocrine system and hormones (not comprehensive).

Hormone System	Hormone	Actions
Ovary	Progesterone Estrogens (converted from androstenedione)	Timing of ovulation
		Supports pregnancy
		Ovulation, secondary sex characteristics, uterine growth
Testes	Androgens	Maturation of sex organs, secondary sex characteristics, body size
Placenta & uterus (during pregnancy)	Progesterone	Supports pregnancy
	Chorionic gonadotropin	Promotes maintenance of corpus luteum.
	Prolactin	Coordinates thyroid function
		Promotes growth of mammary gland & milk production
Thyroid	Thyroxine (T4)	Major product of thyroid gland, metabolism development
	Triiodothyronine (T3)	Hormonally active form of T4
	Calcitonin	Regulates blood calcium levels, stimulates bone construction
Parathyroid	Parathyroid hormone (PTH)	Regulates blood calcium levels
Pituitary gland	Growth hormone (GH)	Stimulates growth
	Thyroid stimulating hormone (TSH)	Stimulates T4 production by thyroid gland
	Follicle stimulating hormone (FSH)	Stimulates follicle maturation in ovary, stimulates spermatogenesis in testes
	Luteinizing hormone (LH)	Stimulates ovulation (females), testosterone synthesis (males)
Hypothalamus	Thyrotropin releasing hormone (TRH)	Promotes secretion of TSH and prolactin by pituitary
	Growth hormone releasing hormone	Stimulates secretion of GH from pituitary
	Growth hormone inhibiting hormone (Somatostatin)	Inhibits release of GH from pituitary
	Gonadotropin releasing hormone (GnRH)	Stimulates secretion of FSH and LH from pituitary
	Corticotropin Releasing Hormone (CRH)	Stimulates ACTH secretion from the pituitary gland
	Vasopressin	Has pressor effect on the cardiovascular system and is a major anti-diuretic hormone
	Oxytocin	Causes smooth muscle contraction including the uterus during parturition and in milk let-down
	Dopamine	Inhibits Prolactin secretion
Stomach	Gastrin	Causes secretion of gastric acid
	Ghrelin	Stimulates appetite
Pancreas	Insulin	Uptake of glucose, regulates glycolysis
	Glucagon	Release of glucose, regulates gluconeogenesis
	Somatostatin	Inhibits release of insulin and glucagon
Liver	Insulin-like growth factor (IGF)	Regulates cell growth, has insulin-like properties
Adipose tissue	Leptin	Decreases appetite, increases metabolism
Kidney	Renin	Regulates blood pressure & fluid balance
	Erythropoietin	Stimulates production of red blood cells
Adrenal gland	Cortisol	Stimulates gluconeogenesis, fat metabolism, inhibits glucose uptake into cells
	Aldosterone	Stimulates water resorption, controls blood pressure & fluid balance
	Adrenaline/epinephrine	Boosts oxygen and glucose to brain & muscles, suppresses non-emergency body responses
	Noradrenaline/Norepinephrine	Boosts oxygen and glucose to brain & muscles
	Dopamine	Regulates heart rate & blood pressure

they are primarily involved in important physiological processes, but they also act on the brain to integrate various behaviours with the specific physiological processes.

In the same way, other hormones control major physiological functions and coordinate these functions between systems (**Table 1.2**). Reproductive hormones, steroids (estrogens, androgens, progestins) and proteins (LH and FSH) control the complex physiological processes associated with reproduction. Thyroid hormones control metabolic processes and coordinate these with the many hormones involved in appetite and body weight regulation and metabolism. The adrenal hormones control the various physiological responses to stress. In addition to their actions on these physiological processes, many hormones also control their own secretion by "negative feedback". For example, thyroid hormone secretion is stimulated by a pituitary protein hormone, TSH (thyroid stimulating hormone), and thyroid hormones in turn suppress TSH. In this way, thyroid hormone levels are maintained within a relatively narrow range for an individual under normal circumstances. All hormone systems are governed to some extent by these processes so that hormone levels are at the appropriate concentration in blood to be effective at controlling physiological process. However, it is also important to note that there are times when tissues can control hormone action locally such that the level of hormone in the blood is not indicative of hormone action in the tissues (see below).

A wide variety of developmental problems and common adult diseases and disorders are well-known to be caused by abnormal endocrine function. For example, diabetes is the result of a defect or defects in insulin action. Defects might be caused by insulin not being present, being present in insufficient or excess amounts, or by a defect in the receptor that mediates insulin action. The insulin itself may be mutated, or there may be a mutation in the receptor or other proteins that are essential for insulin to act properly. Like most non-communicable diseases, diabetes is a result of a complex combination of genetic processes and the environment. Therefore, we need to measure and assess both types of factors to better understand complex disease. The wealth of knowledge that has been gained by studying hormone systems and endocrine-related diseases has enhanced our ability to treat people with these diseases and disorders and forms the basis of our ability to identify environmental chemicals that can interfere with hormone action and evaluate the consequences of exposure to these chemicals.

1.2.2 Hormones act on receptors

Hormones produce effects by acting on specialized proteins called receptors (see **Figure 1.2**), which attract and bind to specific hormones. Hormone receptors provide specificity to hormone actions, both in terms of the time and the place of hormone action. Hormone receptors are always limited in their abundance and cause limited and specific effects downstream of hormone binding. Most hormones do not act in all cells because their receptors are not found in all cells. Most hormones also do not act at all times during the life cycle because their receptors are found only at specific times during development or in adulthood.

Importantly, hormones do not act the same way in all cells or in the same cell at different developmental times, and we are beginning to understand the physiology behind these differences. In some cases, multiple receptor types mediate the actions of a single hormone. For example, there are two receptor classes for thyroid hormones (TRα and TRβ), and they have different temporal and spatial patterns of expression. The spatial distribution of hormone receptors can vary widely and this accounts for the degree to which the hormone has global effects. For example, insulin receptors are found throughout the body and this accounts for the ability of insulin to affect all tissues. In contrast, receptors for the hormone Thyroid Stimulating Hormone (TSH) are found predominantly in the thyroid gland. This limited distribution accounts for the much more restricted impact of TSH in the body.

Likewise, estrogens exert their effects by acting on at least two major nuclear receptor types (Estrogen Receptor alpha and beta; ERα and ERβ), although they also act by specific membrane receptors on some cells. There is still uncertainty about how many different kinds of receptors mediate estrogen actions. In contrast, testosterone exerts its effects by acting on a single Androgen Receptor (AR). Despite the fact that some hormones act through multiple receptors and some hormones act through a single receptor, in all cases the actions of a hormone in one cell type are different from the actions of that hormone in another cell type. There are important processes that contribute to the cell-specific nature of hormone actions, and it is important to understand how this happens so that the effects of environmental chemicals that interfere with these processescan be better predicted.

In the case of nuclear receptors – receptors for steroid and thyroid hormones – the hormone-receptor complex binds to specific regions of DNA to regulate the process of gene transcription resulting in the formation of new proteins (**Figure 1.2**). These hormones regulate different genes in different cell types, or at different times during development. While this flexibility is in part due to having different receptor types in different cells, there are also mechanisms that allow the same receptor to have different effects in different cells. These mechanisms are not completely understood, but they include mechanisms that turn "off" or "on" specific genes independent of the hormone. Thus, a liver cell will produce different proteins than a brain cell, despite the fact that the same hormone (e.g. estrogen) can affect both cells.

Protein hormone receptors can also be located on the cell membrane (**Figure 1.2**). Insulin, for example, binds to its membrane receptor to cause cells to take up and use glucose. After insulin binds to its receptor, there are very specific responses inside the cell to cause glucose to be taken up. In this regard, it is important to recognize that insulin causes glucose uptake by different processes in different cells, even though there is only one insulin receptor. For example, insulin stimulates the production of glycogen in the liver (which sequesters glucose inside liver cells), but it activates a glucose

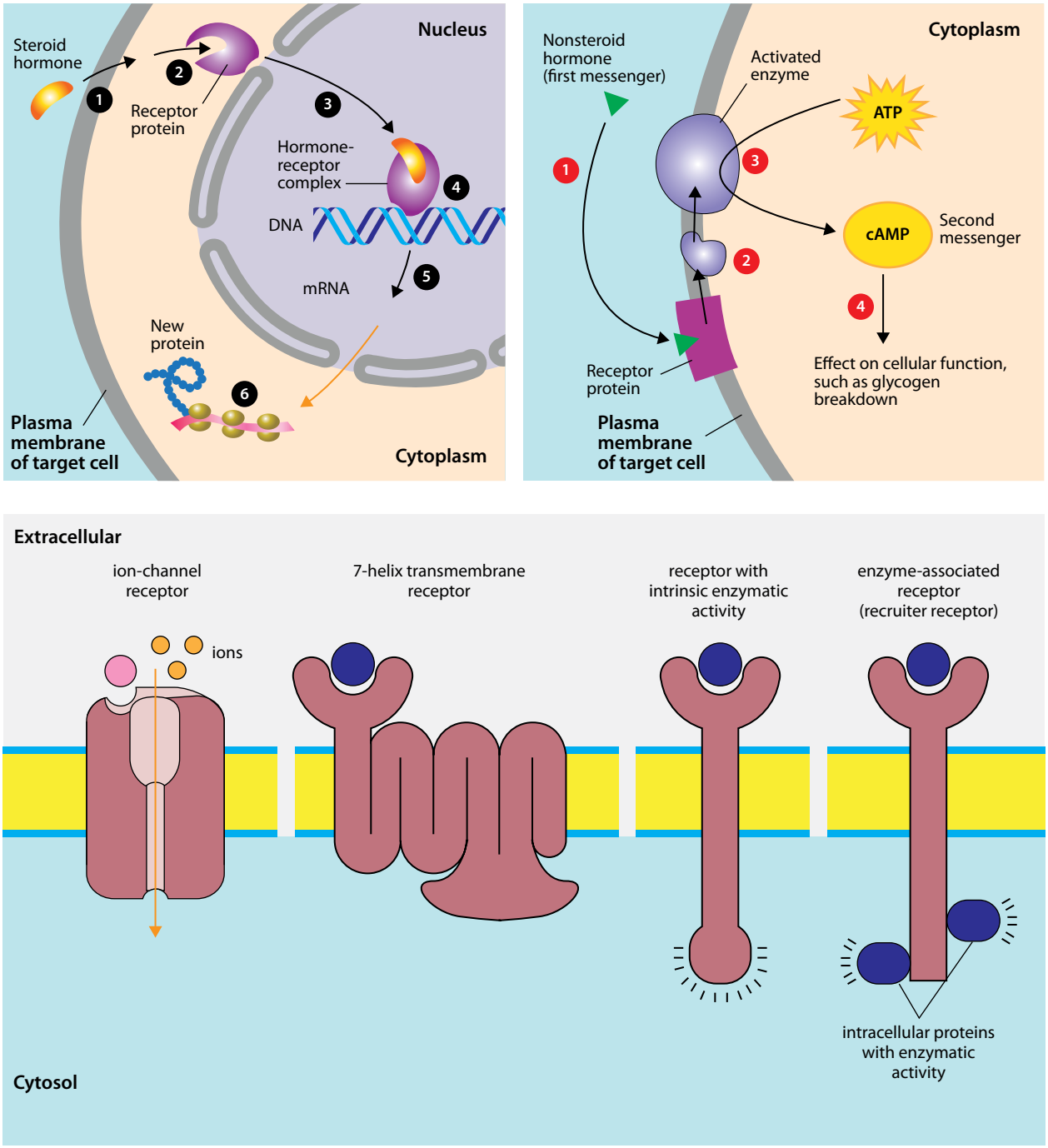

Figure 1.2. Hormones produce effects in the body exclusively by acting on receptors. There are different classes of receptors. A (Upper left): Nuclear receptors bind to steroid and thyroid hormones and act directly to regulate gene expression. B (Upper right): Membrane receptors bind to protein and amine hormones and produce effects inside the cell by a second messenger system. C (lower): Membrane receptors can be linked to a variety of second messenger systems. In addition, there are "co-regulator" proteins that link the hormone receptor to the transcriptional apparatus, and these co-regulators can differ between cells, which can affect the way a nuclear hormone receptor can function. These are important considerations because we know that environmental chemicals can interact directly with some nuclear receptors in ways that change their ability to interact with gene regulatory processes, thereby producing effects that are unexpected. These effects need to be identified and considered when we think about endocrine disruption.

transporter in other tissues, which directly stimulates glucose uptake. This occurs because the insulin receptor is linked to different kinds of cellular machinery in different cells. All protein and peptide hormones act in a similar fashion; there are specialized receptors on the outside of cells that "transduce" the effect of hormone binding to the inside of the cell. These receptors are linked to different sets of proteins in different cells, which cause the cells to respond differently to the same hormone. Interestingly, there are also membrane receptors for some steroid hormones that also act via nuclear receptors. Estrogens and progestins both act through nuclear and membrane receptors. In these cases the membrane receptor is

coupled to fast acting pathways that result in immediate effects, in contrast to the nuclear receptors, which take several hours to stimulate production of new proteins and exert effects.

Importantly, hormone receptors on the cell membrane can interact with hormone receptors in the nucleus by various mechanisms. Thus, different kinds of hormones can interact with each other through both extracellular and intracellular receptors to regulate development and various physiological processes.

1.2.3 Hormones act at very low concentrations

In general, hormones act at very low concentrations. In part, because hormones act through high affinity receptors; that is, very low concentrations of hormone can bind to the receptor population and initiate important biological effects. In addition, hormones produce a sigmoidal dose-response curve (see **Figure 1.3A**). In this case, small changes in hormone concentration at the low end of the dose-response curve produce greater differences in effect than similar changes in hormone concentration at the high end of the dose-response curve. This is important because very low concentrations of environmental endocrine disruptors could add to the endogenous hormone effect to produce a response that is much greater than would be predicted based on the hormone alone. In addition, hormone receptors can be expressed in a single cell at different concentrations, and this will affect the various characteristics of the dose-response curve (see **Figure 1.3B**). In this case, as receptor concentration increases, the dose-response curve is shifted to the left; i.e., the same biological effect is produced at lower hormone concentrations. This can explain both why some endpoints of hormone action are more sensitive to hormones than others, and can explain why some are more sensitive to exogenous chemicals than others.

1.2.4 Responses to hormones are not linear and can be "bi-phasic" (non-monotonic)

Because hormones interact with and activate their receptors in a non-linear fashion, dose-responses are at least sigmoidal, but can also be more complex, including being non-monotonic (**Figure 1.3C**). These dose response curves are often referred to as U-shaped (with maximal responses observed at low and high doses) or inverted U-shaped (with maximal responses observed at intermediate doses). In vitro studies have been instrumental in understanding the mechanisms behind non-monotonic dose responses. Studies using many hormone sensitive cell lines have shown that non-monotonic responses can be produced by a variety of mechanisms. One mechanism involves integrating two (or more) monotonic responses that affect a common endpoint. For example, studies of prostate cell lines have shown that these cells proliferate to the highest degree when provided with intermediate concentrations of androgen. The reason for this inverted U-shaped response is

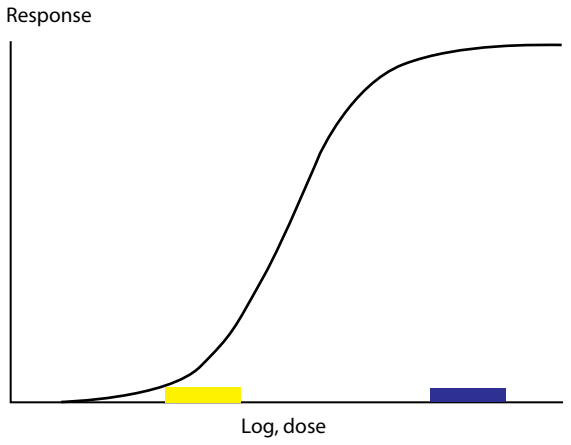

Figure 1.3A. Dose-response curve for hormones. As the dose of hormone increases, the response increases in a logarithmic manner until the point of saturation. Different hormone-receptor interactions will have differences in the dose of hormone or the dynamic range of the log-linear portion of the curve or the maximal response. Some receptors are down-regulated by the hormone, so the dose-response curve will decline at the high dose (this will be a function of both dose and time). Note that a small change in hormone concentration at the low end of the curve (yellow box) will have much greater effects on the response than a similar change in hormone concentration at the high end of the curve (blue box).

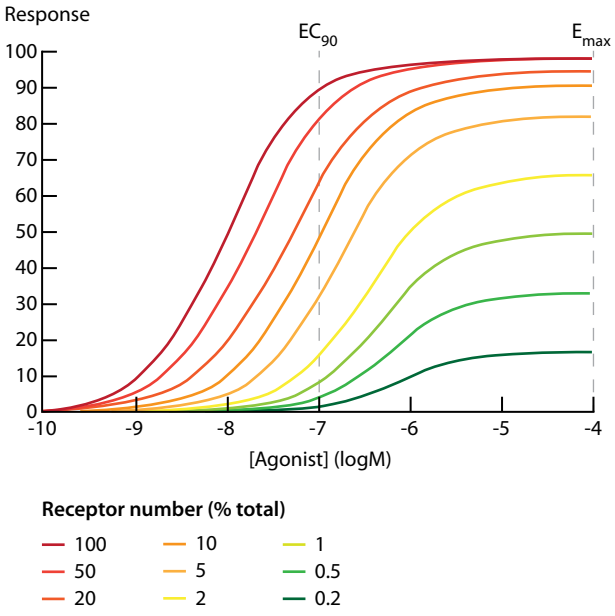

Figure 1.3B. The dose-response to the hormone depends on receptor concentration. These data show clearly that as the receptor concentration increases, the hormone becomes "more potent"; that is, it takes significantly less hormone to produce the same response. In fact, at low hormone receptor levels, the maximum response does not achieve the "EC50" response of the high receptor level. (Figure from Charlton (2009), redrawn; Used with publisher's permission).

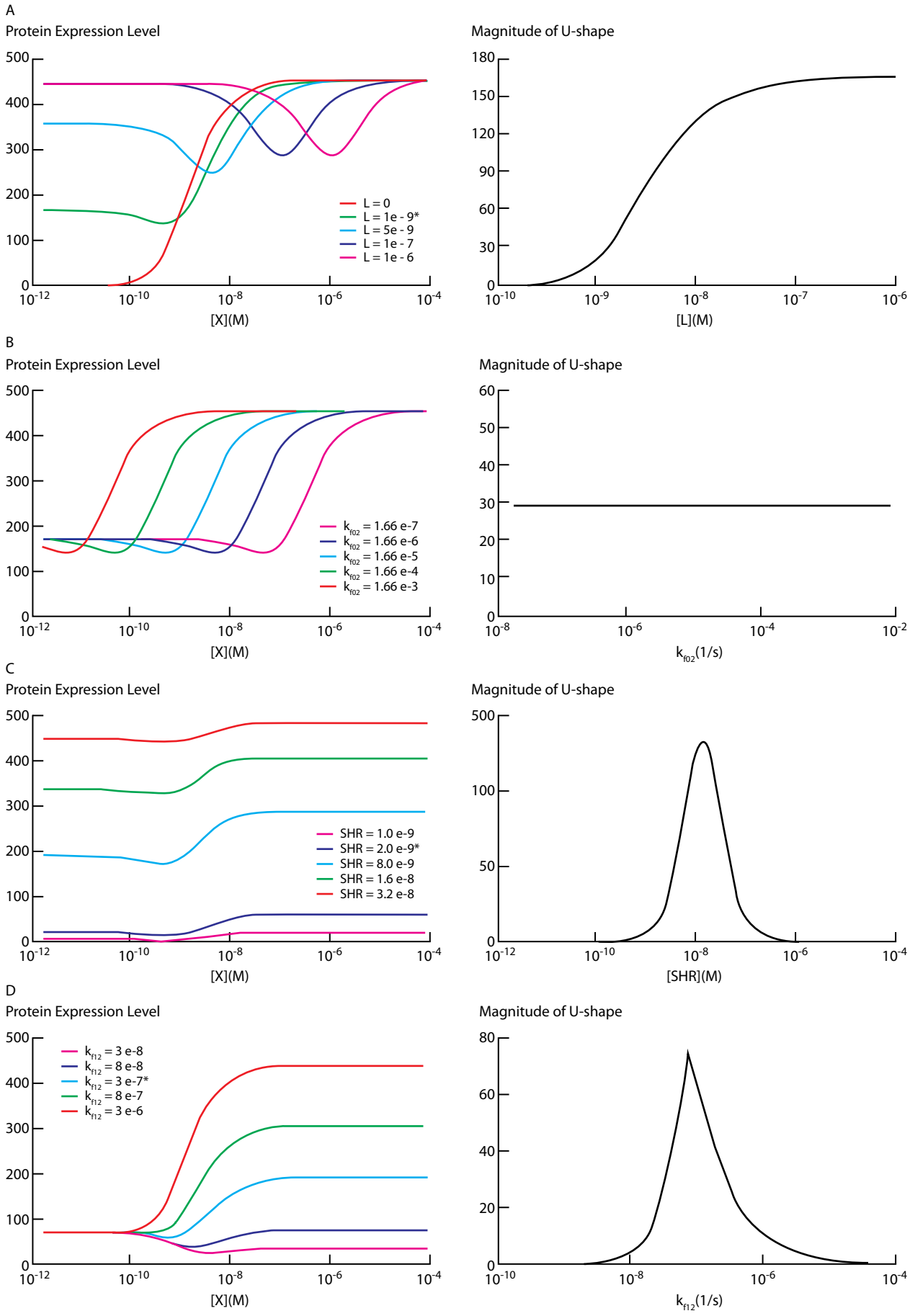

Figure 1.3C. Hormones can produce dose-response curves of various shapes including non monotonic. Non-monotonic dose-response relationship in steroid hormone receptor-mediated gene expression. (Figure from Li et al. (2007), redrawn; Used with publisher's permission).

that the cell line actually contains two populations of cells: one population proliferates in response to testosterone, while testosterone inhibits cell proliferation in the other population. At low doses, the first population has minimal proliferation, and at high doses the second population has a low level of proliferation because it is being inhibited. When looking only at cell number, intermediate doses have the maximal effect because at these concentrations the first population is somewhat proliferative and the second population is only somewhat inhibited. Ultimately, these two cell populations were isolated from each other, and when observed individually, each one had a monotonic response to androgen. Vandenberg et al. (2012) have extensively reviewed these issues.

Non-monotonic dose responses also occur as the result of receptor down regulation. When hormones are present in high concentrations, they bind to their receptors causing a down-regulation of receptor number. The degradation of receptors is increased when the hormone is abundant, and the cell's ability to replace these receptors is slower than the rate at which they are removed from the system. Thus, high concentrations of hormone lead to fewer available receptors, and a natural shift in the receptor-mediated response. In addition to this mechanism, non-monotonic responses can be caused by the increased toxicity of a hormone (cytotoxicity) at high doses. For example, the MCF7 breast cancer cell line proliferates in response to estrogen until high doses are reached (10^{-5} – 10^{-4} M) where it is cytotoxic resulting in cell death. The same toxicity has been observed in a subpopulation of MCF7 cells that no longer express the estrogen receptor, suggesting that 17β-estradiol (the natural estrogen) is not having endocrine effects at these high doses, but is generally toxic.

Finally, non-monotonic dose responses can occur because of differences in receptor affinity at low versus high doses. More specifically, low doses of estrogen bind almost exclusively to estrogen receptors, but at high doses it can also bind weakly to other hormone receptors, like androgen receptor and thyroid hormone receptor. Hormone-receptor complexes are often thought of as a lock-and-key, suggesting that the 'keys' (hormones) can actually fit multiple 'locks' (receptors) at certain concentrations. Thus, the effects seen at high doses can be due to action via the binding of multiple receptors, compared to the effects of low doses, which are only caused by action via a single receptor or receptor family.

1.2.5 When do hormones act?

Hormones act at all times during life – in utero and in early life, in childhood, puberty, adulthood and in aging (**Figure 1.4**). In fact, the timing of hormone action is an important determinant in the potency of hormone action at low doses. In the adult, hormones are thought to have transient effects on target cells and tissues. Thus, the hormone has an effect when it is present, but when the hormone is withdrawn the effect diminishes – much like insulin levels rising when blood sugar is high, and then declining when blood sugar declines. This does not diminish their importance, but contrasts with their effects in the fetus and neonate where a hormone can have permanent effects in triggering early developmental events such as cell proliferation or differentiation. Hormones acting during embryonic development can, cause some structures to develop (e.g. male reproductive tract) or cause others to diminish (e.g. some sex-related brain regions). Once

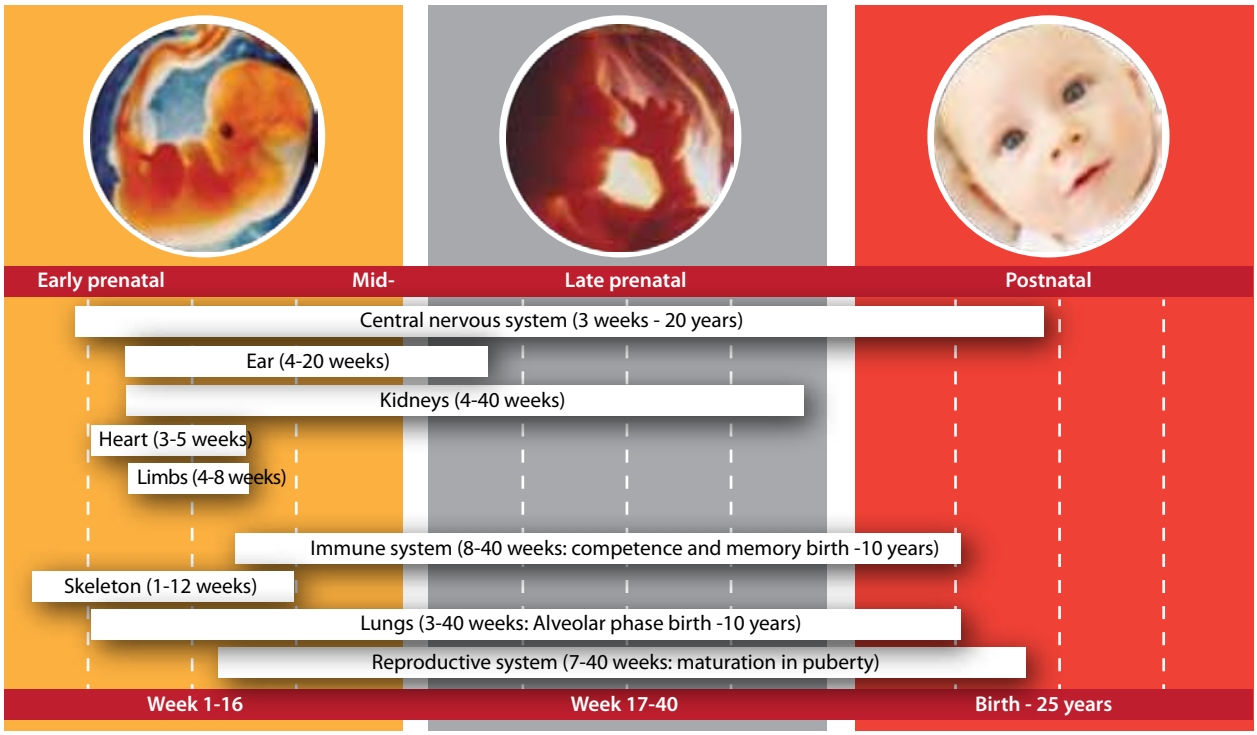

Figure 1.4. Timing of organ development. Hormones affect each of these indicating that they are important, and in different ways, throughout life.

hormone action has taken place, at these critical times during development, the changes produced will last a lifetime.

In some ways, hormone actions during development are considered to be programming events. The term "programming" in this case refers to the ability of hormones to exert effects in the fetal period that influence or dictate the functions of endocrine and physiological systems in the adult. For example, the monthly cycle of changes in hormone levels in adult women that cause ovulation, and upon which female fertility depends, is programmed during fetal development by the actions of estrogens and androgens (reviewed in Mahoney & Padmanabhan, 2010; Sarma et al., 2005)). Thus, small perturbations in estrogen action during fetal development can change the reproductive axis in adulthood and diminish fertility (Mahoney & Padmanabhan, 2010). It is now clear that fetal programming events can predispose the adult to a number of chronic diseases (Janesick & Blumberg, 2011; Hanson & Gluckman, 2011); thus, endocrine disease prevention should begin with maternal and fetal health. In some cases, experimental studies have identified the developmental events that influence adult function. An important example of this is that of thyroid hormone and brain development. It is well established that thyroid hormone is essential for brain development during the fetal and neonatal period in humans, especially as revealed in the disorder known as congenital hypothyroidism (CH) (Klein, 1980). In fact, all babies born in developed countries are universally screened for thyroid function to identify those children with a defective thyroid gland (Klein, 1980). This strategy has been successful in preventing severe mental retardation. Moreover, the fundamental knowledge we now have of the mechanisms by which thyroid hormone acts and the developmental events it controls can further guide clinical management of these and other thyroid disorders.

1.3 What are endocrine disruptors?

In the preface to the 2002 IPCS document, endocrine disruptors were referred to as "*...chemicals that have the potential to interfere with the endocrine system*"; in Chapter 1 of that document, an endocrine disruptor was defined in a generic sense as, "*...an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations.*" (IPCS, 2002). While there have been many modifications of this definition (Kortenkamp et al., 2011), we use the original for this review. However, scientific reports over the past decade support the conclusion from the ICPS 2002 document that endocrine disruptors represents a unique kind of toxicity.

If an endocrine disruptor is an exogenous chemical that alters the function(s) of the endocrine system and thereby causes adverse health effects, it is important to consider the role of hormone action in development and adult physiology as the backdrop for understanding both the potential health consequences in a population (human or wildlife) and the ways that we can observe this activity in experimental systems and in human epidemiology. The mechanism by which a chemical disrupts hormone action has a very large impact on the pattern of effects one would expect to observe. Generally, there are two pathways by which a chemical could disrupt hormone action: a direct action on a hormone receptor protein complex, or a direct action on a specific protein that controls some aspect of controlling hormone delivery to the right place at the right time. This could be a protein that is involved in hormone production (e.g. aromatase), an important transporter (e.g. sodium/iodide symporter), or a carrier protein (e.g. cortisol binding protein). Thus, a chemical could block the synthesis of a hormone, with the result that the blood levels of the hormone would increase or decline. The impact on the downstream action of that hormone would likely be the same as the situation in which hormone levels are changed because of disease or genetic defect in which hormone synthesis is inhibited or stimulated. In contrast, if a chemical interacts directly with a hormone receptor, then the effects could be quite complex and should be expected to follow the mechanisms outlined above for how hormones interact with receptors.

In the following sections, we introduce the evidence that exogenous chemicals can interfere with hormone action and produce adverse effects. It is not our intention to develop a list of known EDCs, or to identify the properties of EDCs. Rather, we provide a description of the logic that must be implemented to identify EDCs and their properties.

1.3.1 Endocrine disruptors act on major physiological systems

Endocrine disruptors interfere in some way with hormone action, and in doing so can produce adverse effects on human and wildlife health. The physiological systems affected by this disruption likely include all hormonal systems ranging from the development and function of reproductive organs to adult onset diabetes or cardiovascular disease. While there are many hormones and hormone systems (see **Figure 1.1**), most studies of endocrine disruptors have focused predominantly on chemicals that interact with estrogen, androgen and thyroid hormone systems. A growing number of studies, however, indicate that environmental chemicals can interfere with other endocrine systems (Casals-Casas & Desvergne, 2011); indeed, no endocrine system should be ruled out for being directly affected by environmental chemicals. Fat development and weight gain is a good example of complex physiological systems that are influenced by endocrine disruptors. There are a number of endocrine disruptors that have been shown to affect weight gain, insulin sensitivity and glucose tolerance (See Chapter 2 for details) indicating a potentially important role for endocrine disruptors in the development of obesity,

Table 1.3. Comparison of hormone and endocrine disruptor action.

Hormones	Endocrine Disruptors
Act via receptors	Some act on hormone receptors
Some have multiple receptors	Will cause abnormal receptors function
Tissue specific receptor classes and subtypes	Likely isoform-specific interactions
Hormones normally bind similarly to all receptor subtypes	
Active at low doses	Some act at low doses, others variable
Blood levels do not always reflect activity	Blood levels do not always reflect activity
May be bound to serum proteins in blood with small % free	May be bound to serum proteins
	Effects on hormone blood levels may not reflect on hormone action
No bioaccumulation	Possible bioaccumulation
Non-linear dose response relationships	Non-linear dose response relationships
Always saturable with variable dynamic range	Always saturable with variable dynamic range
Can exhibit non-monotonic dose-response	Can exhibit non-monotonic dose-response
High dose effects not same as low dose	High dose effects not same as low dose
Tissue and life-stage specific effects	Tissue and life-stage specific effects
Developmental effects permanent	Developmental effects permanent
Programs brain and endocrine system for adult function	Interferes with programming processes
Different end-points vary in sensitivity	Different end-points vary in sensitivity

type 2 diabetes and metabolic syndrome (Casals-Casas & Desvergne, 2011). The elements of the endocrine system that control weight gain and metabolism/energy expenditure include the adipose tissue, pancreas, GI tract, liver, skeletal muscle, bone and brain, and endocrine disruptors could specifically and directly affect each of these tissues by interfering with their various hormone systems.

1.3.2 How do exogenous chemicals interfere with hormone action?

As discussed above, environmental chemicals have been shown to exert direct actions on hormone receptors and receptor function, as well as to exert direct actions controlling hormone delivery to the receptor. Both of these pathways of endocrine disruption can be quite specific to a particular endocrine system in part because hormone receptors have features that might be somewhat unique to that system, and because the process by which a hormone is delivered to the target may be somewhat unique. Thus, there are few rules about the actions of endocrine disruptors that can be generalized to all cells and organs of the body and to all times in the life cycle. Since endocrine disruption represents a special form of toxicity, this must be taken into consideration when interpreting the results of studies of endocrine disrupting chemicals, or when designing studies to clarify the effects of endocrine disrupting chemicals and quantifying the risks to human and wildlife health.

Hormone receptors have a high affinity for their natural ligand, but typically a much lower affinity for endocrine disruptors – with some exceptions. However, it is important not to confuse *affinity* (ability to bind) for the receptor with *potency* (ability to cause effects) of action (Ruenitz et al., 1996). Although we don't fully understand this issue, the part of the chemical that controls its ability to bind to the receptor is not necessarily the same part of the chemical that controls receptor activation. Also, as reviewed in section 1.2.3, receptor abundance will impact the dose at which a hormone activates the receptor, and this will apply to an endocrine disruptor as well. This may underlie in part the tissue- or cell-specific differences in effects of endocrine disruptors. Thus, just like the hormones they interfere with, endocrine disruptors will be receptor and tissue specific. Some endocrine disruptors actually have an affinity that is similar to or greater than that of the natural ligands. An example of this is tributyltin (TBT), which has an affinity for RXR (retinoid-X-receptor) and PPARγ (peroxisome proliferator activating receptor subtype gamma) in the low nanomolar range (Grun & Blumberg, 2006). Indeed it is the most potent agonist known for these receptors.

1.3.3 Endocrine disruptors produce non-linear responses that can be non-monotonic

Because natural hormones are characterized by non-linear dose-responses, it is expected that endocrine disruptors should also produce non-linear responses. The dose-response curve can take several forms: in its simplest form – a sigmoidal shape – non-linear dose responses occur because hormones act on receptors, which are limited in number and the response itself is "saturable". That means that there is a dose of hormone – or endocrine disruptor – beyond which there is no

further response (see **Figure 1.3A**). However, like hormones, endocrine disruptors can also produce non monotonic dose responses in which the slope of the curve changes sign over the course of the dose-response. For example, when fetal mice are exposed to low or high doses of diethylstilbestrol (DES), a synthetic estrogen, their adult prostate weights are relatively low. However, intermediate doses of DES produced significantly heavier prostates (vom Saal et al., 1997). This also occurs for the female reproductive tract (**Figure 1.5**). A review of bisphenol A (BPA) has shown that there are over 50 reports on non-monotonic dose responses in a variety of tissues [see (Richter et al., 2007) and (Wetherill et al., 2007) for a review of some of these studies and (Alonso-Magdalena et al., 2008; Hugo et al., 2008; Jeng & Watson 2011; Jenkins et al., 2011; Cabaton et al., 2011) for several recent examples]. Indeed non-monotonic dose responses have been reported for more than a dozen natural hormones and more than 60 endocrine disruptors in both cell culture and animal experiments [reviewed in (Vandenberg et al., 2012)]. Recent research also suggests that non-monotonic responses can be extended to the population level. For example, individuals in the highest quartile of environmental exposure to dichlorodiphenyldichloroethane (DDE, an estrogenic metabolite of the pesticide, DDT) have decreased BMI and blood triglyceride levels compared to individuals in the third quartile (Lee et al., 2011). Moreover, women exposed to the lowest and highest doses of dioxin after an industrial accident had no changes to the age at which they entered menopause, although those women exposed to intermediate doses had an increased risk of early menopause (Eskenazi et al., 2005). The actual mechanisms to explain these non-monotonic effects at the population level have not yet been identified, but it is important to recognize that these dose-response characteristics are fully within the realm of hormone action and endocrine disruption; indeed they are to be expected.

1.3.4 Do endocrine disruptors act at low doses?

Hormones act at low doses, in part by virtue of their strong affinity for their receptors (see 1.2.3). Some endocrine disruptors also have a very high affinity for nuclear receptors (e.g. tributyltin for PPARγ), and can act at very low doses primarily as a result. However, it is important to recognize that endocrine disruptors can act at low doses even if their affinity for hormone receptors is considerably lower than that of the native hormone. This can happen, in part, because the impact of small changes in hormone action at the low end of the dose-response curve is much greater than at the high end of the dose-response curve (see **Figure 1.3A**). In addition, differences in receptor abundance have a very large effect on the concentration of hormone (or endocrine disruptor) required to produce an effect (see **Figure 1.3B**). In addition, endocrine disruptors may have different potencies on different receptor isoforms (e.g. ERα or ERβ). Therefore, the "potency" of an endocrine disruptor will be highly dependent upon several important factors. This explains why some cells and tissues – or developmental time points – are much more sensitive to endocrine disruptors than others.

Table 1.4. Examples of EDCs with low dose effects (in animals) (Vandenberg et al., 2012).

Insecticides/Fungicides	Industrial/General
Chlordane	Arachlor 1221
Chlorothalonil	Bisphenol A/Genistein/DES
Chlorpyrifos	Dioxin
DDT	4-methylbenzylidene
Heptachlor	Methylparaben
Hexachlorobenzene	Nicotin
Maneb	Nonphenol
Parathion	Octyphenol
Methoxychlor	Sodium Fluoride
Tributyltin oxide	PBDEs/PCBs
Vinclozolin	Perchlorate

There are many examples of low dose effects of endocrine disruptors (**Table 1.4**), (Vandenberg et al. 2012).

We have focused our discussion on the principles of endocrinology described above and from this perspective, environmental chemicals interacting with endocrine systems can exert effects at low, environmentally relevant doses, and will exhibit dose-response curves that are non-linear and potentially non-monotonic. However, this represents one of two perspectives that are currently under debate. The term "low dose" is defined in two ways. The first is a dose below that which is traditionally accepted by toxicologists as the no adverse effect level (NOAEL; Owens & Chaney, 2005). The second is that of a dose that is environmental relevant to humans, (e.g., Owens & Chaney, 2005). One perspective is that chemicals exert toxicologically relevant adverse effects in a manner that is or approximates linearity; i.e., the dose makes the poison. In addition, the endpoints traditionally captured in toxicological studies are sufficient to determine all adverse outcomes (Owens & Chaney, 2005). In contrast, the "low dose" hypothesis posits that exogenous chemicals that interact with hormone action can do so in a manner that is quite specific such that traditional toxicological endpoints are not sufficient to preclude adverse outcome, and they do so with dose responses that are nonlinear and potentially non-monotonic (Vandenberg et al., 2012).

1.3.5 When do endocrine disruptors act?

Endocrine disruptors can act throughout life just as hormones do by interacting with the same pathways as hormones (see **Table 1.2**). When chemicals with endocrine disrupting activity are present during development, they will affect programming of cell and tissue development and thus their effects are expected to be permanent. When the same endocrine disruptor

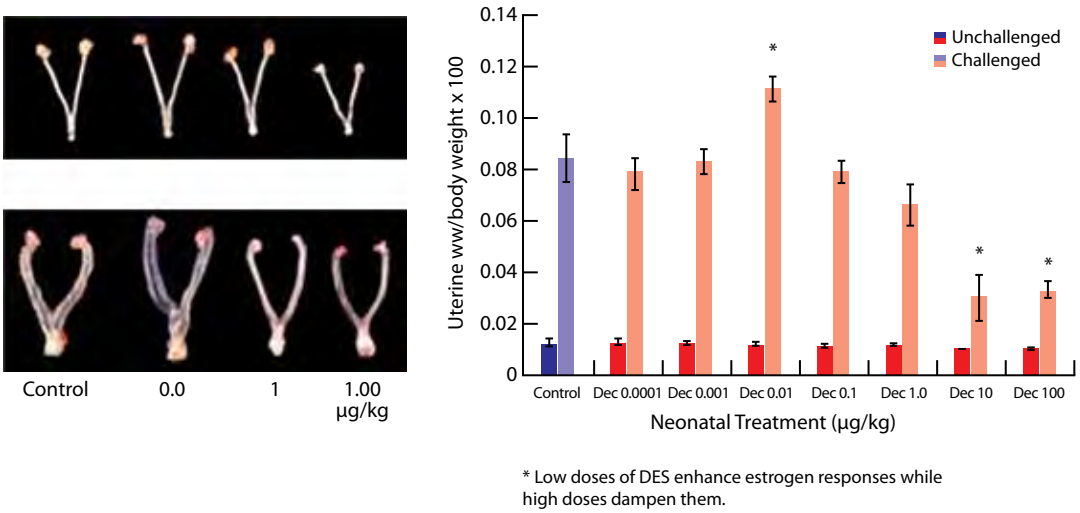

Figure 1.5. Non-monotonic dose response of DES on uterine weight. (Figure from Newbold et al. (2004), redrawn; Used with permission of the publisher).

is present later – in childhood or in the adult – the effects will be different and could be transient. The difference in sensitivity and action of endocrine disruptors over the lifespan has several important implications. First, when studies are designed to link human exposures to specific outcomes, it is important to measure chemical exposures at the developmental time-point that is appropriate for the specific outcome measured. Of course, the outcome may not be visible until adulthood in some cases. This may be more difficult for chemicals that do not persist in the body (e.g. many pesticides), than for chemicals that do (e.g. flame retardants, POPs). Another important implication is that not all endpoints of hormone action will exhibit the same sensitivity to chemical exposures.

The ability of endocrine disruptors to alter the normal hormonal control of development is perhaps the most significant consequence of exposure, because developmental effects will occur at lower doses than are required for effects in adults (Alonso-Magdalena et al., 2010). Additionally, the effects of exposure to endocrine disruptors during development will remain throughout life, due to their effects on programming of cell differentiation and tissue development, resulting in a tissue that has a different predisposition for disease in adulthood to that of a non-exposed tissue. For example, a low dose of bisphenol A during fetal mouse development predisposes the prostate to the cancer-causing actions of low doses of estrogen during adulthood but at birth the prostate "looks" normal and the effect of BPA can only be picked up by an 'omics analysis (Prins et al., 2008). **Table 1.1** shows a list of diseases that have been observed in animal models after acute exposures to endocrine disrupting chemicals during development. This list contains the diseases that were highlighted at the beginning of this chapter as those that have increased in the last few decades. In addition, similar diseases and disorders have been reported in wildlife populations in some cases in relation to exposure to chemical contaminants that are known to be endocrine disruptive. The diseases caused by endocrine disruptors will be described in more detail in Chapter 2.

1.3.6 Endocrine disruptors, the epigenome and transgenerational effects

Parents pass on their genes to their children, and in so doing, pass on various traits associated with those genes. The combination of all genes in a species is referred to as the "genome" and "genomic" studies refer to those studies designed to understand how various patterns of genes are controlled. Cells in the body can pass on heritable traits to their cellular progeny without altering their genome. During development, a single cell – the ovum – will divide, multiply and differentiate and ultimately become an adult. Development from this perspective is a process of "fate restriction" – permanently turning on or turning off different combinations of genes required for a cell to be a functional cell in the liver, kidney, brain, etc. It turns out that this view of development is not complete. Instead it is clear that development is controlled not only by genetics but also by epigenetics, and thus, is subject to changes depending on the "environment". Epigenetics is broadly defined as those heritable changes in the genome not dependent upon changes in genetic sequences (e.g. DNA methylation or histone modification). It is these epigenetic processes that define and control tissue development by controlling gene expression. Thus, a major route by which hormones act during development is by changing the epigenome – the combination of genes that can or cannot be expressed. Though the mechanisms underlying these effects are a relatively new area of study, one manifestation of endocrine disruption is to alter a small subset of hormone-dependent epigenetic mechanisms and thereby alter development. Importantly exogenous chemicals have also been shown to produce heritable "transgenerational"

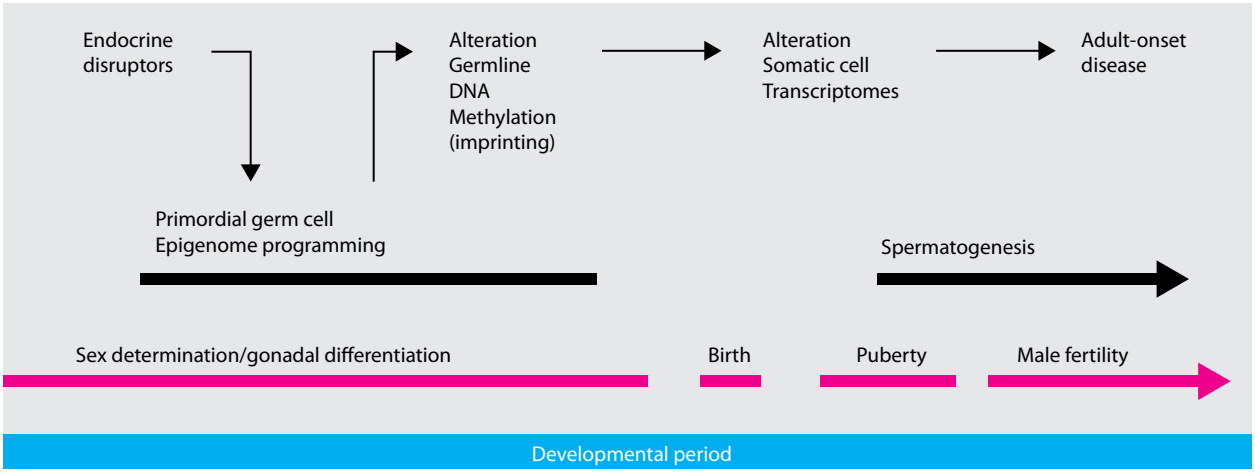

Figure 1.6. Potential mechanism by which EDCs may affect disease transmission across generations. In this scenario, EDC exposure during the period of germ cell programing can alter epigenetic marks which are then transmitted both to germ cells (i.e., gametes) and via an unknown mechanism thereby to future generations as well as in the somatic cells that develop in the embryo thereby altering tissue development. These changes have been shown in animal studies to result in adult disease (Skinner & Guerrero-Bosagna, 2009; Used with publisher's permission).

effects as a result of their ability to alter epigenetic processes. This issue first arose with studies in which an anti-androgenic pesticide (vinclozolin) was given to developing mice at a single time when the testis was in a critical period of development. Vinclozolin produced adverse effects on the developing testis, and this effect was passed on to the following three generations of mice (reviewed in (Skinner, Manikkam & Guerrero-Bosagna, 2011)). This effect is likely to be caused by epigenetic changes that were transmitted with high fidelity from one generation to the next via the germ cells (**Figure 1.6**). A number of exogenous chemicals have now been shown to influence epigenetic mechanisms and to produce effects in several generations of animals. We have a great deal to learn about this issue, but it is plausible that chemical exposures during pregnancy will affect the health of several subsequent generations of people and wildlife that are not themselves exposed.

1.3.7 Evidence for a common mechanism for human/wildlife effects

Estrogen, androgens and thyroid hormones are identical in all vertebrates. However, the receptors are somewhat different among different vertebrate classes and this can influence the ability of exogenous chemicals to interact with them. Endocrine disruptor screening methods for wildlife have been developed using estrogen receptors (ERs) and androgen receptors (ARs) from various animal species, including fish, amphibians, and reptiles (Katsu et al., 2007; 2010). These studies showed species differences in sensitivities of ERs to chemicals. Therefore, in order to protect biodiversity from endocrine disruptors, we need to understand the molecular mechanisms underlying species differences in hormone receptor sensitivity in various wildlife species. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g. hormone synthesis, transport and metabolism, activation of nuclear receptors, gene methylation) have been shown to be equally important (IPCS, 2002; Tabb & Blumberg, 2006). For most associations reported between exposure to endocrine disruptors and a variety of biological outcomes, the mechanisms of action are poorly understood.

The endocrine systems of vertebrates largely share molecular mechanisms such as the ability of particular chemicals to bind to steroid receptors (Iguchi & Katsu, 2008). However, the physiological consequences of these mechanisms - for instance, for sex differentiation - differ in different classes of vertebrates. For example, sex is determined by the *sry* gene in mammals (Sinclair et al., 1990), the *dmy* gene in the medaka fish (Matsuda et al., 2002), whereas temperature-dependent sex determination is common in crocodilians and turtles. Estrogen is quite important in the development of ovaries in fish, amphibians, and reptiles, and very likely plays a role in birds as well. Likewise, critical developmental windows of sensitivity - periods when hormonal or xenobiotic chemicals can act during development - differ among vertebrate species.

In insects and crustaceans, reproduction and development are controlled mainly by novel steroids termed ecdysteroids, such as ecdysone and juvenile hormones. The functions of these are well understood in model species of insect (*Drosophila)* and in some aquaculture species of crustacean. For the remaining invertebrate species, information on the endocrine system and the hormone receptor system is limited. The ecdysone receptor has been cloned in *Daphnia magna* (Kato et al., 2007), but no juvenile hormone receptor or binding protein has been identified.

Of the "vertebrate" steroid hormones and their receptors, ER homolog genes have identified in molluscs such as *Aplysia,* octopus, and a marine snail (rock shell; *Thais clavigera*) showed no ligand binding, but they did display ligand-independent gene activation (Thornton, 2003). Thus, functional

nuclear-type ER may not be present in these invertebrates. In annelids, however, a functional ER activated by estradiol has been isolated (Keay & Thornton, 2009) and in rotifers, a membrane progesterone receptor may also be functional (Stout et al., 2010) Membrane ERs have been found in vertebrates and act as an acute response system to estrogens. Therefore, we cannot rule out the possibility that membrane ERs could be present in invertebrate species.

Despite our lack of knowledge on their fundamental endocrinology, chemicals that affect hormonal activities in vertebrates also appear to affect several invertebrate species, such as *Hydra vulgaris,* copepods, barnacles, nematodes, freshwater mud snails, and sea urchins (Fox, 2005). Juvenile hormone agonists used as pesticides induced a reduction of reproduction in parthenogenic *D.magna* and resulted in 100% male offspring (Oda et al., 2005), as would be expected, but the effects of "vertebrate" steroid hormone antagonists and agonists are less clear. In particular, the susceptibility of molluscs to morphological and physiological disruption by estrogenic compounds is a subject of current debate. Amongst the most extreme effects reported are those exerted by bisphenol A (BPA), 4-tert octylphenol (OP) and ethinylestradiol on reproductive output and morphology of the neo-tropical freshwater snail *Marisa cornuarietis*, including increased oocyte production and egg-laying in females and gross morphological effects on the sex organs in both developing juveniles (e.g. formation of additional sex organs in females) and adults (e.g. reduction in male penis length) (Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2004). In direct conflict with these reports are those in which adult M. cornuarietis were exposed to BPA using a different experimental design (Forbes et al., 2007) showing clearly that these effects were not observed. These conflicting reports have fuelled controversy (Dietrich et al., 2006) surrounding the true sensitivity of this species, and molluscs in general, to estrogen mimics and, also, the perceived safety of the aquatic environment from the impacts of these xenestrogens. BPA, in particular, is purported to be much more potent in molluscs than in other aquatic organisms.

Clarifying the molecular basis of the action of hormones and endocrine disruptors on invertebrates is essential to aid in explaining differences in the responses of vertebrates and invertebrates at the cellular and organismal levels and to elucidate the ecological effects of exposure to endocrine disruptors starting at the bottom of the food chain (Iguchi, Watanabe & Katsu, 2006).

1.3.8 Endocrine disruptors and cocktail effects

When the toxicity of chemicals is evaluated, their effects are usually considered in isolation, with assumptions of "tolerable" exposures derived from data about one single chemical. These assumptions break down when exposure is to a large number of additional chemicals that also contribute to the effect in question. This can be illustrated by considering combined actions between estradiol and other chemicals capable of mimicking the hormone's action. For a long time, the risks associated with these "xenestrogens" have been dismissed, with the argument that their potency is too low to make an impact on the actions of estradiol. But it turned out that xenestrogens, combined in sufficient numbers and at concentrations that on their own do not elicit measureable effects, produced substantial estrogenic effects (Silva, Rajapakse & Kortenkamp, 2002), an observation dubbed "something from 'nothing". When mixed together with estradiol, the presence of these xenestrogens at low levels even led to a doubling of the effects of the hormone (Rajapakse, Silva & Kortenkamp, 2002). All these effects could be predicted accurately from the potency of all single components by making the assumption that the xenestrogens acted together without influencing each other's action (the additivity assumption according to the mixture assessment concept of concentration addition).

The above experiments were conducted with a yeast-based reporter gene system for estrogen action, a test system that lends itself to working cost-effectively with the large sample numbers necessary for conducting multi-component mixture experiments. But for a long time it remained untested, whether the principles of mixture toxicology established in such "test tube" experiments would be applicable to more complicated systems, including experimental animals. A break-through was made with the demonstration that multi-component mixtures of estradiol and other xenestrogens induced vitellogenin in fish in a manner that could be predicted from the effects of the single chemicals (Brian et al., 2005). At first, it seemed counter-intuitive to expect that such experiments would be successful, considering the many sources of variation and error inherent in studies with animals, but the empirical evidence showed that this is not the case.

The predictability of combination effects could be demonstrated with systems even more complex than vitellogenin induction in fish. In a developmental toxicity model in the rat, it was shown that combinations of androgen receptor antagonists worked together additively to produce changes in anogenital distance and retained nipples, effects considered to be the hallmarks of disruption of androgen action in fetal life (Hass et al., 2007). In these models, pregnant female rats were dosed throughout gestation and the effects in male offspring monitored. Similar observations were made with combinations of antiandrogens that work by a variety of different mechanisms (Christiansen et al., 2009, Rider et al., 2008). Studies with thyroid disrupting chemicals also showed additive combination effects at low doses (Crofton et al., 2005).

In many of these in vivo experiments with endocrine disruptors, the "something from 'nothing" principle was shown to apply. These findings challenge current regulatory practice. The experimental doses that are used as a basis for deriving health-based exposure standards (e.g. acceptable daily intakes) cannot be considered safe under all circumstances if exposure is to a large number of chemicals that also produce the effect

of interest. These experiments expose an important knowledge gap that currently hampers progress with the risk assessment of endocrine disrupters (and other chemicals). To truly assess the possible health risks that arise from these chemicals, it is necessary to know the full extent of exposures to exogenous chemicals that exert actions on a specific endocrine pathway, together with their potency of effect. But, we are currently far removed even from having fragmentary information about these issues (Kortenkamp & Faust, 2010). Years of mixing cocktails of endocrine disruptors have shown that the combined effects are largely additive and that the effects of multi-component mixtures can be predicted when the potency of its individual components are known (see the review by Kortenkamp, 2007). The challenge ahead is to define what environmentally relevant mixtures of endocrine disruptors are and to assess their effects.

1.3.9 Endocrine disruptors and toxicity testing methods

Man-made chemicals are an important part of modern life. Human and wildlife populations cannot avoid coming into contact with some chemicals employed in food production (plants and meat), in pathogen control (e.g. insecticides), in the production of modern materials (e.g. plastics), or in the built environment (e.g. insulations, flame retardants) (see Chapter 3 for more details). Considering the importance of these chemicals, and their widespread presence in the environment, it is important that strategies are developed to preclude widespread environmental contamination with endocrine disruptors.

Testing strategies currently employed around the world are based on the premise that endocrine disruptors can be evaluated in the same manner as acute toxicants; this implies that tests at high doses will inform us about low-dose exposures, and it also implies that one endpoint of hormone action can effectively act as a surrogate for all endocrine endpoints. However, as we have introduced in this chapter and will develop further in the following chapters, hormone action is quite complex and depends on the developmental stage and the endpoint being evaluated and that endocrine disruptors act like hormones and not general toxicants. Therefore, it is predictable that endocrine disrupting chemicals will exert effects that are also quite complex and that are not captured using strategies designed to detect acute toxicity with a limited range of exposure paradigms and endpoints evaluated. Specifically, endocrine disruptors will produce non-linear dose responses, sometimes including non-monotonic dose-responses, such that high dose toxicity testing will not be sufficient to predict the effects at low doses. In addition, chemicals that interact directly with hormone receptors may not produce effects on endpoints that are routinely employed in toxicity testing in a manner that extrapolate to other disease endpoints or doses. The sensitivity of different endpoints of hormone action is quite variable and will not be captured by a small subset of endpoints captured in general toxicity studies. Finally, the preponderance of toxicity studies have focused on chemicals that interfere with reproductive and thyroid hormones. Little is known about chemicals that can interact with other endocrine systems, and we should not assume that the current assays employed in toxicity testing will identify those chemicals. Considering this, we cannot be confident that the current system of protecting human and wildlife population from chemicals with endocrine activity is working as well as it should to help prevent adverse health impacts on human and wildlife populations.

It is important to recognize that the identification of human or wildlife health effects of chemical exposures in epidemiological studies is an indication that the pre-market evaluation of chemical effects failed to accurately predict their toxicity. A clear example of this is that of polychlorinated biphenyls (PCBs). These chemicals were produced heavily during the first half of the 20th century and their production was banned in the 1970s because of their potential carcinogenicity and because of their persistence in the environment. However, a large number of studies have now shown that prenatal exposure to these chemicals – even several decades after their production was banned – produces adverse effects on cognitive function in children (Schantz, Widholm & Rice, 2003). Experimental studies have demonstrated the different kinds of effects these chemicals have on important signaling mechanisms including on thyroid hormone, estrogen and calcium signaling in the brain. Despite this, the current guideline studies for identifying chemicals that interfere with thyroid hormone action would not identify PCBs as anti-thyroid agents. Given the importance of thyroid hormone action during development, and the successful screening program for identifying congenital hypothyroidism (CH) at birth, it is paradoxical that PCBs would be missed by these guideline studies. It is critical then to learn from this experience to avoid committing several generations of children to exposures to chemicals that limit their potential (Suvorov & Takser 2008).

1.3.10 Framework for evaluation of evidence for endocrine disruption in humans and wildlife

It is important to use a systematic and transparent approach to evaluating the scientific evidence about the relationship between environmental exposure and health effects. Often, this approach is referred to as weight of evidence (WOE), which is an approach used to characterize the extent to which the available data support the hypothesis that there is a relationship between an exposure and adverse health effect. Various WOE methods have been developed; these methods are often a qualitative process in which reviewers put various strands of evidence together to evaluate whether they are likely to support, or not some relevant associations which can include causality. Descriptors are used to characterize the overall conclusions. For example, IARC uses the terms “sufficient”, “limited”, and “inadequate”, to characterize the evidence related to carcinogenicity (IARC, 2006). Similarly, the US National

Toxicology Program (US NTP) uses the terms "sufficient", "limited" and "insufficient" to characterize the evidence (NTP, 2011). The degree of subjectivity embedded in the WOE process has led to much discussion and there are currently no internationally agreed upon methods with which to perform this process, although many proposals of how to do this exist (see the review in Kortenkamp et al., 2011). One such proposal was presented in the 2002 IPCS document (Chapter 7). Others include Conrad & Becker (2011), Woodruff & Sutton (2011; 2010) Linkov et al., 2009, ANSES (2011), and SCENIHR (2012).

WOE of evidence evaluations, and current systematic review approaches generally focus on an individual chemical exposure and a specific outcome. In contrast, WOE evaluations have not been adapted to answer the broad, more open-ended questions addressed in this report, such as "Is there evidence of endocrine disruption in wildlife?") (EFSA, 2010). In this context, narrative reviews can serve an important first step in compiling scientific evidence that addresses critical questions.

The European Environment Agency has stated that methods for evaluating evidence need to be modified to reflect reality more clearly, by including consideration of multicausality, thresholds, timing of dose, mixtures and delayed negative impacts, especially when evaluating EDCs (Gee 2006; 2008). Similarly for temporality, which says that the putative cause *X* of harm *Y* must come before *Y* appears. This is not robust in a multicausal, complex world of common biological endpoints that can have several biological pathways leading to an adverse health effect. For example, falling sperm counts can have multiple, co-risk factors and resulting overall sperm count trends could be rising, falling, or static, depending on the combined direction and strengths of the co-risk factors and the time lags of their impacts" (Gee, 2006). Chlorine chemicals may or may not be co-risk factors in falling sperm counts, but the previous use of the temporality argument in the IPCS (2002) document does not provide robust evidence that they are not a contributing factor (Gee, 2006; EEA, 2012).

Finally, the evaluation of the strength of the evidence is not the same as the strength of the recommendation. Specifically, different strengths of evidence can be appropriate to justify action to reduce exposures, or other measures, in specific cases depending on their circumstances including the cost of being wrong in both direction (i.e., in acting or not acting). This is analogous to" iatrogenic risk in medicine (risk "caused by the doctor") (NAS, 2009). In the same way that a delay in diagnosis by a physician can increase risk to the patient, delays in the process of assessing risks may increase overall exposure to risk when decisions are delayed" (NAS, 2009). Hill (1965) recognised the case of specificity of different strengths of evidence when deciding whether to act on the evidence, or not, observing "*that, it almost inevitably leads us to introduce differential standards before we convict. Thus on relatively slight evidence we might decide to restrict the use of a drug for early-morning sickness in pregnant women. If we are wrong in deducing causation from association no great harm will be done. The good lady and the pharmaceutical industry will doubtless survive. On fair evidence we might take action on what appears to be an occupational hazard, e.g. we might change from a probably carcinogenic oil to a non-carcinogenic oil in a limited environment and without too much injustice if we are wrong. But we should need very strong evidence before we made people burn a fuel in their homes that they do not like or stop smoking the cigarettes and eating the fats and sugar that they do like."* Indeed, Bradford Hill himself recognised the shortcomings of his framework, stating that *"none of my nine viewpoints can bring indisputable evidence for or against the cause and effect hypothesis and none can be required as a sin qua non……what they can do is help us to make up our minds on the answer to the fundamental question – is there another way of explaining the set of facts before us".* (Hill, 1965).

In the example above, Bradford Hill recognized the need to separate strengths of evidence for causality from strengths of recommended actions, an approach subsequently taken up by the GRADE scheme in clinical medicine, which also recognises this separation (GRADE 2011). As noted by GRADE, "*Not all grading systems separate decisions regarding the quality of evidence from the strength of the recommendations. Those who fail to do so create confusion. High quality evidence doesn't necessarily imply strong recommendations, and strong recommendations can arise from low quality evidence*". (Guyatt et al., 2008). These considerations were not taken into account in the IPCS (2002) document. Further, the use of the Bradford Hill criteria was intended to evaluate evidence with only a few examples given in Tables 7.1 and 7.2 of that document and it was not a suggestion for a general way forward.

In the current document, features specific to endocrine disrupting chemicals were considered. These have been articulated by the Endocrine Society (Zoeller et al., 2012). Their perspective emphasizes the non-linearity of hormone action, the temporal and spatial specificity of hormone action, and the myriad of known ways in which chemicals can interfere with hormone action to produce adverse outcomes. Inherent in any approach employing endocrine principles to assess WOE is a careful evaluation of the science itself, rather than a simple "count" of negative and positive findings for a certain chemical. Approaches have been developed in clinical medicine that applies a systematic, transparent approach to evaluating evidence that provides an overall evaluation of the evidence (similar to WOE approaches in environmental health). Further, more recent development of these approaches (GRADE) allows for both assessing the strength of evidence and strength of recommendations separately to allow decision-making even when scientific evidence is uncertain. While these approaches have been developed for clinical medicine and evaluating randomized control trials, they are not currently amenable to evaluating EDCs, as they do not account for evidence from ecological and toxicology literature and are still evolving in relationship to observational epidemiology studies – all evidence that is critical to environmental health. While these approaches are being developed and tested (Woodruff

& Sutton, 2011), and particularly their application to EDCs, conventional narrative.

A central issue common to all WOE approaches is that the reliability of the data must be evaluated. This, in itself, is a very difficult issue and different authors propose different standards. For example, Conrad & Becker (2011) propose that the use of Good Laboratory Practice (GLP) is an important – if not the essential – element of evaluating laboratory studies. This, however, eliminates almost all modern scientific studies and it seems unreasonable to employ state-of-the-art (non GLP) science to guide public health protection in some domains (e.g. clinical), but not in chemical safety. Many of the standardized protocols carried out to GLP standards are no guarantee of quality if they do not include the most sensitive relative endpoints or exposures during critical windows in development. In contrast, Woodruff & Sutton (2011) and ANSES (2011) propose ways of evaluating the strength of evidence based on scientific standards, independent of the method applied. Experience in clinical medicine with systematic reviews suggests that rigorous, empirically tested evaluation of the methods and study quality will provide the least biased evaluation.

It is also noteworthy that there is no universally accepted scheme for the classification of the results of a WOE assessment for endocrine disruptors (e.g. probable, possible or unlikely), like those developed for carcinogens (by IARC) or for air pollution (by WHO) or for Climate Change (by IPCC). It is also not straightforward to adopt the approaches worked out for carcinogenic modes of action under the auspices of WHO IPCS (Sonich-Mullin et al., 2001, Boobis et al., 2006) for application to endocrine disrupting chemicals. Uniquely, WOE approaches for endocrine disruptors will have to deal with the issues of adversity and mode-of-action at the same time which is currently without precedent. Such methods will have to be elaborated for endocrine disruptors, as recommended by Kortenkamp et al. (2011).

With this in mind, Chapter 2 takes a narrative review of the state of the science on endocrine disruption in humans and wildlife, considering each part of the endocrine system in turn. Emphasis was placed on the literature that appeared after 2000, with a cut-off data by March 2012. Best professional judgment was used to make expert assessments of the data linking exposure to chemicals with each disease/dysfunction. The literature on disease and disorder trends was aggregated, biological plausibility, relevant exposures (considering possible multiple exposures), consistency of the data across species (where endocrinology is similar), dose responses and temporality (considering possible latent effects and multi-causality). Wherever relevant, evidence was integrated from human and ecotoxicological species. The reviewers of the evidence were the expert authors of each of the sub-chapters. Their reviews were then peer-reviewed by an external panel of experts carefully chosen for their knowledge of each of the relevant areas. Both non-GLP and GLP studies were included as long as they were considered to be reliable and relevant.

Concerns about the hazards and risks following widespread exposures to EDCs arise from what is known of the role of hormones in organizing critical events during early development. Consequently, evidence was assessed from a viewpoint that is sensitive to the evidence that EDCs may exert low dose effects and effects during critical windows of susceptibility. Although debates relating to low dose phenomena are likely to go on for the foreseeable future, authoritative bodies (such as the US National Academy of Sciences) have proposed that given multiple chemical exposures and variability in response, that, like for carcinogens, a "threshold" should not be assumed (NAS, 2009). Rather, it is more likely that risks increase linearly also in the low dose range, but this will be difficult to prove because methods for detecting effects at low doses are insufficiently sensitive to observe these thresholds, if they exist. Moreover, because in almost all situations, pre-existing endogenous hormone levels exist, any additional exposure will increase this load in a threshold-independent manner.

Statements of the strength of the evidence are generic (e.g. sufficient or insufficient, strong, moderate or weak). Characterizing the strength of evidence is an integral step in decision-making; however, equally important is to characterize the other aspects critical to decision-making including values and preferences, and consequences of different choices. As pointed out by Bradford Hill and articulated here, weak evidence might be sufficient if there was a possible teratogenic effect of a pregnancy pill prescribed to millions of women worldwide whilst stronger evidence might be needed if there was a probable carcinogen in the workplace. Our goal was that these descriptors provide guidance seeking the goals of protecting wildlife and human health from hormonally active chemicals; however we suggest that a methodology and framework for evaluating evidence for endocrine disruption is further developed together with guidance for future directions for applying transparent, consistent and systematic concepts and terminology on the nature of the EDC-effect relationships.

1.4 Main messages

- Endocrine disruptors are exogenous chemicals or chemical mixtures that can interfere with any aspect of hormone action.
- Endocrine disruptors can act directly on hormone receptors or can act directly on any number of proteins that control the delivery of a hormone to its normal target cell or tissues.
- The affinity of an endocrine disruptor for a hormone receptor is not equivalent to its potency. Chemical potency on a hormone system is dependent upon many factors including receptor abundance.
- Endocrine disruptors produce nonlinear dose responses both in vitro and in vivo; these non linear dose responses can be quite complex and often include non-monotonic dose responses. They can be due to a variety of mechanisms; because endogenous hormone levels fluctuate, no threshold can be assumed.

- Endocrine disruptors show tissue specific effects.
- Endocrine disruptors can act on membrane or nuclear receptors.
- Environmental chemicals can exert endocrine disruptor activity on more than estrogen, androgen and thyroid hormone action. Some are known to interact with multiple hormone receptors simultaneously.
- Sensitivity to endocrine disruption is highest during tissue development; developmental effects will occur at lower doses than are required for effects in adults. Endocrine disruptors can work together to produce combination effects when combined at low doses. The extent of combination effects is governed by the sheer number of endocrine disruptors and their individual potency.
- Testing for endocrine disruption must encompass the developmental period and include lifelong follow-up to assess latent effects.
- Not all endpoints of hormone action will exhibit the same sensitivity to chemical exposures (example, uterine response to BPA).
- Endocrine disruption represents a special form of toxicity, and this must be taken into consideration when interpreting the results of studies of endocrine disrupting chemicals, or when designing studies to clarify the effects of endocrine disrupting chemicals and quantifying the risks to human and wildlife health.
- Endocrine disruptors will exert effects that are also quite complex and that are not captured using strategies designed to detect acute toxicity.
- Efforts are needed to develop systematic and transparent approaches to identify, evaluating and synthesizing the scientific evidence for endocrine disruptors that consider the science of endocrine action.

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Chapter 2

Evidence for endocrine disruption in humans and wildlife

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2.0 Introduction

At the time of the publication of the Global Assessment of the State-of-the-science of Endocrine Disruptors (IPCS, 2002), there was evidence that the health of some wildlife populations had been adversely affected by exposure to EDCs, but very weak evidence that human health was similarly affected. This was mainly due to an insufficient number of rigorous studies carried out on this issue, rather than to a lack of likelihood that EDCs could cause health effects in human populations. There were multiple experimental animal studies showing chemicals could interfere with hormone production and/or action and that this could result in adverse effects. Abnormalities in male reproductive health and development in fish and other wildlife species exposed to contaminants were the early leads for this research.

Over the last decade, scientific understanding of the relationship between the environment and health has advanced rapidly. Of greatest significance is that we now know that there are particularly vulnerable periods during fetal and postnatal life when EDCs alone, or in mixtures, have strong and often irreversible effects on developing organs, whereas exposure of adults causes lesser or no effects. Consequently, there is now a growing probability that maternal, fetal and childhood exposure to chemical pollutants play a larger role in the etiology of many endocrine diseases and disorders of the thyroid, immune, digestive, cardiovascular, reproductive and metabolic systems (including childhood obesity and diabetes) than previously thought possible.

Many of the internationally agreed upon and validated test methods for the identification of EDCs address only a very limited range of the known spectrum of EDC effects. For many effects, adequate test methods do not exist. This introduces uncertainties with potentially serious consequences for human and wildlife populations and also suggests a failure in environmental protection that should be addressed. Although there are genetic, anatomical and physiological constraints, large parts of the endocrine system are highly conserved among vertebrates and, therefore, wildlife should be considered effective and important sentinels of human health. Moreover, the protection of wildlife is vital in ensuring the conservation of biodiversity and safeguarding the important role that biological communities play in preserving ecosystem services and ecological sustainability.

The aim of this chapter is to provide a critical review of the evidence for endocrine disruption in wildlife and humans and to highlight the gaps in knowledge and in chemical test methods that need to be filled if we are to protect humans and wildlife from potential effects of endocrine disrupting chemicals. The focus is on identification of the characteristics of the hazards posed by endocrine disruptors rather than risk assessment. As risk assessment is largely undertaken on a chemical by chemical basis with little or no account taken of other concurrent exposures, it is not appropriate for assessing the combined effect of mixtures of similarly acting endocrine disruptors described here in. Moreover, human exposure data are limited for most EDCs, making accurate risk assessment difficult or impossible for many chemicals.

Plans to comprehensively evaluate chemicals for their endocrine-disrupting activities are already part of the regulatory agenda in the USA and Europe, however, so it may be possible to count the number of chemicals to be taken into account in a “mixtures risk assessment " in the future.

A brief account is given of the historical case of diethylstilbestrol (DES), a “pharmaceutical endocrine disruptor” that caused a variety of unexpected reproductive disorders and diseases in the sons and daughters of the women who took it during pregnancy. These problems arose because of ignorance of the actions of hormones on the programming of tissue structure and function during fetal life. Although DES was a pharmaceutical drug given at relatively high doses, this case study illustrates the spectrum of possible effects that endocrine disrupting chemicals could cause when exposures occur at critical times during the early development of an organism.

2.1 An introduction to endocrine disruption of the reproductive system

2.1.1 The diethylstilbestrol case

DES is a potent synthetic estrogen that was originally synthesized in 1938. It was used extensively to treat pregnant women from the 1940s-1970s to prevent miscarriages and other pregnancy complications (Giusti Iwamoto & Hatch, 1995; Bamigboye & Morris, 2003). It was initially given to women with at-risk pregnancies, but ultimately it was also prescribed to women with normal pregnancies to make babies “healthier”. It is still not known how many people were DES-exposed

worldwide. Anyone born or pregnant in the USA between 1938 and 1971, and until the mid-'80s in some European countries (until 1977 in France), could have been exposed. In the USA alone, there were an estimated 10 million people (mothers, daughters and sons). Subsequently, DES was found ineffective in reducing miscarriages. More importantly, it was linked to a rare form of vaginal cancer in a small number (< 0.1%) of adolescent daughters who were exposed to it during in utero development (Herbst, Ulfelder & Poskanzer, 1971). DES was later associated with more frequent benign reproductive problems in ~90-95% of DES-exposed daughters; reproductive tract malformations and dysfunction, miscarriage, preterm delivery, and low birth weight, ectopic pregnancies, and premature labour and births were reported (for review, Giusti, Iwanioto & Hatch, 1995; Bamigboye & Morris, 2003) and there were also effects on DES sons (described fully in section 2.3). Research found that in utero exposure to DES alters the normal programming of gene families that play important roles in reproductive tract differentiation (Pavlova et al., 1994; Taylor, Vanden, Heuvel & Igarashi, 1997; Miller, Dagenhardt & Sassoon, 1998). As a result, women exposed to DES *in utero* were at increased risk of clear cell adenocarcinoma of the vagina and cervix, structural reproductive tract anomalies, infertility, and poor pregnancy outcomes (Schrager & Potter, 2004). Moreover, developmental exposures may have played a role in increased risk of adult onset of fibroids and endometriosis. Two epidemiologic studies evaluated increased risk of uterine fibroids in women who were prenatally exposed to DES. One study using a more sensitive method to detect fibroids found an elevated risk from prenatal DES exposure, whereas a second study did not find an association with fibroids, but did find an association with paraovarian cysts (Baird & Newbold, 2005; Wise et al., 2005). In utero exposure to DES is associated with an 80% increased risk of endometriosis (Missmer 2004).

As DES-daughters age, they are more susceptible to breast cancer than unexposed, age-matched women. DES-exposed daughters > 40 years exhibit a statistically significant increase in risk of developing breast cancer (Hatch et al., 1998; Palmer et al., 2002; Palmer et al., 2006; Troisi, Potishman & Hoover, 2007); this increased risk is more pronounced in DES-women over 50 years old, though this association did not reach statistical significance (Palmer et al., 2006). DES-exposed mothers also have an increased risk of breast cancer (Giusti, Iwanioto & Hatch, 1995). Prenatally DES-exposed sons suffer a range of reproductive tract problems including malformations (urethral abnormalities, epididymal cysts, and undescended testes) and increased genital/ urinary inflammation (Herbst & Bern, 1981; NIH, 1999; CDC, 2003; Titus-Ernstoff et al., 2010).

These observed human effects of DES have been confirmed in numerous animal models, and predict changes found in DES-exposed humans, such as oviductal malformations (Newbold et al., 1983) and increased incidence of uterine fibroids (McLachlan et al., 1977; Newbold et al., 1998; Baird & Newbold 2005; Hoover et al., 2011), particularly in Eker rats genetically predisposed to uterine fibroids (Cook et al., 2005). There are also second-generational effects (Newbold et al., 1998; Newbold et al., 2000) such as increased menstrual irregularities (Titus-Ernstoff et al., 2006) and possibly ovarian cancer (Blatt et al., 2003) in DES grandaughters. Prenatal DES exposure resulted in hormonal imprinting of the developing uterine myometrium in both wild -type and genetically susceptible rats.

Based on a wealth of accumulated scientific information from humans and experimental animals, DES is well-documented to be a "transplacental carcinogen"; it crosses the placenta, reaches the fetus, adversely affects developing tissues/organs, and causes a myriad of problems including cancer (Herbst & Bern, 1981; NIH, 1999; CDC, 2003; Diamanti-Kandarakis et al., 2009; Hoover et al., 2011). DES caused a major medical catastrophe that continues to unfold today.

DES was eventually banned for use during pregnancy, but experimental studies continue to explore mechanism(s) through which DES causes its adverse effects. The murine model using prenatally DES-exposed outbred mice has been particularly successful in duplicating and predicting abnormalities reported in prenatally DES-exposed humans (for reviews see Newbold, 1995; Newbold, 2004). DES taught us the following four important lessons that can guide our investigations on endocrine disrupting chemicals now and in the future. Lessons are further discussed in detail in Chapter 2.43:

1. Exposure to endocrine disruptors during early (fetal) development can induce disorders of the endocrine system in the fetus, whilst the mother may appear healthy.
2. The risk of health impacts from exposure to hormone disruptors is especially high during early development when multiple developing tissues may be affected.
3. An endocrine disease or disorder induced during early development might only be apparent decades later, and exposure to this one chemical could lead to multiple health risks in exposed individuals and in subsequent generations.
4. Since all the effects shown in animal models with DES have also been shown in human situations (indeed animal models even predicted human outcomes), DES is also a good example of the need for extrapolation of animal data on EDCs to humans.

2.1.2 Endocrine disruptors in the reproductive system – experimental results

There is a large body of literature from experimental studies with rodents on the adverse effects of DES and of other EDCs on the reproductive system, examples of which are compiled in **Table 2.1.** Unlike human studies, animal studies enable the investigator to measure all indices of hormone action at various times during development and thus to accurately interpret the relationship between exposure and all of the effects on the

endocrine system. Although most of the chemicals listed would appear to be weaker (less potent) estrogens than DES, their effects can be equally undesirable when the exposure occurs in early development where potency seems less important (Diamanti-Kandarakis et al., 2009). These data present a compelling case for chemically-induced endocrine disruption as one factor in the causation of male and female reproductive disorders and diseases in both human and wildlife populations. The use of some of these chemicals is now prohibited in many countries (e.g. alkylphenols, some of the phthalate plasticizers, PCBs and the pesticide DDT, in addition to DES; see Chapter 3 for a description of these chemicals), but others are still in wide use. Moreover, we now recognize that an increasing number of chemicals in modern commerce can interfere with male and female reproductive function (see Chapter 2.2 & 2.3 for a current review of effects).

There are currently many gaps in the available chemical test methods for screening chemicals for endocrine disrupting effects. Regulatory tests for many wildlife taxa are currently not developed and of the mammalian assays available, most do not cover endocrine endpoints adequately enough to detect the effects of endocrine disrupting chemicals described in this chapter. Perhaps most importantly, the exposure periods do not cover critical developmental windows of increased susceptibility now known to exist. Delayed effects that can manifest themselves with ageing are not included either.

Table 2.1. Effects of endocrine disruptors observed in the reproductive system of animals (adapted from WHO, 2012).

Contaminant	Sex	Observation	References
Diethylstilbestrol (DES)	Male	Sterility	McLachlan, 1977
		Epididymal cysts	McLachlan, 1977
		Cryptorchidism	McLachlan, 1977
		Reduction in testis weight	Fisher et al., 1999; Lewis et al., 2003; McKinnell et al., 2001
		Testicular lesions	McLachlan, 1977
		Inflammatory disease of the accessory sex glands	McLachlan, 1977
		Reduction in the number of spermatogonia with multinucleate cells in lumina of testis	McLachlan, 1977
		Nodular enlargements of the seminal vesicles and/or prostate	McLachlan, 1977
		Distension and overgrowth of the rete testis	Fisher et al., 1999; McKinnell et al., 2001; Rivas et al., 2002
		Distension and reduction in epithelial height of the efferent ducts	Fisher et al., 1999; McKinnell et al., 2001; Rivas et al., 2002
		Underdevelopment of the epididymal duct epithelium	McKinnell et al., 2001
		Reduction in epithelial height in the vas deferens	McKinnell et al., 2001; Rivas et al., 2002
		Convolution of the extra-epididymal vas	McKinnell et al., 2001;
		Decreased testosterone levels	Rivas et al., 2002; Yamamoto et al., 2003
		Increased gonadotrophin levels	Yamamoto et al., 2003
		Decreased AR expression in testis, epithelium of the rete testis, caput and cauda epididymis and vas deferens	McKinnell et al., 2001
	Female	Decrease in reproductive capacity	McLachlan, 1977
		Impaired ovarian function	McLachlan, 1977
		Increased uterus weight	Lewis et al., 2003
		Squamous metaplasia in the oviducts, uterus and cervix	McLachlan, 1977
		Increased the size of sexually dimorphic nucleus of the preoptic area	Faber & Hughes 1991; Lewis et al., 2003
		Cystic hyperplasia of the endometrium and uterine adenocarcinoma	McLachlan, 1977
		Epidermoid tumours of the cervix and vagina	McLachlan, 1977
		Glandular elements and cellular atypia in the vaginal epithelium	McLachlan, 1977
		Advanced development of primary and secondary follicles in the ovary	Yamamoto et al., 2003
		Decreased pituitary responsiveness to GnRH	Faber & Hughes 1991
		Increased pubertal FSH levels	Yamamoto et al., 2003
Tributyltin	Male	Increased anogenital distance	Adeeko et al., 2003
		Reduced the number of Sertoli cells and gonocytes in fetal testis	Kishta et al., 2007
	Female	Reduced the number of germ cells in fetal ovaries	Kishta et al., 2007
		Increased post-implantation loss	Adeeko et al., 2003

Table 2.1. (Continued)

Contaminant	Sex	Observation	References
Phytestrogens (Genistein, Daidzein)	Male	Impaired erectile function	Pan et al., 2008
		Decreased plasma testosterone levels	Pan et al., 2008
		Increased testis weight	Fisher et al., 1999
		Reduction in epithelial height of the efferent ducts	Fisher et al., 1999
		Increased pituitary response to GnRH	Faber & Hughes, 1991
	Female	Decreased pituitary responsiveness to GnRH	Faber & Hughes, 1991
		Increased the size of sexually dimorphic nucleus of the preoptic area	Faber & Hughes, 1991; Lewis et al., 2003
		Increased the weight of uterus	Lewis et al., 2003
		Decreased the weight of uterus	Awoniyi et al., 1998
		Decreased the weight of ovaries	Awoniyi et al., 1998
		Reduced serum estradiol levels	Awoniyi et al., 1998
		Reduced serum progesterone levels	Awoniyi et al., 1998; Lewis et al., 2003
		Irregular estrous cycle	Nagao et al., 2001
		Histopathological changes in the ovaries and uterus	Nagao et al., 2001
		Induced permanent estrous	Lewis et al., 2003
		Decreased the age of vaginal opening	Lewis et al., 2003
Alkyl phenol ethoxylates (*p-tert*-octylphenol, *p*-nonylphenol)	Male	Increased testis weight	Fisher et al., 1999
		Decreased testis weight	de Jager, Bornman & Oosthuizen, 1999; Pocock et al., 2002
		Decreased seminiferous tubule diameter	de Jager, Bornman & Oosthuizen, 1999; Pocock et al., 2002
		Decreased epididymal weight	de Jager, Bornman & Oosthuizen, 1999
		Decreased total cauda epididymal sperm count	de Jager, Bornman & Oosthuizen, 1999
		Reduction in epithelial height of the efferent ducts	Fisher et al., 1999
	Female	Post-implantation embryonic loss	Harazono & Ema, 2001
		Irregular estrous cycle	Katsuda et al., 2000; Pocock et al., 2002
Alkylphenolethoxylates (*p-tert*-octylphenol, *p*-nonylphenol)	Female	Increased sexual motivation towards a female teaser	Pocock et al., 2002
		Decreased the weight of ovaries	Pocock et al., 2002
		Increased the size of sexually dimorphic nucleus of the preoptic area	Herath et al., 2001
		Decreased the age of vaginal opening	Katsuda et al., 2000
		Persistent estrus	Katsuda et al., 2000
		Increased relative uterine weight	Katsuda et al., 2000
		Decreased serum gonadotropin levels	Katsuda et al., 2000
		Decreased serum progesterone levels	Katsuda et al., 2000
		Increased serum inhibin levels	Katsuda et al., 2000

Table 2.1. (Continued)

Contaminant	Sex	Observation	References
Phthalate esters (DEHP, BBP, DiNP, DBP)	Male	Nipple retention	Barlow, McIntyre & Foster, 2004; Borch et al., 2004; Gray et al., 1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000
		Decreased testis weight	Gray et al., 1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000; Parks et al., 2000
		Reduced anogenital distance	Borch et al., 2004; Barlow, McIntyre & Foster, 2004; Gray et al., 1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000; Parks et al., 2000
		Cryptorchidism	Gray et al., 1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000
		Reduced accessory sex organ weights	Andrade et al., 2006; Barlow, McIntyre & Foster, 2004; Gray et al.,1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000
		Lesion of the rete testis	Barlow, McIntyre & Foster, 2004
		Hemorrhagic testis	Gray et al., 1999b; Gray et al., 2000
		Cleft phallus and hypospadias	Barlow, McIntyre & Foster 2004; Gray et al., 1999b; Gray et al., 2000; Mylchreest et al., 1999; Mylchreest et al., 2000
		Multinucleated gonocytes	Gray et al., 2000; Parks et al., 2000
		Agenesis of the seminal vesicles and coagulating glands	Gray et al., 2000; Mylchreest et al., 2000
		Agenesis of bulbourethal glands	Gray et al., 2000
		Agenesis of ventral prostate	Barlow, McIntyre & Foster 2004; Gray et al., 2000
		Agenesis of gubernacular cords	Gray et al., 2000
		Agenesis of epididymis and vas deferens	Barlow, McIntyre & Foster, 2004; Gray et al., 1999b; Mylchreest et al., 1999; Mylchreest et al., 2000
		Histopathological changes of testis	Barlow, McIntyre & Foster, 2004; Mylchreest et al., 1999; Mylchreest et al., 2000; Parks et al., 2000
		Delayed preputial separation	Gray et al., 1999b; Mylchreest et al., 1999
		Reduced fertility	Gray et al., 1999b
		Reduced fecundity	Gray et al., 1999b
		Reduced cauda epididymal sperm numbers	Gray et al., 1999b
		Reduced daily sperm production	Andrade et al., 2006
		Reduced plasma and/or testicular testosterone levels	Borch et al., 2004; Parks et al., 2000
		Increased serum testosterone levels	Andrade et al., 2006
		Reduced serum inhibin B levels	Borch et al., 2004
		Increase plasma LH levels	Borch et al., 2004; Grande et al., 2007
	Female	Uterine abnormalities	Gray et al., 1999b
		Reduced fertility	Gray et al., 1999b
Chlorinated pesticides (DDE [1])	Male	Nipple retention	Gray et al., 1999b; Kelce et al., 1995; You et al., 1998
		Hypospadias	Gray et al., 1999b
		Reduced accessory sex organ weights	Gray et al.,1999b; Kelce et al., 1995
		Reduced anogenital distance	Kelce et al., 1995; You et al., 1998
		Delayed preputial separation	Kelce et al., 1995
		Abnormally small penis	Guillette et al., 1994
		Poorly organized testis	Guillette et al., 1994
		Decreased plasma testosterone levels	Guillette et al., 1994
	Female	Increased plasma estradiol levels	Guillette et al., 1994
		Abnormal ovarian morphology with large number of polyovular follicles and polynuclear oocytes	Guillette et al., 1994

Table 2.1. (Continued)

Contaminant	Sex	Observation	References
Dioxins	Male	Reduced accessory sex organ weights	Gray et al., 1995; Mably et al., 1992a; Mably et al., 1992b; Ohsako et al., 2001; Simanainen et al., 2004
		Decreased testis weight	Gray et al., 1995; Mably et al., 1992b
		Delayed preputial separation	Gray et al., 1995a
		Reduced anogenital distance	Gray et al., 1995; Mably et al., 1992a; Ohsako et al., 2001; Simanainen et al., 2004
		Delayed testis descent	Mably et al., 1992a
		Epididymal malformations	Gray et al., 1995; Simanainen et al., 2004
		Altered sex behaviour	Gray et al., 1995
		Decreased sperm numbers	Gray et al., 1995; Mably, Moore & Peterson 1992b; Simanainen et al., 2004
Dioxins	Male	Decreased daily sperm production	Mably, Moore & Peterson, 1992b
		Dose-related tendencies to decrease plasma testosterone and DHT	Mably et al., 1992a
	Female	Delayed puberty	Gray et al., 1995
		Clef phallus	Gray et al., 1995
		Vaginal thread	Gray et al., 1995
		Reduced ovarian weight	Gray et al., 1995
		Enhanced incidences of constant estrus	Gray et al., 1995
		Cystic endometrial hyperplasia	Gray et al., 1995
		Decreased fertility rate	Gray et al., 1995
		Reduced fecundity	Gray et al., 1995
Polychlorinated biphenyls (PCBs; PCB 77, 118, 126, 132, 169)	Male	Reduced accessory sex organ weights	Faqi et al., 1998; Gray et al., 1999b; Hsu et al., 2007; Kuriyama & Chahoud, 2004
		Decreased testis weight	Gray et al., 1999b; Kuriyama & Chahoud, 2004
		Increased testis weight	Faqi et al., 1998
		Increased epididymis weight	Faqi et al., 1998
		Reduced anogenital distance	Faqi et al., 1998
		Increased anogenital distance	Kuriyama & Chahoud, 2004
		Delay in onset of spermatogenesis, preputial separation and sex accessory growth	Gray et al., 1999b
		Decreased sperm number and total motile sperm count	Gray et al., 1999b; Hsu et al., 2007; Kuriyama & Chahoud, 2004
		Increased daily sperm production	Faqi et al., 1998
		Decreased serum testosterone levels	Faqi et al., 1998
		Increased the number of abnormal sperm	Kuriyama & Chahoud, 2004
		Altered sex behaviour	Faqi et al., 1998
	Female	Vaginal thread	Gray et al., 1999b
		Mild hypospadias Delayed the timing of vaginal opening	Gray et al., 1999b Faqi et al., 1998
Dicarboximide Fungicides (Vinclozolin, Procymidone)	Male	Hypospadias with cleft phallus	Gray, Ostby & Kelce 1994; Gray et al., 1999a; Gray et al., 1999b; Hellwig et al., 2000; Ostby et al., 1999
		Reduced anogenital distance	Cowin et al., 2010; Elzeinova et al., 2008; Gray, Ostby & Kelce 1994; Gray et al., 1999a; Gray et al., 1999b; Hellwig et al., 2000; Ostby et al., 1999
		Decreased testis weight	Elzeinova et al., 2008; Hellwig et al., 2000
		Cryptorchidism	Gray, Ostby & Kelce, 1994; Hellwig et al., 2000; Ostby et al., 1999

Table 2.1. (Continued)

Contaminant	Sex	Observation	References
Dicarboximide Fungicides (Vinclozolin, Procymidone)	Male	Increased the number of apoptotic germ cells in testis	Cowin et al., 2010
		Nipple retention	Gray, Ostby & Kelce, 1994; Gray et al., 1999a; Hellwig et al., 2000; Ostby et al., 1999
		Reduced accessory sex organ weights	Cowin et al., 2010; Elzeinova et al., 2008; Gray, Ostby & Kelce, 1994; Gray et al., 1999a; Gray et al., 1999b; Hellwig et al., 2000; Ostby et al., 1999
		Glandular atrophy and chronic inflammation of prostate	Cowin et al., 2010; Gray et al., 1999b; Hellwig et al., 2000; Ostby et al., 1999
		Reduced secretion and chronic inflammation of seminal vesicles	Hellwig et al., 2000
		Epididymal granulomas	Gray, Ostby & Kelce, 1994; Gray et al., 1999a; Ostby et al., 1999
		Chronic inflammation of epididymis	Hellwig et al., 2000
		Agenesis of prostate	Gray, Ostby & Kelce, 1994
		Spermatogenic granuloma	Hellwig et al., 2000
		Decreased sperm number and daily sperm production	Elzeinova et al., 2008; Gray et al., 1994; Gray et al., 1999a
		Increased sperm head abnormalities	Elzeinova et al., 2008
		Reduced elongated spermatid content per testis	Cowin et al., 2010
		Low ejaculated sperm count	Gray et al., 1999a
		Abnormal morphology of seminiferous tubules	Elzeinova et al., 2008; Gray, Ostby & Kelce 1994
		Decreased fertility	Gray, Ostby & Kelce, 1994
		Reduction of erections during the ex copula penile reflex test	Colbert et al., 2005
		Increase in seminal emissions during the ex copula penile reflex tests	Colbert et al., 2005
		Decreased serum testosterone levels	Gray, Ostby & Kelce, 1994
Herbicides (Linuron)	Male	Nipple retention	Gray et al., 1999b
		Reduced accessory sex organ weights	Gray et al., 1999b
		Delayed preputial separation	Gray et al., 1999b
		Decreased testis weight	Gray et al., 1999b
		Reduced spermatid number	Gray et al., 1999b
		Decreased anogenital distance	Gray et al., 1999b
		Epispadias	Gray et al., 1999b
		Testicular and epididymal malformations	Gray et al., 1999b
Lead	Male	Reduced accessory sex organ weights	Ronis et al., 1996
		Decreased testis weight	Ronis et al., 1996
		Enlarged prostate weight	McGivern, Sokol & Berman, 1991
		Reduced serum testosterone levels	Ronis et al., 1996
		Decreased sperm counts	
		Reduced serum LH levels	Ronis et al., 1996
		Reduced volume of the sexually dimorphic nucleus of the preoptic area	McGivern, Sokol & Berman, 1991
		Less masculine sex behaviour	McGivern, Sokol & Berman, 1991
		Irregular release pattern of gonadotropins	McGivern, Sokol & Berman, 1991
	Female	Delayed the timing of vaginal opening and the day of first diestrus	Dearth et al., 2002; Kimmel et al., 1980; McGivern, Sokol & Berman, 1991; Ronis et al., 1996
		Prolonged and irregular periods of dioestrus	McGivern, Sokol & Berman, 1991;
		Disruption of estrous cycling	Ronis et al., 1996
		Suppressed serum levels of IGF-I, LH and/or oestradiol	Dearth et al., 2002; Ronis et al., 1996
		Irregular release pattern of gonadotropins	McGivern, Sokol & Berman, 1991

Table 2.1. (Continued)

Contaminant	Sex	Observation	References
Cadmium	Male	Time- and dose-dependent decrease in sperm motility	Benoff et al., 2008
		Partial or entire evacuation of the seminiferous tubules	Toman, Massanyi & Uhrin, 2002
		Increased the diameter of seminiferous tubules	Toman, Massanyi & Uhrin, 2002
		Reduced epithelial volume and increased lumen of tubule in the epididymis	Toman, Massanyi & Uhrin, 2002
		Hyperemic testes with extensive haemorrhaging, destruction of all of the presperm spermatogenic cells, and general necrosis and shrinkage of the seminiferous tubules	Foote, 1999
		Decrease in sperm output	Foote, 1999
		Reduced size of the testis	Tam & Liu, 1985
		Reduced number of differentiating germ cells in 16.5-day embryos	Tam & Liu, 1985
		Spermatozoa had poor ability to capacitate in vitro and showed a low fertilizing capability	Tam & Liu, 1985
	Female	Perturbed estrous cycles	Ishitobi & Watanabe, 2005
		Reduced number of differentiating germ cells and the size the ovary in 16.5-day embryos	Tam and Liu, 1985
		Tendency towards delayed timing of vaginal opening	Ishitobi & Watanabe, 2005
		Earlier onset of vaginal opening	Johnson et al., 2003
		Increased the epithelial area and the number of terminal end buds in the mammary glands and decreased the number of alveolar buds	Johnson et al., 2003
Manganese	Male	Increased serum gonadotrophin levels	Lee et al., 2006
		Increased serum testosterone levels	Lee et al., 2006
		Increased daily sperm production and efficiency of spermatogenesis	Lee et al., 2006
	Female	Increased serum gonadotropin levels	Pine et al., 2005
		Increased serum estradiol levels	Pine et al., 2005
		Earlier onset of vaginal opening	Pine et al., 2005

[1] Human health aspects of indoor spraying of DDT have been extensivly reviewed in IPCS, 2011. The document includes chapters on endocrine and reproductive effects, neurological effects and cancer.

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2.2 Endocrine disrupting chemicals and female reproductive health

2.2.1 Overview of female reproductive health trends in humans and wildlife and evidence for endocrine disruption

The knowledge gained from the case of the synthetic estrogen, diethylstilbestrol, can now be used to protect humans and wildlife from the effects of endocrine disrupting chemicals in widespread use. Given that endogenous estrogens participate in female reproductive development and function, it is biologically plausible that exposures to endocrine disrupting chemicals are influencing female reproductive health. If they are, then it is possible that females today could be suffering from higher rates of reproductive problems than their ancestors did. Whilst historical data that could definitively answer this question do not exist, currently-available data in human populations from all countries that have been studied show that millions of women are today affected by the following reproductive disorders:

- ***Polycystic ovary syndrome (PCOS) can affect 3 to 15% of women of reproductive age*** (Teede, Deaks & Moran, 2010; Broekmans et al., 2006). PCOS is the leading cause of sub-fecundity and anovulatory infertility, and women with this disorder are more likely to have gestational diabetes, endometrial cancer, preterm labour, and pre-eclampsia. There are no secular trend data, although the prevalence of PCOS is a comorbidity with increasing rates of obesity (Mason et al., 2008; Burt Solorzano & McCartney, 2010).
- ***Uterine fibroids (also termed leiomyomata) are the most common tumour of the female reproductive tract, possibly affecting up to 25-50% of pre-menopausal women*** (Walker & Stewart, 2005; Baird et al., 2003). They are a significant cause of pelvic pain, abnormal uterine bleeding, menorrhagia, infertility and complications of pregnancy including preterm labour (Rice, Kay & Mahony, 1989; Carlson, Miller & Fowler, 1994a;1994b; Kjerulff et al., 1996; Rowe et al., 1999; Coronado, Marshall & Schwartz, 2000). Fibroids are the leading cause of hysterectomies, accounting for over 200 000 of these surgeries annually in the USA alone (Buttram & Reiter 1981) at an estimated cost of $1.7 billion per year. Risk factors include age, obesity, race, metabolic syndrome and early age at menarche.
- ***Endometriosis occurs in 10-15% of women of reproductive age (15-49) and a minimum of 176 million women worldwide, and in up to 50% of women with infertility and/or chronic pelvic pain*** (Rogers et al., 2009; Adamson, Kennedy & Hummelshoj, 2010). The prevalence of endometriosis is higher in infertile or sub-fertile women than in the general population and has been also been linked to increased risk of endometrial and clear cell ovarian cancer, non-Hodgkin's lymphoma, and atopic disorders (Giudice, 2010). The pelvic pain associated with endometriosis is a major cause of disability and compromised quality of life. Early menarche, short and heavy menstrual cycles, and cycle irregularity are risk factors for endometriosis.

These three disorders, described above, are causes of infertility or sub fertility.

- ***Data from the United States show that the percentage of women who have difficulty in achieving and maintaining pregnancy has increased between 1982 to 2002*** (Swan et al., 1999; NSFG, 2013), ***and is slightly lower in 2006-2010 (though still higher than 1995 and earlier).*** While some of this increase is likely due to people starting families later in life (fertility decreases with age and miscarriage rates increase with age), this does not explain why the sharpest increase in reported infertility is seen in younger women between 1982 and 2002 (Crain et al., 2008).
- ***In the United States, United Kingdom and Scandinavia, the preterm birth rate has increased by more than 30% since 1981*** (Institute of Medicine, 2007; Martin et al., 2009). Since 1990, the percentage of infants born in the USA with low birth weight also rose 16% to 8.1% of births in 2004 (Hamilton et al., 2005). This is of concern because infants born preterm and/or with low birth weight experience significantly higher rates of morbidity and mortality, including respiratory and neurological conditions, during the perinatal period than term and normal birth weight infants. They are also more likely to suffer from cardiovascular disease, obesity, lung disease, and type 2 diabetes in adulthood (Resnik & Creasy, 2004).
- ***There is a secular trend toward earlier onset puberty among American and European girls*** (Euling et al., 2008; Castellino et al., 2005; Semiz et al., 2008). Premature puberty can lead to reduced adult height and is also associated with a higher risk of breast cancer and polycystic ovary syndrome (DiVall & Radovick 2009). It can also have psychological consequences such as greater likelihood of engaging in risky behaviours (smoking, unprotected sex, alcohol and drugs; Cesario & Hughes, 2007).

Genetic and environmental factors (including diet, age, exercise habits, sexually transmitted diseases, and access to good health care) play a role in a woman's overall reproductive health and thus could contribute to these disorders. Moreover, effects of chemicals seen in exposed wildlife and in laboratory animals, similar to those seen in human populations and in DES-exposed individuals, have caused the scientific community to consider whether endocrine disruptors could also cause an increasing variety of reproductive health problems in women, including altered mammary gland development, irregular or longer fertility cycles, and accelerated puberty (Crain et al., 2008; Diamanti-Kandarakis et al., 2009; Woodruff et al., 2008). These changes indicate a higher risk of later health problems such as breast cancer, changes in lactation, or reduced fertility.

Alongside the evidence of female reproductive health diseases and disorders in humans, data describing patterns of reproductive dysfunction in female wildlife have expanded over the past ten years, albeit effects on female reproductive health have been little studied compared with those on the male. In many cases, these patterns appear to mirror those observed in humans, in that the affected wildlife populations appear to exhibit a suite of symptoms that is consistent with exposure to estrogen/anti-androgen and/or androgens. The symptoms recorded often reflect the comparative endocrinology of humans and wildlife, indicating that the human and wildlife evidence for endocrine disruption should perhaps be considered in parallel when assessing whether EDCs contribute to the etiology of female reproductive disorders (for example, see Guillette & Moore, 2006; Edwards, Moore & Guillette, 2006).

There are some well-known and closely-studied examples of female reproductive system disorders in wildlife, which are discussed in the following sections. In many of these wildlife examples, the available evidence supports the involvement of chemicals in the causation of reproductive dysfunction and disease. Evidence includes:

- ***Population declines in Baltic grey seals during the 1950s*** and their more recent recovery (Olsson, Karksson & Ahnland, 1994; O'Hara & Becker, 2003). A high incidence of uterine fibroids (leiomyomata) was found to be correlated with the body burden of organochlorine contaminants in these seals (especially PCBs; Bergman & Olsson, 1986; Bergman, 1999).
- ***Dramatic declines in juvenile recruitment in a population of American alligators*** exposed to chlorinated pesticides, concomitant with abnormal ovarian morphology, large numbers of polyovular follicles and polynuclear oocytes (Guillette & Moore, 2006).
- ***Reproductive endocrine disruption across a range of bird species*** correlated with high concentrations of persistent organic pollutants (e.g. Bosveld & Van Den Berg 2002)
- ***Reduced fecundity, alterations in the timing of sexual maturity and reproduction and premature atresia*** (or degeneration and reabsorption of preovulatory follicles) in some populations of fish in rivers receiving sewage treatment works effluent (reviewed in Tyler & Jobling, 2008).
- ***Masculinisation of female snails*** exposed to the antifoulant tributyltin (TBT), causing blockage of the oviduct resulting in sterility and leading to population declines (Galante-Oliveira et al., 2011; Gibbs & Bryan, 1986; Ellis & Pattisina, 1990).

In addition to wildlife studies, there are data from domestic animal studies that are pertinent here. For example, one of the earliest documented cases of female reproductive dysfunction through exposure to estrogenic compounds concerns breeding problems reported in adult sheep grazing on a type of phytestrogen (isoflavone)-rich clover (Bennetts, Underwood & Shier, 1946). Several biological effects were subsequently associated with reduced fertility in these female sheep, including increased teat length, gestational period and uterine weight, as well as an increased rate of prolapsed vagina, cervix and rectum (Cox & Braden, 1974; Trenkle & Burroughs, 1978). Thus the sheep have provided a useful model for the effects of phytoestrogens. Similarly, it is noteworthy that in some farmed and domesticated animals such as dogs, cats and guinea pigs, as in humans, fibroids are the most common tumour of the genitalia. However, this tumour is normally rare in other species. Human and animal populations may be affected by specific disorders because they share the same habitat (e.g. urban, rural, indoor, and outdoor) and are exposed to similar types of contaminants. Further studies on these domesticated and companion animal species could provide opportunities to learn more about causation and also serve to highlight the similarities between effects occurring in humans and in other vertebrate species.

Hormonal mechanisms underlying female reproductive disorders and diseases

Mechanistic evidence suggests that a proportion of female reproductive endocrine disorders in both humans and wildlife are likely caused by exposures to estrogens, androgen excess or insufficiency, and/or by an imbalance between estrogens and androgens during critical times during the life cycle (e.g. when the ovaries and genitalia are differentiating and/or during puberty when the organs are maturing). Normal hormonal signalling at these times is critical to future reproductive health. For example, at birth the early development of the ovarian follicles depends on the balance between estrogen and other hormones within the developing ovary and, if this is disrupted, ovarian follicle formation and function can be impaired (Dupont et al., 2000). This is believed to lead to ovarian disorders in women and vertebrate wildlife species, like premature ovarian failure (POF). Pre- and/or post-natal exposure to androgens, e.g. in sheep (Hogg, McNeilly & Duncan, 2011), primates (Abbott Tarantal & Dumesic, 2009) and rats (Tyndall et al., 2011) can lead to a polycystic ovary syndrome-like phenotype, including a metabolic component. Importantly, these disorders, both of which can impair fertility, would not be seen until after puberty. It has also been suggested that a changing endocrine environment underlies the age-related increase in human aneuploidy (Hunt & Hassold, 2008). High concentrations of estradiol are required for normal meiotic maturation, although the existence of an endocrine mechanism in the onset of meiosis in the fetal ovary has not yet been explored.

A further example is given by the development of the Müllerian ducts that irreversibly differentiate and proliferate in utero into the oviducts, uterus, cervix and upper vagina (in humans between 9.5 and 11.5 weeks of gestation; Neill, 2006). In vertebrate wildlife species and in laboratory models for human health, differentiation of the external and internal reproductive organs is known to be controlled mainly by the secretion of sex steroid hormones. Abnormal differentiation of the external genitalia has been observed in numerous species

due to exposures to androgens or anti-androgens prenatally (e.g. Jackson, Timmer & Foster, 2008; and c.f. below). As in humans, activation of both the androgen and estrogen receptors (and genes downstream of these) play critical roles in normal female development and the balance of androgen and estrogen may be more important than the action of either of these hormones alone.

Evidence for endocrine disruption of the female reproductive system in humans and wildlife

Apart from the historical case of DES (Chapter 2.1), most of the available evidence concerning the relationship between environmental chemical exposures and female reproductive disorders comes from studies of adults rather than neonates, and often from exposures to persistent organic pollutants (e.g. DDT, PCBs and dioxins), rather than to more modern chemicals (see Chapter 3 for discussion of exposure). The understanding of the contribution of other EDCs beyond legacy persistent and bioaccumulative chemicals has only recently expanded to other chemicals that may influence these outcomes. As previously discussed, it is well established scientifically that environmental exposures during critical periods of growth and development can contribute to an increased risk of future disease or dysfunction later in life (Newbold & Heindel, 2010). In particular, an ovarian dysgenesis syndrome has been proposed which posits that alterations in ovarian structure or function could lead to a syndrome of various gynaecologic disorders, impaired fecundity or later onset adult disease (Buck Louis, Cooney & Peterson, 2011). There is evidence that reproductive dysgenesis in females occurs in response to high environmental exposure levels, such as those encountered occupationally or during chemical accidents or spills (reviewed in Crain et al., 2008). However, evidence is lacking as to whether lower levels of exposure, such as those encountered by the majority of human and wildlife populations, pose a risk to female reproductive development; these studies have not been done.

2.2.2 Evidence for endocrine disruption of the female reproductive system in humans and in mammalian models of humans (rodents and primates)

2.2.2.1 Puberty

Human puberty can be divided into several stages, the first of which involves breast development. This is closely followed by the formation of pubic hair and completes, approximately two years later, with the occurrence of menstruation (menarche).

The average age of menarche has been 13 years of age for the last several decades, whereas some 200 years ago it occurred around 17 years of age (Aksglæde et al., 2008; 2009a). Whilst it is generally accepted that changes in general health and nutrition have most likely caused this advancement (Parent et al., 2003), the more recently reported increased proportion of young girls that develop breasts at aged 7-8 when compared with 10-20 years ago is not so easily explained. Both American (PROS, NHANES III, BCERC) and European studies document this earlier breast development (Biro et al., 2010; Herman-Giddens et al., 1997; Sun et al., 2002; Wu, Mendola & Buck, 2002; Chumlea et al., 2003; Aksglaede et al., 2009b; Semiz et al., 2008; Castellino et al., 2005), as compared to previous studies (e.g. Euling et al., 2008; Reynolds & Wines, 1948; Nicolson & Hanley, 1953).

Up to 86% of the variance in pubertal timing can likely be explained by genetic factors (Parent et al., 2003; Wehkalampi et al., 2008), and a role for increased body mass index and childhood obesity are also indicated by several studies (e.g. Kaplowitz 2001; Bau et al., 2009). The most recent studies, however, suggest that obesity alone cannot explain earlier puberty onset (Aksglaede et al., 2009a) and that other environmental factors are involved (Mouritsen et al., 2010).

Hormonal mechanisms underlying puberty

Pubertal onset is regulated by gonadotropin releasing hormone (GnRH) neurons in the central nervous system. Puberty starts when pulsatile GnRH secretion stimulates the pituitary cells to secrete other hormones (the gonadotropins, follicle stimulating hormone, FSH, and luteinizing hormone, LH) that act on the gonads. The testes and ovaries then start to secrete sex steroid hormones (estrogens and androgens) that induce secondary sexual characteristics such as breast development in females and facial hair in males. Endocrine disruptors could affect puberty through affecting the neuronal circuits and interactions in the brain or directly on the gonads as steroid hormone agonists or antagonists. Furthermore, the same compound can be an agonist when the endogenous hormone level is very low (childhood) and an antagonist when the endogenous hormone level is high (adulthood; see Chapter 2.3).

In recent years, there have been large advances in our understanding of the endocrine control of puberty, particularly in neuroendocrine mechanisms shared with metabolic control, thus providing a mechanistic explanation for the association of obesity with early pubertal onset. The major breakthrough was the discovery of the kisspeptins, hormones that stimulate secretion of GnRH at puberty via interaction with their receptors in GnRH neurons (Navarro et al., 2004). A year later, the regulation of kisspeptin and its receptor by estrogens and androgens was demonstrated in rodent animal models, thus establishing the GnRH-producing neurons as putative key targets of endocrine disruptors affecting the timing of puberty (Navarro et al., 2005; Tena-Sempere, 2010).

Evidence for the role of EDCs in causing early puberty in mammalian models of humans (rodents and primates)

Despite differences in the neuroendocrine control of initiation of puberty, there is convincing evidence from experimental studies with both rodents and primates that prenatal and/or neonatal treatment with estrogen receptor agonists accelerates pubertal onset (GnRH release) in a dose-dependent fashion, whilst the aryl hydrocarbon receptor (AhR) agonists such as

dioxins result in delayed vaginal opening in the female rat (reviewed in Buck Louis et al., 2008). In female rodents, there is evidence for several critical developmental windows that are particularly sensitive to the accelerating influence of estrogenic chemicals on vaginal opening (reviewed in Rasier et al., 2006). These are:

1) The prenatal/postnatal period of reproductive tract development
2) The prenatal period of brain differentiation, and
3) The prepubertal period of brain development.

A few chemicals (exposure to which is described in Chapter 3) have been studied in both animals and humans (e.g. dioxins (TCDD), lead, DDE (metabolite of the pesticide DDT), PCBs and pharmaceutical estrogens), and similar findings tend to be observed across these species. There is recent evidence that neonatal administration of estradiol benzoate or BPA to rodents impairs the release of kisspeptin from the hypothalamus of the brain during pre-puberty, resulting in a dose dependent decrease in luteinizing hormone concentrations in the pituitary (Navarro et al., 2009). This same effect can be seen in offspring from female dams who were undernourished during gestation compared with those that received normal nutrition (Iwasa et al., 2010), thus illustrating a similar effect of chemical exposure and nutrition on puberty in this case.

Epidemiological evidence for EDCs causing early puberty (reviewed in Toppari & Juul, 2010)

Several local epidemics of precocious (early) puberty have been reported (Comas, 1982; Fara et al., 1979). Normal puberty is started by the activation of the whole hypothalamic-pituitary-gonadal axis. In central precocious puberty (CPP) this activation occurs abnormally early. In peripheral precocious puberty, the hypothalamus and the pituitary are not activated, but the hormonal stimulation of pubertal development comes from exogenous agents or autonomously functioning gonads, adrenals or endocrine tumours. Endocrine disruptors with intrinsic sex hormone activity are typical exogenous agents causing precocious puberty. There have not been signs of CPP, but rather of peripheral precocious puberty that has been reversible in many instances. Unfortunately the causes were not identified with sufficient certainty. There are also some regions with a high incidence of CPP, e.g. in Northwest Tuscany (Massart *et al.,* 2005), but the possible causes remain speculative (pollution from local greenhouses and several small navy yards).

Children from developing countries who move to industrialized and rich environments have an increased risk of developing CPP (Parent et al., 2003) and endocrine disruptors have been hypothesized to contribute (Krstevska-Konstantinova et al., 2001). Twenty six immigrant girls with CPP had relatively high levels of the organochlorine contaminant p,p'-DDE, whereas only 2 of 15 native patients in Belgium had detectable serum DDE concentrations (Krstevska-Konstantinova *et al*., 2001). Early and temporary exposure to weakly estrogenic DDT might stimulate both hypothalamus and pituitary maturation at the same time as it has a direct negative feedback effect on the pituitary gonadotropin secretion, preventing sexual maturation. After moving to a new environment where DDT is no longer used, the decrease in exposure may allow the onset of puberty (Rasier et al., 2006). DDT has a long half life, however, making a sudden decline in internal exposure unlikely. Nevertheless, DDT affects GnRH activity in experimental settings and it is likely that other chemicals with similar mechanisms of action will also (Rasier et al., 2006).

There are many case reports of peripheral precocious puberty in children exposed to pharmaceutical drugs or ointments or food containing sex steroids (Henley et al., 2007). Estrogens stimulate breast development, while androgens induce growth of pubic hair and changes in skin (oily skin and hair, adult-type sweat odour). If the exposure can be stopped, peripheral puberty does not advance and pubertal signs can disappear slowly. Peripheral puberty can also induce central puberty although association of endocrine disruptors with the onset of CPP is less well documented than with peripheral puberty. A summary of the epidemiological studies investigating a role for endocrine disruptors in causing early puberty are summarized in **Table 2.2.**

In summary, there is consistent evidence that exposure to lead is associated with a slight delay in puberty, whereas all other exposures studied so far do not show any clear association with the timing of puberty except for polybrominated biphenyls that were linked to an early age at menarche and pubic hair development. Taking all of the evidence together, whilst there is biological plausability that exposure to endocrine disruptors could contribute to changes in pubertal onset, demonstrated epidemiological associations are absent and warrant further investigation. One of the difficulties concerns the complexity of relating this endpoint with exposures that may have occurred at different times during development and for different durations. Exposure to mixtures have not been considered. There are also many other factors known to influence timing of puberty (e.g. nutrition) that may vary between individuals and populations.

2.2.2.2 Low fecundity, sub fertility, infertility, adverse pregnancy outcomes

Between 3.5 and 16.7% of couples in developed countries and 6.9 to 9.3% of couples in less developed countries experience an inability to conceive (Boivin et al., 2007). Paternal exposures to chemicals resulting in reduced semen quality could have an effect on fecundity (capacity to conceive) as well as specifically on male fertility, which is discussed in the next section. Here we focus on female fecundity and fertility (ability to deliver a live born infant). Sub fertility/infertility can carry increased risks of adverse pregnancy outcomes such as spontaneous abortion, preterm delivery, low birth weight, and fetal death. Causes of low female fecundity and of sub fertility/infertility include ovulatory disturbances, premature ovarian

Table 2.2. Overview of epidemiological studies investigating the effects of endocrine disruptors on onset of human puberty. Adapted from WHO (2012).

Contaminant	Sex	Observation	References
Chlorinated pesticides (DDT and DDE)	Male	No association with pubertal development	Gladen, Ragan & Rogan, 2000
	Female	Younger age at menarche	Vasiliu, Muttineni & Karmans, 2004
		Precocious puberty	Krstevska-Konstantinova et al., 2001
		No association with breast stage or pubic hair development	Wolff et al., 2008
		No association with pubertal development	Gladen, Ragan & Rogan, 2000
Dioxins	Male	No association with sexual maturation	Den Hond et al., 2002
	Female	Later onset of breast development	Leijs et al., 2008
		No association with the onset of menarche	Warner et al., 2004
		Lower stage of breast development	Den Hond et al., 2002
Polychlorinated biphenyls (PCBs)	Female	Slowed breast development	Staessen et al., 2001
		No association with menarche or pubertal stages	Den Hond et al., 2002; Vasiliu, Muttineni & Karmans, 2004
		No association with breast stage or pubic hair development	Wolff et al., 2008
		No association with pubertal development	Gladen, Ragan & Rogan, 2000
	Male	Late first ejaculation	Leijs et al., 2008
		Reduced penile length	Guo et al., 2004
		Slowed genital development	Den Hond et al., 2002; Staessen et al., 2001
		No association with the development of puberty	Mol et al., 2002
		No association with pubertal development	Gladen, Ragan & Rogan, 2000
Polybrominated biphenyls (PBBs)	Female	Earlier age at menarche and pubic hair development	Blanck et al., 2000
Bisphenol A	Female	No association with breast stage or pubic hair development	WHO, 2011
Lead	Female	Delayed breast and pubic hair development	Selevan et al., 2003
		Inversely associated with inhibin B levels	Gollenberg et al., 2010
		Delayed breast development, pubic hair growth and age of attainment of menarche	Naicker et al., 2010
	Male	Delayed onset of puberty on the basis of testicular volume of > 3 mL, genitalia staging and pubic hair staging	Williams et al., 2010
Cadmium	Female	High levels of both cadmium and lead is inversely associated with inhibin B levels	Gollenberg et al., 2010

insufficiency, implantation disorders, aneuploidy and uterine abnormalities such as fibroids. It is important to note here that exposure of the male parent to chemicals can also cause sub fertility, albeit manifest in the female (Silbergeld & Patrick, 2005).

Premature birth rates and low birth weight

Preterm birth is the single largest factor worldwide in infant mortality and morbidity, and the frequency of preterm birth has seen a dramatic rise in developed countries over the last two decades. In the United States, the preterm birth rate has increased more than 30% since 1981, and 8% since 1990 (Institute of Medicine, 2007; Martin et al., 2009; **Figure 2.1**). Most of the increase has been in moderately preterm births (32-36 weeks), though the very early preterm birth rate (less than 32 weeks) has also risen in recent years. Since 1990, the percentage of infants born with low birth weight in the United States has also risen 16% to reach 8.1% of births in 2004 (Hamilton et al., 2005). Low birth weight is defined as birth weight less than or equal to 2500g and encompasses pre term infants and those born at term but whose growth was restricted in utero (intrauterine growth restriction, IUGR), as well as those who are both growth retarded and premature (small for gestational age; GA). These increases in low birth weight and preterm delivery in the USA cannot be explained by increases in multiple births or in vitro fertilisation, changes in medical practice, or other demographic factors (Davidoff et al., 2006; Donahue et al., 2010). Further, there is a persistent racial disparity in adverse birth outcomes, with African Americans having higher rates of low birth weight and preterm delivery (Hamilton et al., 2005; Institute of Medicine, 2007).

Trend data indicate that preterm delivery rates are also increasing in the United Kingdom and in Scandinavian countries (Morken et al., 2008; Beck et al., 2010), and as

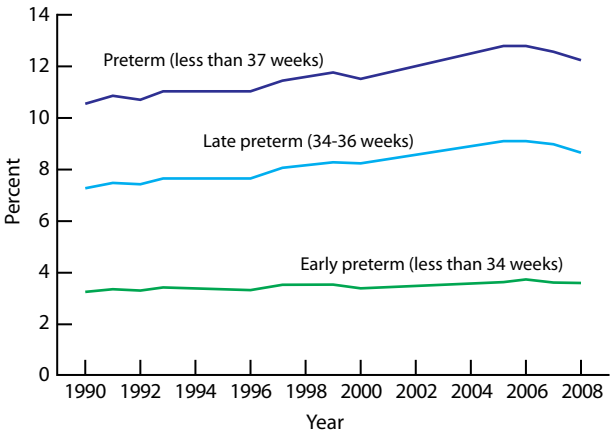

Figure 2.1. Preterm birth rates: United States, final 1990-2006 and preliminary 2007 and 2008. Source CDC/NCHS National Statistics System.

with the USA, are not necessarily explained by changing demographic or medical/delivery characteristics, although social inequalities such as rented and crowded homes, smoking, alcohol consumption and intake of saturated fatty acids are reported to be predictive of preterm delivery in some populations (Morken et al., 2008; Niedhammer et al., 2011).

Birth weight and gestational age at delivery are important predictors of neonatal and infant health. Infants born preterm and/or with low birth weight also experience significantly higher rates of morbidity and mortality during the perinatal and neonatal periods than term and normal birth weight infants, including respiratory and neurological conditions. Low birth weight infants experience longer hospital stays at birth and a greatly increased risk of respiratory distress syndrome. IUGR has been identified as a significant risk factor for chronic hypertension, cardiovascular disease, obesity, lung disease, and type 2 diabetes in adulthood (Resnik & Creasy, 2004). Premature deliveries and low birth weight pose significant challenges to the children born from these pregnancies and women during these pregnancies, and also have a major financial impact on health care systems. For example, average hospital charges for premature births in 2003 in the USA have been estimated to be $18.1 billion, about half the total infant hospital charges for all USA births (March of Dimes, 2006).

Hormonal mechanisms underlying fecundity and fertility

Achieving pregnancy requires a normally functioning hypothalamic-pituitary-ovarian axis, a normal female reproductive tract, normal endocrine homeostasis, and normal semen parameters. The structure and function of the oviduct relies heavily on the coordinated regulation and interaction of progesterone and estrogens (Hess, Nayak & Giudice, 2006). The same hormones also prepare the endometrium for implantation through an orderly process that enables attachment of the embryo to the endometrial epithelium, passage through the epithelium, and invasion of the trophoblast into the maternal decidual compartment (Hess, Nayak & Giudice, 2006). Dysfunction of the Müllerian tissues (oviduct, uterus, cervix and upper vagina) can occur as a result of disturbances in hormonal action and/or production during the preparation of the uterus for pregnancy (Crain et al., 2008). This can result in miscarriage as a result of disturbances in implantation. If the fetus survives beyond the first trimester, then there can be difficulties in pregnancy, such as pre-eclampsia, which can contribute to adverse pregnancy outcomes such as preterm delivery and intrauterine growth restriction. Even a partial withdrawal of progesterone, for example, in late pregnancy can result in reduced fetal growth (Bowman, Streck & Chapin, 2010).

The female menstrual cycle is highly regulated by a variety of hormones. According to Small et al., (2006), 84% of cycle variability is due to variation in the length of the follicular phase. In ageing women, changes in hormonal levels, including increased follicular phase estradiol, results in decreased cycle length and decreased fertility whilst decreased follicular phase estrogen results in lower fecundity (Small et al. 2006). As with puberty, kisspeptins have now emerged as major triggers for ovulation and are also involved in the metabolic control of reproductive function in overweight and underweight women (Roa et al., 2008). Hormone disruptors could interfere with menstrual cyclicity through multiple pathways, resulting in irregular periods, shorter or longer cycles and changes in duration of bleeding and/or pain (Mendola, Messer & Rapazzo, 2008; Mendola & Buck Louis, 2010).

Evidence for a role for EDCs in causing lowered fecundity and/or fertility in mammalian models of humans (laboratory rodents)

Hormonal balance, a proper level of sex hormones, is important to preserve female reproduction and regular estrous cycles, and to maintain fertility in rodent models, as with other vertebrates including humans. This balance can be disturbed by changing concentrations of estrogen, androgen or progesterone or by altering the expression of steroid hormone receptors (e.g. Ortega, Salvetti & Padmanabhan, 2009). Several studies have shown that circulating estradiol concentrations in rodents can be decreased by exposure to several pesticides, including heptachlor, lindane, atrazine, simazine or hexachlorobenzene (see Chapter 3 for a review of exposures to these chemicals). Progesterone concentrations also can be decreased by exposure to methoxychlor, especially during the estrous phase of the estrous cycle in rats. It should be noted, however, that the estrous cycle in non-primate mammals only partly corresponds with the ovarian cycle in humans; estrus is the period of greatest female sexual responsiveness, usually coinciding with ovulation; whereas, diestrus is the luteal phase of the estrous cycle when the female is not receptive to the male.

Organochlorine compounds, atrazine and simazine are all known to interrupt the estrous cycle in rodents through altering hormone synthesis or action. Some pesticides also decrease the number of healthy follicles and increase the number of atretic follicles, potentially reducing fertility. Adverse effects

of endocrine disruptors on the female reproductive system are summarized in Chapter 2.1, **Table 2.1.** Some studies suggest that in utero exposure to endocrine disruptors may alter the mouse estrous cycle, and prematurely end cyclicity altogether (Rubin et al., 2001; Markey et al., 2003; Nikaido et al., 2004; Jefferson, Padilla-Banks & Newbold, 2005).

Several EDCs have been found to cause urogenital dysmorphogenesis in mammalian laboratory models, leading to infertility/sub fertility. TCDD and chlordecone inhibit events responsible for the regression of the Wolffian ducts, which then causes interference with development of the genitourinary system (Silbergeld & Patrick, 2005). Neonatal exposure to BPA, or DES, but not phytoestrogen isoflavones, has been shown to alter Hoxa-10 and Hoxa-11 uterine expression in the rat and thus impair the response of the endometrium to steroid treatment (Varayoud et al., 2008).

Follicles containing two or more oocytes have been observed in wildlife and in animal models in lab studies following prenatal exposure to estrogenic substances, indicating that EDCs can interfere with follicle formation (Crain et al., 2008) and suggesting that this effect might precede the formation of primordial follicles. The transition from primordial follicle to primary follicle is inhibited by estrogen and EDCs such as methoxychlor can inhibit folliculogenesis (Uzumcu et al., 2006).

Evidence for EDCs affecting menstrual cyclicity in women

A review of the results from epidemiology studies evaluating adult environmental chemical exposures and menstrual cycle effects published by Mendola, Messer & Rapazzo (2008) states that there are a number of published studies, but they cover differing types of exposures, and reported effects are contradictory (e.g. longer versus shorter menstrual cycles for different chemicals). This is perhaps not surprising considering that exposures are never to one chemical, but rather to a large number of additional chemicals that may also contribute to the effect in question (Chapter 1.3.8 for a discussion of cocktail effects). Thus, it is difficult to assess the overall effect of environmental chemical exposures on menstrual cycles, given the current limited data, although individual chemicals have been associated with particular effects on menstrual cycles (Mendola, Messer & Rapazzo, 2008). For example, shorter menstrual cycles have been observed among lead-battery plant workers (Tang & Zhu 2003), in women exposed to chlorodibromomethane in drinking water (Windham et al., 2003), or those exposed to DDT (Ouyang et al., 2005; IPCS, 2011). In contrast, longer menstrual cycles have been observed in association with exposure to dioxins (Eskenazi et al., 2007) and hormonally-active pesticides (Farr et al., 2004), elevated serum PCBs (Cooper et al., 2005), or working in the semiconductor industry (Hsieh et al., 2005). Many of these studies also observed associations with other menstrual disorders such as abnormal and/or painful bleeding, and missed periods (Tang & Zhu, 2003; Farr et al., 2004; Cooper et al., 2005). Some studies, however, find no relationship between menstrual abnormalities, cycle length, or other menstrual characteristics and PCB, metals, DDE, or DDT exposure (Yu et al., 2000; Chen et al., 2005). In studies with biomarker data, women exposed to pentachlorophenol had lower follicle-stimulating hormone levels (Gerhard et al., 1999a). Women exposed to DDT and DDE had reduced progesterone and estrogens, but PCBs were not associated with changes in hormone profiles (Windham et al., 2005; Perry et al., 2006).

Epidemiological evidence for EDCs causing lowered fecundity or fertility

Epidemiological evidence of effects of EDCs on female fecundity has been the subject of three recent reviews (Buck Louis et al., 2008; Mendola, Messer & Rapazzo, 2008; Woodruff & Walker, 2008). These studies have generally been restricted to occupational and accidental exposures, mainly to metals and pesticides, as well as to perfluorinated compounds (Fei et al., 2009). Effects include those on menstrual cycles (mirrored by effects on fecundity) and on time to pregnancy (which can be confounded). Interestingly, effects often only become significant in population subsets with other known risk factors such as age or smoking, suggesting that interacting (perhaps additive) effects are occurring. Moreover, opposite effects on female fecundity of prenatal exposure to DDT appear to depend on whether high concentrations of DDT or its metabolite DDE were measured in maternal serum (IPCS, 2011; Law et al., 2005; Harley et al., 2008; Cohn et al., 2003; Gerhard et al., 1999b). This raises interesting questions regarding maternal differences in metabolism and genetic susceptibility to endocrine disruption.

Epidemiological evidence for EDCs causing adverse pregnancy outcomes including pre term delivery

Exposure to endocrine disruptors and other chemicals have also been associated with a variety of adverse pregnancy outcomes, including miscarriage, preeclampsia (characterized by hypertension during pregnancy), IUGR, poor weight gain during fetal development, and preterm delivery (Stillerman et al., 2008; Slama & Cordier, 2010). Sufficient evidence exists that prenatal exposure to lead and glycol ethers can increase the risk of miscarriage (Slama & Cordier, 2010). There is limited evidence that other metals (e.g. mercury and cadmium), chlorinated chemicals (e.g. DDT/DDE) and solvents can increase the risk of miscarriage (Slama & Cordier, 2010; IPCS, 2011). For fetal growth, most evidence is limited, including for metals, PCBs and DDT/DDE, although there is stronger evidence that exposure to perfluorinated chemicals can affect fetal growth outcomes (Slama & Cordier, 2010).

Limited evidence exists that exposures to metals or organochlorine and organophosphate pesticides can increase the risk of preterm delivery (Slama & Cordier, 2010). There are several studies evaluating the relationship between phthalates and gestational length. However, studies reporting both increased or decreased gestational length have been found, and the current epidemiologic evidence is insufficient to draw a conclusion about the mechanisms underlying such a relationship (Slama & Cordier, 2010). Similarly, it has been hypothesised that BPA can adversely affect pregnancy outcomes

(Ranjit, Siefert & Padmanabhan, 2010), and a small nested case control study from Mexico City found an association between BPA and preterm delivery (Cantonwine et al., 2010). Conclusions based on this single small study are not possible; more studies are needed.

The importance of steroidal hormones in implantation means that adverse pregnancy outcomes are a plausible consequence of endocrine disruption, albeit they are difficult to study because of the possibility that trends may be obscured by confounders for maternal age and weight and by the quality of the prenatal care available. Over the last 10 years, there have been advances in our understanding of the endocrinology underlying implantation, however, and there are now suggestions that chemical exposures could influence birth outcomes other than urogenital abnormalities.

Menopause

There are a few studies of the relationship between chemical exposure and effects on menopause. Three studies of PCB exposures have found no relationship with changes in age at menopause (Yu et al., 2000; Blanck et al., 2004; Cooper et al., 2005). However, some studies have found a relationship between DDT/DDE or dioxin exposures and early age at menopause (Cooper et al., 2002; Akkina et al., 2004; Eskenazi et al., 2005; IPCS, 2011). In contrast, DDT exposure in the Agricultural Health Study was associated with slightly older age at menopause (Farr et al., 2006). Most of these studies are cross-sectional, which could be insufficient to detect relevant exposures if they occur earlier or during critical periods of development.

2.2.2.3 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a disorder affecting both metabolism and reproduction and occurs in 3 to 15% of women of reproductive age, depending on the population studied and the diagnostic criteria applied (Teede, Deeks & Moran, 2010; Broekmans et al., 2006). It has had multiple diagnostic criteria, although it is typically characterized by hyperandrogenism (abnormally high circulating testosterone concentrations), oligo/amenorrhea (irregular or absent periods or abnormal bleeding), and polycystic ovaries (i.e. those with an overabundance of maturing follicles) (Mendola & Buck Louis, 2010). A recent definition of PCOS requires two of the following three criteria: (1) clinical or biochemical evidence of hyperandrogenism; (2) intermittent or absent menstrual cycles; and (3) polycystic ovary morphology as visualized by ultrasound (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). PCOS is the leading cause of sub-fecundity and anovulatory infertility, and women with this disorder are more likely to have gestational diabetes, preterm labour, and preeclampsia, endometrial cancer and infertility (Azziz et al., 2004); up to 10% of women with this disorder develop diabetes during the third or fourth decade (Norman et al., 2007). Many body systems are affected resulting in ovulatory dysfunction, infertility, obesity, acne and metabolic syndrome, all of which have personal, social, and economic consequences. The prevalence of PCOS has risen with increasing rates of obesity (Mason et al., 2008; Burt Solorzano & McCartney, 2010).

Impaired fetal growth as well as a higher weight at birth have been associated with the subsequent development of PCOS during adulthood. It is also associated with early puberty, although associations with age at menarche are unclear (Hart, Hickey & Franks, 2004).

Hormonal mechanisms underlying PCOS.

Various mechanisms underlying this disorder have been proposed, although the origins and etiology remain largely unknown (Mendola & Buck Louis, 2010; Goodarzi et al., 2011). Hypothesised etiologies include in utero environmental exposures leading to fetal reprogramming during critical windows of follicle formation and follicle activation (Mendola & Buck Louis, 2010), primary ovarian abnormality, or deregulation of fat metabolism (Mendola & Buck Louis, 2010). The latter has received the least attention, albeit obesity–related hyperinsulinemia may lead to hyperandrogenism during puberty and promote progression towards a PCOS phenotype. Evidence for a fetal origin for the development of PCOS phenotypes stems from animal models and epidemiological studies. For example, excessive prenatal testosterone (T) exposure is hypothesized to be a mechanism underlying PCOS (Dumesic, Abbot & Padmanabhan, 2007), with evidence from experimental animal studies of rhesus monkeys, rats and sheep (West et al., 2001; Abbott et al., 2005; Forsdike et al., 2007) showing that exposure of fetuses to testosterone when target organ systems are differentiating alters their ontogenic development and phenotypic expression such that they mimic characteristics of the phenotypes characteristic of PCOS in women. The full spectrum of symptoms associated with PCOS is not apparent until puberty and thus a "two hit" hypothesis has also been proposed in which prenatal testosterone exposure leads to hyperandrogenism which then impairs the sensitivity of the brain to feedback mechanisms such that sustained hyperandrogenism is maintained, eventually leading to PCOS (Bremer, 2010). Yet another mechanism of fetal programming by androgen excess is epigenetic changes in gene expression, recently demonstrated in a PCOS mouse model (Zhu et al., 2010). This may explain the heterogeneous phenotypic expression of PCOS that occurs in sisters with the same genotype (Diamanti-Kandarakis et al., 2006).

Epidemiological and animal studies linking EDCs exposure to PCOS

There is definitely a genetic basis for PCOS but the heterogeneity of its features even within families suggests that the gestational environment and lifestyle are of prime importance (Norman et al., 2007). Few studies have been carried out to evaluate the relationship between PCOS and exposure to EDCs. One EDC that has been associated with PCOS, however, is BPA where two studies of adults found a relationship between serum BPA levels and women with PCOS

(Takeuchi et al., 2004; Kandaraki et al., 2011). It is possible that the elevated BPA is a consequence, and not a cause, of PCOS as women with PCOS have higher circulating testosterone levels than healthy women and these elevated androgen concentrations decrease BPA clearance (Takeuchi et al., 2006). However, at least one animal study supports the case for BPA exposure playing a role in PCOS in rodents (Fernandez et al., 2010).

Overall, genetic markers for PCOS are still not established and its etiology is not known, albeit it seems consistent with an interaction between genetic susceptibility and environmental factors, including lifestyle. Given the hormonal basis for this disease, a role for EDCs as risk factors should be considered. Prospective epidemiological studies have not been conducted and chemical test methodologies have not been devised.

2.2.2.4 Uterine fibroids

Uterine fibroids (leiomyomata), benign tumours that arise from the uterine myometrium, are the most common tumour of the female reproductive tract (Walker & Stewart, 2005). They are a significant cause of pelvic pain, abnormal uterine bleeding, infertility and complications of pregnancy including preterm birth (Rice, Kay & Mahony, 1989; Carlson, Miller & Fowler, 1994a; 1994b; Kjerulff et al., 1996; Rowe et al., 1999; Coronado, Marshall & Schwartz, 2000). Uterine leiomyomatas occur in 25-50% of all women in countries in which this has been studied, although the prevalence estimates are mostly based on clinical cases, and may not, therefore, reflect the true incidence (Baird et al., 2003). A study in the USA of randomly selected women from the northeast, independent of clinical symptoms, found that cumulative incidence of fibroids by age 50 was almost 70% for Caucasian women and greater than 80% for African-American women (Baird et al., 2003). One other published study has reported prevalence of fibroid tumours using transvaginal ultrasound screening from a random sample of women from Sweden (Borgfeldt & Andolf, 2000). Prevalence was much lower, with 5% of women aged 25 to 40 and 8% of women 33 to 40 years old having fibroids. Prevalence for a comparable age range in the Baird et al. study is 26% for white women and 53% for black women.

Time trend data are not available on fibroid incidence. However, they are the leading cause of hysterectomies, accounting for over 200 000 of these surgeries annually in the USA alone (Buttram & Reiter, 1981) at an estimated cost of $1.7 billion per year. The rates of hysterectomies in the United States have declined slightly, although they have increased for certain reproductive conditions (e.g. bleeding and pain) (Farquhar & Steiner, 2002). While these tumours rarely metastasize, they can have a significant and negative impact on a woman's health (Rice, Kay & Mahony, 1989). This is because even in fertile women, fibroids have been implicated in recurrent pregnancy loss and first and second trimester miscarriage. In late pregnancy, fibroids can enlarge and cause obstetric complications.

Hormonal and anatomical changes associated with menstruation and pregnancy influence uterine fibroid incidence, as does obesity and metabolic syndrome. Earlier and later age at menarche have been associated with increased and decreased risk, respectively (Crain et al., 2008; Okolo 2008; Terry et al., 2010). The risk of fibroids also increases with age during premenopausal years but thereafter disappears.

Hormonal mechanism underlying fibroids

Fibroids have significantly higher concentrations of estrogen receptors compared with normal uterine tissue, despite the fact that circulating levels of steroid hormones in women with clinically detectable fibroids are no different from those measured in normal women (Blake, 2007; Okolo, 2008). In in vitro cultures, rodent fibroid cells proliferate in response to estrogen and this response is inhibited by estrogen antagonists (Othman & Al-Hendy, 2008). There is also a role for progesterone in promoting fibroid growth and recent literature indicates that fibroid growth is stimulated in response to a mixture of progesterone and estrogens, rather than one or the other (Ishikawa et al., 2010).

Laboratory evidence for EDCs causing fibroids in rodent models of humans

A small collection of studies in laboratory rats and/or mice has demonstrated that exposure to some EDCs can increase the incidence of uterine fibroids (reviewed in Crain et al., 2008). These effects reported in rats and mice inform human epidemiology studies and add credence to the hypothesis that fibroids in humans can be induced by EDC exposure (McLachlan, Newbold & Bullock, 1980).

In addition to these in vivo studies, in vitro studies also suggest that EDCs contribute to the growth of uterine fibroids. Rat uterine leiomyoma cells are extremely sensitive to estrogenic EDCs, with physiologically relevant concentrations of kepone, α-endosulfan, or 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, a breakdown product of the pesticide methoxychlor) stimulating cell proliferation, and HPTE, methoxychlor, kepone, α-endosulfan, β-endosulfan, toxaphene, or dieldrin increasing transcription in an estrogen-sensitive reporter gene assay (Hodges et al., 2000; for a review of human exposure to these chemicals, see Chapter 3). DES also induces proliferation of rat uterine leiomyoma cells, indicating that such cells are sensitive to estrogenic pesticides and pharmaceutical agents (Hodges et al., 2001).

Although evidence for a fetal origin of uterine fibroids in humans is controversial, in rodent models (CD-1 mice and Eker rats), fetal exposures to both DES and bisphenol A during particular periods in development have been shown to cause increased risk of fibroids in adulthood (Newbold, Jefferson & Padilla-Banks, 2007; Crain et al., 2008).

Epidemiological evidence for EDCs causing fibroids

There are scant data from humans evaluating EDCs and fibroids, and these studies have mostly focused on adult exposures (Mendola, Messer & Rapazzo, 2008). One study demonstrated that women exposed to high levels of TCDD from an industrial accident were less likely to have fibroids,

leading the authors to speculate that this was because TCDD may have an anti-estrogenic effect (Eskenazi et al., 2007). Another study of women who were sport fishers or sport fish consumers from the Great Lakes area and potentially exposed to higher organic pollutants including DDE and PCBs found that while the activity itself was associated with a modest increased risk of fibroids, circulating levels of the chemicals themselves were not associated (with the exception of PCBs in a subpopulation) with higher disease outcomes (Lambertino et al., 2011). Finally, two studies of women exposed to phthalates found an association with fibroids. In a relative small study of women in Taiwan, researchers found higher levels of the metabolites of the phthalate plasticizers diethylhexylphthalate (DEHP) and dibutylphthalate (DBP) in women with fibroids compared to controls, and a higher risk for women who were glutathione S-transferases M1 null, suggesting genetic susceptibility to phthalate effects (Huang et al., 2010). A larger, cross-sectional study of USA women also found an association with uterine fibroids and the metabolite of DBP (Weuve et al., 2010). Human exposure to phthalates is discussed in Chapter 3.

2.2.2.5 Endometriosis

Endometriosis is a major cause of infertility and chronic pelvic pain in women, and has also been linked to increased risk of endometrioid and clear cell ovarian cancer, non-Hodgkin lymphoma, and atopic disorders (Giudice, 2010). Endometriosis is characterized by the presence and growth of endometrial glands and stroma outside the uterus, primarily in the pelvic and abdominal cavities, but can also rarely be found in the thoracic cavity and the brain (Giudice, 2010). The growth produces hemorrhages or vesicles appearing as brown, black or blue lesions (Mendola & Buck Louis, 2010). Pelvic pain associated with endometriosis is a major cause of disability and compromised quality of life. Estimates for the incidence of endometriosis vary, often by study population. While most studies find that between 10% and 15% of reproductive-age women have endometriosis (Leibson et al., 2004; Vigano et al., 2004), this could be influenced by difficulties in diagnosis. A study of USA women in 2007-09 found that while MRI-visualized incidence was 11%, 34% of women had endometriosis as diagnosed by surgical, MRI and histological confirmation (Buck Louis et al., 2011), indicating that the true incidence among the population could have been previously underestimated. Incidence is much higher (between 35% and 50%) in women with pelvic pain, infertility, or both (Cramer & Missmer 2002). In the USA in 2009, endometriosis was estimated to cost $69 billion in health care and loss in productivity (Hummelshoj & O'Hooghe, 2012).

Early menarche, short and heavy menstrual cycles, and cycle irregularity have fairly consistently been associated with an increased risk of endometriosis (Vigano et al., 2004; Matalliotakis et al., 2008; McLeod & Retzloff, 2010) and late age at menarche has been suggested to be protective (Treloar et al., 2010). Family history, genetic predisposition, race and social status have all been explored as possible factors in the etiology of endometriosis with conflicting results (Falconer, D'Hooghe & Fried 2007; Vigano et al., 2007; Montgomery et al., 2008; Guo 2009; Siristatidis 2009). The most recent study (Guo, 2009) argues that genetic polymorphisms contribute little to disease risk, and that endometrios may be largely an epigenetic disease (for a discussion of epigenetics see Chapter 1.3.6).

Hormonal mechanism underlying endometriosis

Endometriosis is at least partially dependent on estrogen. Uterine endometrial gland development (adenogenesis) in humans begins in utero and is completed during puberty (Yin & Ma, 2005). Adenogenesis is influenced by growth factors, tissue remodeling factors, and steroid hormones that alter cell proliferation and extracellular matrix remodelling (Gray et al., 2001). All of these can thus be affected by altered hormonal signalling during early development, which can, in turn, adversely affect adult uterine morphology and function. One condition that could result, albeit not until adult life, is endometriosis. An in utero origin for this disorder has, however, only recently been proposed; until relatively recently, the primary hypothesis for endometriosis was that it was initiated by retrograde menstruation (backward movement of menstrual fluids through the fallopian tubes and into the peritoneal cavity; Sampson 1927). However, as retrograde menstruation occurs in the majority of women (Halme et al., 1984) and fewer women develop endometriosis, other initiation pathways have since been explored and it is now hypothesised that influences on in utero development can contribute to future endometriosis risk (Messmer, 2004; Bulun, 2009; Mendola & Buck Louis, 2010). Moreover, the immune system is also thought to play a role (see Chapter 2.11; Hompes & Mijatovic, 2007; Crain et al., 2008; Giudice, 2010; Mendola & Buck Louis, 2010). In either case, however, it appears that estrogen is necessary for progression of endometriosis (Dizerega, Barber & Hodgen, 1980), and aromatase in endometriosis lesions converts androgens to the estrogen estrone (E1) which is then converted to estradiol (E2) by other steroidogenic enzymes. Prostaglandin E2 is also a potent inducer of aromatase activity (Bulun, 2009).

Evidence for EDCs causing endometriosis in mammalian models of humans (rodents and primates)

Endometriosis occurs spontaneously only in primates, as estrous animals do not shed their endometrial tissue and do not develop endometriosis. Several studies in primates have found a relationship between adult exposures to dioxins and endometriosis. This includes a study of twenty rhesus monkeys dosed and followed for 15 years, which reported an increase in incidence and severity with higher dioxin exposures (Rier et al., 1993; Rier et al., 2001). Another study in the cynomolgus monkey found implants of endometrial tissue in the pelvic cavity survived longer and grew larger in animals exposed for one year to high doses (17.86 ng/kg per day) of TCDD (Yang, Agarwall & Foster, 2000).

Rodent models (mainly rats and mice) of endometriosis have been developed, where the disease is induced by

surgical transplantation or endometrial tissue into ectopic sites. Studies with these models find that endometriosis can be promoted by many organochlorines, including the dioxin TCDD, the pesticides methoxychlor and DDT, or many PCBs with dioxin-like effects (Birnbaum & Cummings, 2002). In several studies, fetal exposures also have been found to promote future endometriosis. For example, mice exposed to TCDD on gestational day 8 had increased size of implanted endometriotic lesions when combined with an adult exposure (Cummings, Hedge & Birnbaum, 1999). There is increasing evidence that epigenetic changes (see Chapter 1.2.5) are involved in endometriosis and there is convincing evidence that such changes can be induced by in utero exposure to exogenous chemicals (Guo 2009; Cakmak & Taylor, 2010). A recently published study showed that dioxin exposure of mice during fetal development led to a progesterone resistant phenotype that persisted for several generations (Bruner-Tran, Ding & Osteen, 2010).

Epidemiological evidence for endocrine disruptors causing endometriosis

Most research on the role of environmental exposures has focused on adults. While some studies find that dioxins are associated with endometriosis, several studies find no relationship (Mayani et al., 1997; Pauwels et al., 2001; Eskenazi et al., 2002; Heilier et al., 2005; Porpora et al., 2009; Rozati et al., 2009; Simsa et al., 2010). Reviews point out that often these epidemiologic studies are limited to specific or uniquely exposed populations (e.g. infertile women) (Mendola, Messer & Rapazzo, 2008). Most studies have found a relationship between PCB exposures and increased risk of endometriosis, or higher levels of serum PCBs among cases when compared to controls (Gerhard & Runnebaum, 1992; Mayani et al., 1997; Birnbaum & Cummings, 2002; Heilier et al., 2004; Buck Louis et al., 2005; Porpora et al., 2006). Several studies have also found a relationship between circulating phthalate (and phthalate esters) and endometriosis (Lebel et al., 1998; Pauwels et al., 2001; Buck Louis et al., 2005; Porpora et al., 2006; Hoffman et al., 2007; Porpora et al., 2009; Rozati et al., 2009; Cooney et al., 2010; Trabert et al., 2010), although the associated phthalate ester varies among the study (primarily metabolites of DEHP and DBP) (Cobellis et al., 2003; Reddy et al., 2006; Huang et al., 2010; Weuve et al., 2010). One cross-sectional study of USA women found a relationship between endometriosis and cadmium (Jackson, Zullo & Goldberg, 2008). Human exposure to these chemicals, particularly phthalates, can be significant (for a review of human exposures, including in breast milk, see Chapter 3).

There are fewer data on human populations examining the relationship between endometriosis in adulthood and EDC exposures during the fetal or early life period. However, a large, prospective cohort study found that daughters of women who took DES during pregnancy had an increased risk of endometriosis (Missmer et al., 2004), suggesting that exposure to EDCs during fetal development can increase risk of future endometriosis. This is biologically plausible because the development of the uterus and endometrium from the uterine mesenchyme depends on specific developmental pathways involving estrogen receptor alpha (ER-alpha) and specific transcription factors, such as HoxA10, bone morphogenetic proteins (BMPs), and leukemia inhibitory factor (LIF) (Bulun, 2009).

There is a strong genetic component in endometriosis in some patients and there may be specific genotypes that are more susceptible to developing the disorder (Painter et al., 2010). This is an area of great importance for future research on fetal, neonatal/childhood, adolescent, and adult exposures to endocrine disrupting chemicals.

2.2.3 Evidence for endocrine disruption of the female reproductive system in wildlife

2.2.3.1 Wild mammals

There are several correlative studies indicating that EDCs could have an impact on reproductive hormones in both seals and polar bears (Haave et al., 2003; Oskam et al., 2003; Oskam et al., 2004). High concentrations of hydrophobic contaminants (particularly PCBs, organochlorine pesticides and brominated flame retardants) have been measured in the marine mammals which, with the exception of plankton-feeding whales, are top predators (Ross et al., 2000; Aguilar, Borell & Reijnders, 2002; Hansen et al., 2004; Lie et al., 2004; Noel et al., 2009; see also Chapter 3.2.1). In Baltic grey seals, for example, population declines during the 1950s were related to exposure to these chemicals (Olsson, Karlsson & Ahnland, 1994). Critical to this conclusion was the fact that the high incidence of uterine fibroids (up to 65% of females 22–41 years old) was found to be positively correlated with the body burden of organochlorine contaminants (especially PCBs; Bergman & Olsson, 1986; Bergman, 1999). Moreover a 60% decrease in the number of females becoming pregnant was observed among ringed seals from PCB contaminated Bothnian Bay. Decreases in fecundity concurrent with lesions of the female reproductive organs were also found in both species of seals (Bäcklin, Bredhult & Olovsson, 2003). No prevalence of uterine fibroids was reported in grey seals outside the Baltic Sea. Some types of PCBs were subsequently shown to have proliferative effects on cells in the middle layer of uterine wall (myometrium) of the seal in vitro, thus suggesting that PCBs could take part in the growth of the uterine fibroids (Bäcklin, Eriksson & Olovsson, 2003), as also observed in rodent models of humans (see Chapter 2.2.2.4).

More recently, O'Hara & Becker (2003) increased the weight of evidence for chemical causation of fibroids with a very thorough study of grey seals during a period when organochlorine concentrations were decreasing in the Baltic. Decreasing organohalogen concentrations in seals were associated with a reduction in uterine obstructions and an increase in pregnancies, providing additional evidence of a causal link between EDCs and fibroids in the grey seal.

It is also noteworthy that reproductive success among other pinnepid species has become a matter of recent concern. Several Alaskan populations of northern fur seal, the Galapagos sea lion (Alava et al., 2009) and the Steller sea lion (Trites & Donnelly, 2003) have experienced recent declines, attributed to reduced pupping rates. The causes and timing of these reproductive failures are unknown, but in the fur seal are suspected to be linked to bioaccumulation of environmental contaminants in maternal body tissues (e.g. Beckmen et al., 2003; Towell, Ream & York, 2006). Moreover, in the contaminated St. Lawrence estuary (Canada) fibroids were recorded in 8 of 12 adult female beluga whales examined from 1996 to 1998 (Mikaelian et al., 2000). Also in other marine mammals, fibroids, as well as ovarian tumours (dysgerminoma), were observed in 5% of a large sample (n=502) of dusky dolphins caught off of Peru in 1985-87 and 1992-1994 (Van Bressem et al., 2000). Of 11 mature females with ovarian tumours or cysts or uterine tumours, only one (9.1%) was pregnant; this is significantly less than the expected pregnancy rate (53.3% in a random sample of Peruvian dusky dolphins).

Recently, in a European Commission funded study (Murphy et al., 2010), the relationship between PCB exposure and female reproductive health was examined in European harbour porpoise and in the short-beaked common dolphin. High PCB concentrations were reported in immature porpoise, of which 42% had contaminant loads above a threshold level of 17 µg/g lipid for adverse health effects based on experimental studies of both immunological and reproductive effects in seals, otters, and mink. Interestingly, all pregnant porpoise sampled had contaminant loads below 20 µg/g lipid compared with much higher levels in resting (not pregnant or lactating) mature females. Moreover, dolphins with the highest PCB burdens (and above the threshold level) were also resting mature females. These individuals also had the highest number of ovulatory scars on their ovaries. This suggests that, due to high contaminant burdens, female common dolphins may be unable to reproduce and, thus, continue ovulating; or females are not reproducing for some other reason, either physical or social, and are accumulating higher levels of contaminants in their blubber because they have fewer loss mechanisms (such as lactation). The high associated PCB burden may thus be either (or both) the cause of infertility or the consequence of infertility. In contrast, in harbour porpoises, once the effect of age and nutritional condition were taken into account, the data so far suggest that higher POP concentrations (PCB, HBCDD and DDE) tended to be associated with lower numbers of corpora scars, possibly indicating that high contaminant levels were inhibiting ovulation. However, as discussed earlier with the seal studies, associations between contaminant loads and reproductive problems found in adult female marine mammals of breeding age could easily be confounded by the fact that much of this contaminant load is transferred to the offspring during lactation. Therefore, lower contaminant loads found in breeding females could simply be reflective of this maternal transfer, rather than any true causative association with the reproductive status of these females.

2.2.3.2 Non-mammalian vertebrates

Examples of female reproductive system disorders, occurring concomitantly with chemical exposure, can be provided for several non-mammalian vertebrate classes. Perhaps one of the most reported cases is that of a reptile exposed to high concentrations of pesticides (dicofol (a pesticide chemically related to DDT), DDT itself and metabolites DDD and DDE) following an accidental spill into a tributary of Lake Apopka (Florida, USA) in 1980, which is reviewed in Guillette, & Crain (2000). This had a profound effect on the resident American alligator population. Although not seen in any of the other Florida lakes studied, there was a dramatic decline in juvenile recruitment in Lake Apopka alligators. Moreover, Guillette and colleagues reported Apopka female alligators with abnormal ovarian morphology, large numbers of polyovular follicles and polynuclear oocytes (Milnes & Guillette, 2008; Hamlin & Guillette, 2011) and plasma estradiol concentrations almost twice as high as those in female alligators from a reference lake.

Laboratory studies supported the hypothesis that contaminant exposure was the most likely explanation for the reproductive abnormalities in female alligator from Lake Apopka, and that these effects would compromise the reproductive capabilities of wild animals (Milnes & Guillette, 2008; Hamlin & Guillette, 2011). Multiple eggs per follicle and abnormal hormone levels can be induced in alligators by embryonic exposure to hormonally-active pesticides. Other estrogenic chemicals, such as DES, have also been shown to cause multi-oocyte follicles in laboratory mice (Guillette & Moore, 2006). In both alligators and mice, adult reproduction appears to be impaired by this condition and, in women, multi-oocyte follicles (MOFs) or polyovular follicles are associated with diminished in vitro fertilisation success and increased early miscarriage as well as with ovarian teratomas (a type of ovarian tumour present from birth; Guillette & Moore, 2006). Gene expression profiles of ovaries from neonates hatched from eggs collected on Lake Apopka are similar to profiles seen in laboratory animal models and women with premature ovarian failure and PCOS (Moore et al., 2010a; 2010b; 2011).

Taken together, data indicate that more research is needed to understand the role endocrine disruptors might play in causing MOF formation, as well as other reproductive disease states in humans and wildlife. Test methods for this endpoint have not been developed.

Reproductive endocrine disruption has also been reported across a range of bird species since the 1950s. Historically, investigations centred on the issue of eggshell thinning in predatory birds in relation to organochlorine pesticide exposure (Ratcliffe 1967; 1970; Lundholm 1997), which ultimately prompted the ban on the use of DDT, in particular, in North America and Europe. Although this led to a reduction in body burdens in birds and an improvement in eggshell thickness, with the subsequent recovery of many of the affected populations, other compounds linked to reproductive failure, such as dioxins and polybrominated diphenylethers (PBDEs), continue to be found at elevated concentrations in wildlife, especially near

urban centres (see Chapter 3.2.1.3). Some are very persistent, bioaccumulating to levels capable of causing toxic effects in predatory species (Bosveld & Van Den Berg, 2002). For example, American kestrels fed a PBDE-contaminated diet had delayed times to egg laying, eggs with thinner shells and reduced weights, and fewer hatchlings (Fernie et al., 2009). In addition to raptors, adverse effects on black-crowned night herons, herring gulls, double-crested cormorants and common terns (Grasman, Scanlon & Fox, 1998) have been reported. Adverse effects of PBDEs include eggshell thinning, embryonic deformities of the foot, bill and spine as well as chick death and retarded growth (e.g. Bowerman et al., 2000). These effects could be directly related, and chick survival inversely related, to the concentration of the contaminants present (Burgess & Meyer, 2008). The effects reported in the field are supported by data generated in the laboratory, which have revealed that estradiol administration during early development can cause reproductive impairments in adulthood, such as the retention of ovulated yolks within the body cavity, egg shell thinning and reductions in the number of eggs laid and in egg length and width. The production of thinner shells is thought to be at least partly due to reductions in the shell gland expression of carbonic anhydrase, an enzyme essential for the formation of the egg shell (Brunström et al., 2009; Rochester et al., 2008).

The masculinization and feminization of male fish living near the outfalls of paper mills and some sewage treatment works (STW) plants is a well known phenomenon that appears to be consistent across investigators, geographical regions, species and habitats (Tyler & Jobling, 2008). Although the effects of estrogenic sewage effluent exposure are most widely reported in male fish, many laboratory and several field studies clearly show that estrogen exposure is also consistent with effects in females, including reduced fecundity, alterations in the timing of sexual maturity and reproduction and premature atresia (or degeneration and reabsorption of preovulatory follicles; reviewed in Tyler & Jobling, 2008; Jobling et al., 2002). Masculinizing effects of pollution on female fish, though apparently less prevalent, have also been reported. In fact, the first evidence of ED in fish was provided by the discovery of masculinized female mosquitofish living in a stream receiving pulp mill effluent (Howell, Black & Bortone, 1980), an observation that has been confirmed in other countries (e.g. Larsson, 2000). Another source of effluent, that of intensive animal husbandry, has also been shown to masculinize wild fish due to the presence of anabolic steroids in the effluent (Orlando et al., 2004). Furthermore, the masculinization of females has also been reported in developing fish exposed to aromatase inhibitors, such as fadrozole (Fenske & Segner, 2004). This is of potential significance due to the widespread use of azole derivatives in agriculture. It is important to recognize, however, that masculinization can also be induced in fish in response to other, non-chemical stressors such as hypoxia, as demonstrated in the Atlantic croaker (*Micropogonias undulates*) (Thomas and Rahman, 2012). This highlights the need for caution when interpreting data from field-based studies.

2.2.3.3 Invertebrates

Compared with vertebrates, little is known about the manifestation of endocrine disrupting effects on the reproductive system of female invertebrates. However, there are some historical reports in which females have exhibited signs of masculinization, apparently in association with exposure to EDCs. For example, it has been shown that potent androgen receptor (AR) agonists and aromatase inhibitors, as well as tributyltin (TBT), induce imposex in prosobranch female snails, a condition in which the penis "imposes" on the normal female reproductive anatomy. The associated development of the sperm duct (vas deferens) can, in extreme cases, lead to blockage of the oviduct of the female, resulting in sterility and population declines (Gibbs & Bryan, 1986). The effects of TBT are the best known example of endocrine disruption in invertebrates, the occurrence of which has been reported in a number of gastropod molluscs (Ellis & Patissina, 1990; Titley-O'Neal, Munkittrick & Macdonald, 2011).

Although the effects of TBT on the reproductive system of female gastropods are well established, the underlying mechanisms are not yet understood. Since testosterone itself appeared to induce imposex in some species, it was suggested by various authors (Spooner et al., 1991; Bettin, Oehlman & Stroben, 1996; Santos et al., 2002) that TBT and testosterone may competitively inhibit P450 aromatase activity, thereby preventing the conversion of androgens to estrogens (and consequently increasing testosterone levels) or by inhibiting testosterone excretion or decreasing the esterification of testosterone (Gooding et al., 2003). Whilst androgens have been identified in several mollusc species, attempts to isolate an androgen receptor from molluscs have so far been unsuccessful and so the mechanism through which these apparent "androgenic" effects are occurring is unknown and controversial (Sternberg, Hotchkiss & Leblanc, 2008). Indeed, the only convincing body of evidence for mechanisms of masculinization in molluscs indicates that tributyltin-induced imposex involves the abnormal modulation of the retinoid-X receptor (RXR; Nishikawa et al., 2004; Kanayama et al., 2005; Castro et al., 2007; Lima et al., 2011), also known to be involved in male reproductive differentiation and external genitalia formation in mice (Kastner et al., 1996; Ogino et al., 2001).

In addition to the masculinization of female gastropods, there is also some laboratory-based evidence to suggest that EDC exposure may be associated with a phenomenon known as "superfeminization". Oehlmann et al. (2000) reported that the exposure of ramshorn snails to BPA induced a superfeminization syndrome at all concentrations tested (from 1-100ug/L BPA). Super-females were characterised by additional sex organs, enlarged accessory sex glands, gross malformations of the pallial oviduct, enhanced egg production outside the main spawning season, and increased female mortality. Effects were concentration dependent (except for mortality) and statistically significant at every test concentration. Limitations on this first study included the lack of analytical confirmation of the exposure regimes, a lack of replication, and the absence of

a positive control. However, two follow-up exposures with expanded experimental and quality assurance procedures confirmed the original findings (Schulte-Oehlmann et al., 2001; Oehlmann et al., 2006) and were also able to demonstrate the importance of the stage of the reproductive cycle and water temperature in the outcome of such studies. Nevertheless, the effects reported by Oehlmann and colleagues have not been reported by other laboratories performing similar, but not identical, studies (e.g. Forbes et al., 2007a; 2007b), leaving the important question of whether or not BPA is likely to cause adverse effects on molluscs at concentrations that are widely reported in the aquatic environment.

Taken together, it would appear that, in all of the wildlife cases of endocrine disruption of the female reproductive tract, there is some evidence to support chemical causation of the disorders. Moreover, there are extensive laboratory-based data that support the chemical causation of these female reproductive abnormalities. In this respect, the developmental exposure of fish to estrogenic chemicals has been associated with delayed reproductive development, aberrations in gonad morphology and increases or decreases in fecundity, depending on the dose and timing of the exposure. Whilst these effects may not be classified as "disorders", but rather the manifestation of physiological perturbations, they may still significantly impact upon female reproductive capacity and success.

2.2.3.4 Interspecies extrapolations

It is important to recognize that some female reproductive disorders are not comparable across all species. Using endometriosis as an example, it is clear that whilst widely reported in humans and many other mammals, this condition is not universally applicable to all species. All vertebrates, reptiles, fishes, birds or amphibians, except some teleost fishes, have well developed uterine and tubal structures derived embryologically from the Müllerian duct, but they do not exhibit the same degree of endometrial growth. Thus the symptoms of endocrine disruption may be deceptively different in these species compared with mammals, although the underlying mechanisms may be the same.

Alternatively, it is important to note that, due to the greater reproductive plasticity observed in wildlife, additional endpoints may be affected that have no clear analogy in humans. However, these symptoms could still form part of the same underlying syndrome and, thus, have the capacity to inform our overall understanding of reproductive dysfunction in other forms of wildlife, as well as in the human population.

2.2.4 Main messages

- Recent studies indicate that a number of female reproductive disorders that can impair fertility or fecundity are prevalent in some human populations.
- Evidence from animal studies with rodents indicates that exposure to endocrine disrupting chemicals during gestation can lead to reproductive health problems in female offspring, as their eggs are exposed whilst they are developing.
- Increased understanding of endocrine pathways governing female reproductive processes suggests that a role for EDCs in the multi causality of female reproductive disorders is biologically plausible.
- There is limited and conflicting experimental and epidemiological evidence to support a role for EDCs in premature puberty and breast development and in causing fibroids (PCBs) and endometriosis (phthalates and dioxins), and almost no evidence for causation of PCOS or infertility; few studies have, however, examined chemical causation of these diseases directly and very few chemicals have been investigated. There is limited evidence that potential endocrine disruptors such as mercury and cadmium, and organochlorine and organophosphate pesticides are associated with increased risk of miscarriage, preterm delivery and reduced fetal growth.
- There are not enough historical data to state whether or not the incidence of the many disorders of female reproductive function are correlated with one another, and therefore no obvious suggestion of a common etiology. It is clear that alterations in steroid hormone levels (principally estrogens) during prenatal life are critical factors.
- Symptoms similar to those seen in women occur in a variety of wildlife species and have been linked to exposure to contaminants (particularly PCBs and organochlorine pesticides) in some cases, e.g. fibroids in seals.
- Vertebrate wildlife are important sentinels for women's reproductive health, as the hormonal control and underlying genetic and cellular responses of female reproduction are well conserved amongst the vertebrates. Further, wildlife species often live in direct contact with similar or the same complex mixtures of anthropogenic environmental contaminants to which humans are exposed.
- There are many gaps in our knowledge of endocrine disruption of the female reproductive system. Many of the mechanisms are poorly understood and the number of chemicals that have been investigated for endocrine disruption in females is limited.
- There are many gaps in the available test methods for screening chemicals for endocrine disrupting effects on female reproduction. Regulatory tests for many wildlife taxa are currently not developed and in mammalian assays, endocrine endpoints measured are sometimes not adequate to detect possible roles of endocrine disrupting chemicals in inducing many of the female reproductive disorders and diseases described here.

2.2.5 Scientific progress since 2002

Since the IPCS (2002) was published, major advancements in our knowledge of endocrine disruption have occurred. These include:

- Downward trends in the age at breast development in Europe to substantiate the USA data.
- Greater understanding of endocrine pathways governing female fecundity and fertility, the timing of puberty, and regulation of menopause.
- Increasing experimental evidence that chemicals can interfere with endocrine signaling of pubertal timing, fecundity and fertility and with menopause.
- There are more suggestions that consequences of maternal exposures during fetal life might influence birth outcomes.
- More evidence linking phthalate exposure to endometriosis.
- Subtle effects of chemicals may only be seen when combined with other risk factors such as genetic susceptibility, age, alcohol consumption and smoking.
- More evidence now exists that reduced reproductive success in female seals, birds, alligators and gastropods are related to exposure to several persistent organic pollutants. When exposure to these EDCs decreases, reproductive effects in wild populations also decline.
- A wide variety of assays have been developed for the study of endocrine disruption. Many of these could be turned into validated regulatory tests to cover aspects of the endocrine system that are outside current testing strategies. Some modifications to existing tests have been made but even the latest of these has considerable gaps with respect to covering endpoints relevant for the detection of endocrine disrupting chemicals.

2.2.6 Strength of evidence

Female reproductive disorders are prevalent in some wildlife and human populations. In humans, they are a major cost to the health-care services in which they have been studied. These diseases include polycystic ovary syndrome (PCOS), fibroids, endometriosis, premature ovarian failure, and disorders associated with poor pregnancy outcomes. There is also sufficient evidence that breast development occurs earlier in girls in the USA and Europe. In some wildlife species, there is sufficient historical evidence that female reproductive disorders were caused by exposure to endocrine disrupting persistent organic pollutants (e.g. imposex in gastropods, fibroids in seals, and egg shell thinning in raptors) and associated with population declines. It is possible that these population-level effects are associated with adverse ecological consequences and that other emerging endocrine disrupting chemicals play a role in the causation of current diseases and disorders and declines in biodiversity in wildlife (see also Chapter 2.12).

Although it is likely that there is a role for endocrine disrupting chemicals as causal factors of female reproductive disorders in humans, the actual experimental and epidemiological evidence to support this hypothesis is limited for puberty/breast development, fibroids and endometriosis, and insufficent for PCOS, infertility and irregular menstrual cycles. In Africa where spraying of DDT is done in homes for prevention of malaria, although studies are conflicting, the WHO concluded that the combined human and animal data available do raise concern for effects of exposure to DDT on female reproductive health, albeit further data are needed (IPCS, 2011). The case for chemical causation of fibroids and of infertility/reduced fecundity, in particular, are made stronger by the existence of wildlife populations with reduced pupping rates and high rates of fibroids inhabiting particularly polluted aquatic environments, and of the recovery of these populations following the decline in the concentrations of the suspected chemical causes (e.g. PCBs and organochlorine pesticides). In addition, laboratory experiments provide sufficient evidence that a range of these types of chemicals can cause these effects. Moreover, for endometriosis, there is limited evidence to support the hypothesis that exposures to PCBs and/or phthalates are an important part of the multifactorial etiology of this disease.

Taking the wildlife and human evidence together, it is likely that exposure to PCBs and perhaps also other endocrine disrupting chemicals (e.g. phthalates and dioxins) that act in the same/similar ways play a role in the causation of fibroids and endometriosis in humans, and it is biologically plausible that for their involvement in PCOS, early age at menarche, and/or breast development and irregular menstrual cycles. In some populations of wildlife, a role for EDCs in causing fibroids is very likely or certain.

2.2.7 References

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2.3 Endocrine disrupting chemicals and male reproductive health in humans and wildlife

2.3.1 Overview of male reproductive health trends in humans and wildlife and evidence for endocrine disruption

Male reproductive health has been a major focus of research on endocrine disrupting chemicals (EDCs) since the early 1990s, when evidence of adverse secular trends in sperm counts first came to light. Subsequent research also showed similar trends in other male reproductive and developmental abnormalities that appeared to have occurred in a manner concomitant with the rapid expansion of the chemical industry, and the associated release of thousands of anthropogenic chemicals into the environment. This has led to growing speculation that chemicals with endocrine disrupting properties may be partially responsible for the decline in male reproductive health over the past several decades. The following diseases and disorders are suspected to be caused, at least partially, by exposure to EDCs during early life:

- **Testicular Germ Cell Cancers (TGC)** have been increasing over the past 40 to 50 years (by as much as 400%) in the majority of industrialised countries, where they are the most common cancers in young men aged 20-45 (Huyghe, Matsuda & Thonneau, 2003; Richiardi et al., 2004). The distinct increase in TGC over a couple of generations is suggestive of environmental causation, although genetic causes may also play a role.
- **Congenital Cryptorchidism:** The incidence of congenital cryptorchidism (where one or both testes do not descend into the scrotum) varies between 1-9 % and has increased in many countries (Toppari et al., 2010) The intra-abdominal temperature is toxic for germ cells, and so cryptorchidism often leads to infertility and is associated with increased testicular fibrosis. It is also associated with an increased risk of testicular cancer later in life.
- **Hypospadias:** This is a birth defect in which the urethra opens on the underside of the penis instead of the tip. Its incidence has increased in several regions of Australia, Europe, and the USA (Källen et al., 1986; Paulozzi, 1999; Toppari, Kaleva & Virtanen, 2001; Nassar, Bower & Barker, 2007; Lund et al., 2009), although its rate is commonly underestimated because of problems with clinical diagnosis and reporting (Toppari, Kaleva & Virtanen, 2001).
- **Semen Quality:** Prospective studies on the general population of Nordic, Baltic, German, Spanish and Japanese men show that 20-40% of young men have semen quality below what andrologists would consider to be compatible with good fecundity (below 40 mill/mL) (Bonde et al., 1998; Guzick et al., 2001; Skakkebæk, 2010). Whilst this cut off differs from the WHO reference value of 15 mill/mL, the fecundity of a man decreases steeply with sperm concentrations below 40 mill/mL, and men with sperm counts below 40 mill/mL would be sub fertile, even though conception could well occur, particularly in the 15-40 mill/mL range.
- **Testicular Dysgenesis Syndrome:** Testicular Germ Cell Cancer is often found in association with hypospadias, cryptorchidism and low semen quality, suggesting that they are risk factors for one another (Boisen et al., 2004; Jacobsen et al., 2006) and that they could be related components of a single underlying condition, termed 'testicular dysgenesis syndrome' (TDS), originating during fetal life (Skakkebæk et al., 2007; Møller & Skakkebæk, 1999) and caused by exposure to contaminants. Some experimental and epidemiological data now exist to support this hypothesis.

Alongside the evidence of continued adverse trends in male reproductive health in humans, data describing patterns of reproductive dysfunction in male wildlife have also expanded considerably over the past ten years. In many cases, these patterns appear to mirror those observed in humans, in that the affected wildlife populations exhibit a suite of symptoms consistent with a demasculinizing and/or feminizing mode of action. There are several examples (some closely studied) of male reproductive system disorders in wildlife:

- **Cryptorchidism, infertility and abnormal antler formation in large numbers (68% in some populations) of Sitka black-tailed deer on Kodiak Island, Alaska** (Bubenik & Jacobson, 2002) and in white-tailed deer sampled in the Bitterroot Valley of west-central, Montana, U.S.A. between 1996-2000 (Hoy et al., 2002). Although genetic causes cannot be ruled out completely, a recent analysis of the Sitka deer favours exposure to environmental contaminants as a likely contributing cause (Latch et al., 2008).
- **Reduced blood testosterone levels and testis size in polar bears** in East Greenland (Sonne et al., 2006) and Svalbard (Oskam et al., 2003). These problems have been correlated with tissue concentrations of persistent organic pollutants (POPs).
- **Reduced phallus size, changes in the structure of the testes, and depressed testosterone concentrations in American alligators** exposed to high concentrations of pesticides (the DDT-like pesticide dicofol, DDT itself and its metabolites DDD and DDE) following an accidental spill into a tributary of Florida's Lake Apopka in 1980 (reviewed in Guillette et al., 2000).
- **Ovarian tissue in the testes of male birds** in a breeding colony of terns on Bird Island, Massachusetts (USA); the prevalence of this intersex condition was associated with PCB and dioxin levels in the developing eggs (Hart et al., 1998).

- **Relationship between EDC burden and depressed testosterone levels in the eggs of wild birds** (Arctic-breeding glaucous gulls; Verboven et al., 2008).
- **Intersex in several species of frogs and toads correlated, in some cases, with exposure to various pesticides in agricultural areas** (Reeder et al., 2005; Hayes et al., 2003; McDaniel et al., 2008; McCoy et al., 2008).
- **Feminization of male fish living near sewage treatment works (STW) in many geographical regions of the world, species and habitats** (Cheek, 2006). Feminized male fish have reduced testosterone levels, feminization of external genitalia (e.g. Jobling et al., 2002a) and are less successful than normal males in competitive spawning experiments (e.g. Harris et al., 2011).
- **Intersex in the estuarine bivalve, *Scrobicularia plana*, in UK estuaries:** varying degrees of intersexuality were reported in over 20% of individuals sampled from 17 out of 23 populations and this has been putatively linked with exposure to EDCs (Chesman & Langston, 2006).
- **Data from many laboratory-based studies also support the chemical causation of testicular abnormalities in wildlife.**

The apparent parallels between the effects reported in humans and in various wildlife populations are not surprising given that there is often considerable overlap between their environments and food chains, as well as between the endocrine control of the male reproductive system. Based on a review of currently available data, a common causative mechanism for human TDS and feminization in vertebrate wildlife is hypothesised. In studying endocrine disruption in wildlife, we may begin to understand the effects of endocrine disrupting chemicals on human development within the same framework.

Hormonal mechanisms underlying male reproductive disorders and diseases

In all vertebrates, and some invertebrates, both the differentiation and development of the male reproductive system, including the differentiation of the Wolffian duct system and the masculinization of the external genitalia, are under the influence of the androgen testosterone. In rodents, Carruthers & Foster (2005) and Welsh et al. (2008) recently showed that there is a "male programming window", when the fetal testes begin to synthesise testosterone, during which the entire programme of development of the male reproductive tract is set up. It is during this time that the distance between the anus and the genitals (the so-called anogenital distance; AGD; shorter in females than in males) is fixed for life. This period is also essential for proliferation of Sertoli cells, often thought of as the conductors of the orchestra of other testis-specific cells in the testis in all animals. Sertoli cell number is determined and, in turn, determines the final sperm count of a male during adult life (see Sharpe, 2010).

Testosterone is synthesised by cells within the testes and adrenal glands. Local concentrations of testosterone are also modulated within target tissues as a result of expression of enzymes such as aromatase (which converts testosterone to estradiol). Estrogenic steroid hormones also regulate certain functions of the male reproductive system important for the maturation of sperm, and may be necessary for a healthy libido. Any disturbance in androgen action or reduction in its production has demasculinizing effects. If there is no functional androgen receptor, for example, androgens cannot relay their signals to developing organs and systems, such that genotypic males develop into phenotypic females.

It is, therefore, biologically plausible that EDCs that mimic or interfere with the action or synthesis of these hormones could play a central role in the causation of disorders associated with declining male reproductive health.

Deregulation of androgen production or action causes male reproductive disorders

Mechanistic evidence suggests that a proportion of male reproductive endocrine disorders are caused by male hormone (e.g. androgen) insufficiency and/or by an imbalance between female and male hormones during critical times during the life cycle (e.g. when the testes and genitalia are differentiating and/or during puberty when the organs are maturing). This can lead to malformations such as cryptorchidism and hypospadias, as well as changes in anogential distance. Indeed, it has recently been proposed that exposure to endocrine disrupting chemicals may cause this imbalance and cause adverse effects which may only become evident later in life. Furthermore, these disorders commonly occur simultaneously, which prompted Skakkebæk, Rajpert-De Meyts & Main (2001) to hypothesise that the increasing frequency of this suite of effects in the human population in recent decades may reflect a single underlying condition, termed TDS.

The concept of TDS is based on the premise that the associated symptoms have a common origin in fetal development, and that the extent and severity to which they are manifested is dependent on the degree to which normal developmental processes have been perturbed. In addition, it assumes that any perturbations occurring during the male programming window are irreversible and have lifelong implications for the affected individual and, potentially, also for their offspring. Although there is good evidence that each of the diseases comprising TDS have strong genetic components, Skakkebæk, Rajpert-De Meyts & Main (2001) noted that the majority of baby boys born with these symptoms lacked the expected genetic aberrations, indicating that environmental factors must play an important role in the etiology of these phenomena. Hormonal perturbations, arising from EDC exposures, have been widely implicated in the causation of TDS in humans, and also in the widespread reports of reproductive dysgenesis in wildlife, between which there are obvious parallels, as well as clear distinctions.

Animal studies suggest endocrine disrupting chemicals could cause TDS

In rat studies, fetal exposure to the phthalate plasticizers diethyl hexyl phthalate or dibutyl phthalate, to various chemicals with androgen receptor antagonist properties (e.g. the fungicides vinclozolin, procymidone or prochloraz), or to chemicals that interfere with steroidogenic enzymes (e.g. finasteride, prochloraz, linuron) causes disturbances of testosterone production and results in hypospadias (termed cleft phallus in rodents), cryptorchidism, impaired fertility and dysgenetic testicular histology a TDS-like outcome (Fisher et al., 2003; Foster, 2005; Foster, 2006; Gray et al., 2006; Hass et al., 2007, Wilson et al., 2008). Other chemicals with estrogenic activity cause similar effects, as do prostaglandin synthesis inhibitors, such as paracetamol (Kristensen et al., 2010). Furthermore, these compounds act in an additive fashion when occurring together (Hotchkiss et al., 2010; Rider et al., 2010; Christiansen et al., 2009). There is a large and convincing body of literature on adverse effects of EDCs on the male reproductive system in rat models, some of which is compiled in section 1 of this chapter, **Table 2.1.**

The next section presents an overview of male reproductive endocrine diseases and disorders seen in humans, followed by some relevant examples taken from the extensive wildlife literature, which are discussed in terms of the evidence to support the case for causation of male reproductive disorders by EDCs. For information on the types and levels of EDCs found in wildlife and human tissues, see Chapter 3.2.1 & 3.2.2, respectively. Use of the chemicals is also described in Chapter 3.1.

2.3.2 Evidence for endocrine disruption of the male reproductive system in humans and in mammalian models of humans (rodents and primates)

2.3.2.1 Testicular germ cell cancer (TGC)

In countries with cancer registries since the middle of the 1900s, the incidence of TGC can be seen to have increased up to 400% (see **Figures 2.2-2.4**). Moreover, remarkable differences in TGC incidence have been demonstrated between different countries, with the highest rates seen in countries with Caucasian populations and high activities

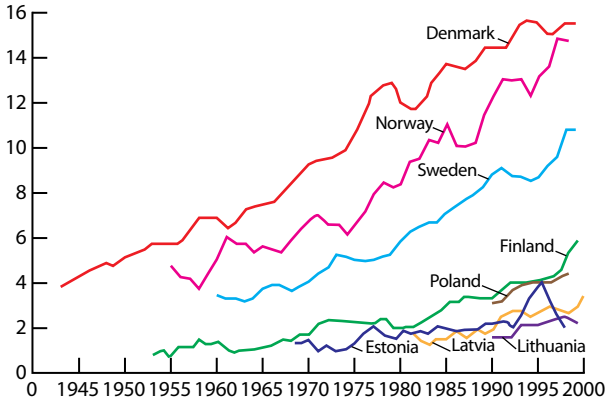

Figure 2.2. Trends in incidence of testicular cancer in northern Europe. Age-standardized (World standard population) by year of diagnosis and country. From Richiardi et al. (2004; Used with publisher's permission).

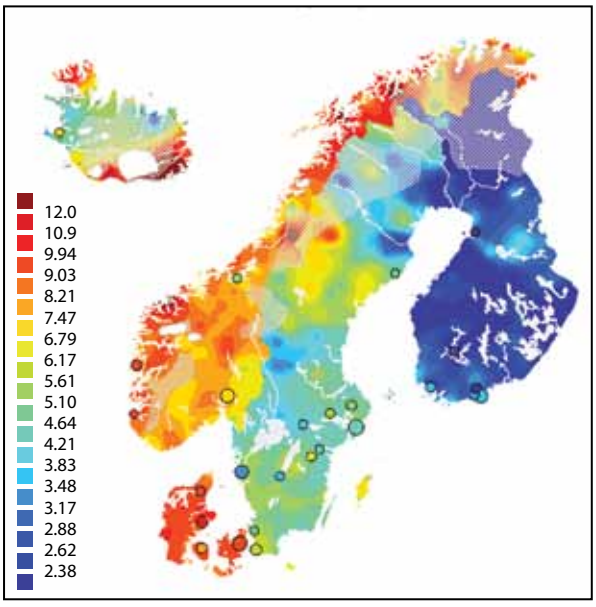

Figure 2.3. Heat map showing the incidence (per 100 000) of testicular cancer in nothern Europe, 1989-1994. The "hotter" the colour, the greater the incidence of testicular cancer. Source: *Finish* Cancer Registry 21.06.2007.

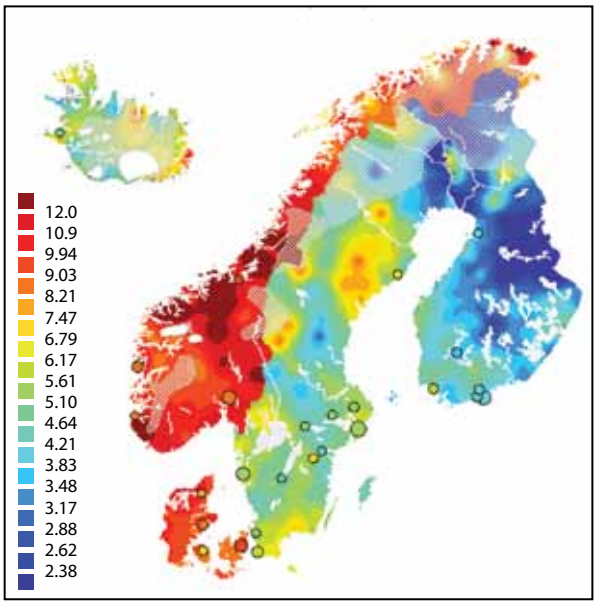

Figure 2.4. Heat map showing the incidence (per 100 000) of testicular cancer in nothern Europe, 1998-2003. The "hotter" the colour, the greater the incidence of testicular cancer. Source: *Finish* Cancer Registry 21.06.2007.

in the industry and farming sectors, including Switzerland, Denmark and New Zealand. Recently, very high rates of TGC have also been documented in Norway and Chile (Jacobsen et al., 2006; Chia et al., 2010). In contrast, low incidences and smaller increases in TGC rates have been seen in countries with Asian populations (Bray et al., 2006) and in countries with mixed populations, where African Americans have much lower incidences of TGC than Caucasians living in the same areas. Thus, although the increase in TGC over a couple of generations is suggestive of environmental causation, genetic susceptibility genes clearly have some involvement in its etiology. Increasing rates of TGC are of special interest to this review as they are also associated with impaired semen quality (Jacobsen et al., 2000) and lower fertility, even prior to the development of cancer (Møller & Skakkebæk, 1999).

Mechanisms of TGC

Although the etiology of TGC is unknown, there is abundant evidence that carcinoma in situ testis (CIS), which is a precursor for all types of TGC, is generated during fetal development; in other words TGC seems to have a prenatal origin (Rajpert-De Meyts, 2006). Numerous studies have shown that the precursor cells of TGC, the CIS cells, are like the primitive reproductive cells of the embryo (the gonocytes or primordial germ cells; PGCs) and share gene expression factors with embryonic stem cells. A current hypothesis is that the CIS develops because the somatic compartment of the fetal testis (composed of Sertoli and Leydig cells) fails to drive the normal differentiation of the primordial germ cells into spermatogonia (Rajpert-De Meyts, 2006; Looijenga et al., 2011). Such failures of Sertoli and Leydig cells during development may not only result in TGC, but also in spermatogenic disorders, cryptorchidism, hypospadias and other disorders of sexual development (see later). In all these disorders, besides CIS cells, other persisting testicular changes reflecting dysgenesis have been described (Chemes, 2001; Høi-Hansen et al., 2003; Skakkebæk et al., 2003). A hypothesis that maternal hormone levels were associated with TGC in sons was first presented in the 1970s by Henderson and his group (Henderson et al., 1979).

Epidemiological evidence for EDCs causing testis germ cell cancer

Follow up studies on the sons of women exposed to the synthetic estrogen diethylstilbestrol during pregnancy (see section 2.1) have indicated that they have a slightly increased risk of developing TGC (Strohsnitter et al., 2001). The hypothesis that maternal exposures could be important for development of TGC was also supported by epidemiological studies from Scandinavia showing that men born during World War II had a decreased risk of developing TGC as they grew up as compared with those born either before or after the war (Bergstrom et al., 1996). The hypothesis that internal sex hormone action plays a role in the pathogenesis of TGC is supported by a recent study showing that baldness, acne and increased androgen levels during puberty were negatively associated with development of TGC (Trabert et al., 2011).

Relating maternal exposures to tumours occurring 20-40 years later is a difficult task (Cook, Trabert & McGlynn, 2011) and no consistent results have indicated that external exposures postnatally are associated with TGC. A single epidemiological study found that prenatal exposures to POPs via the mother, was a risk factor for TGC, although they did not find evidence for links between postnatal exposures to POPs and TGC (Hardell et al., 2006). Whilst there is little doubt that the increase in TGC incidence during the last half century is linked to environmental factors, the possible roles of EDCs remain to be determined.

2.3.2.2 Cryptorchidism

The incidence of congenital cryptorchidism, a condition in which one or both testes are not located at the bottom of the scrotum at the time of birth, varies between 1% and 9% according to cohort studies (Toppari et al., 2010). Accurate diagnosis of cryptorchidism requires careful clinical examination. Prospective clinical studies and registry-based studies can give very different incidence figures because the latter ones usually pick up only those who have been operated on, and this introduces yet another confounding factor; a large proportion of operated boys have had so-called acquired cryptorchidism, i.e. their testes have been descended at birth, but ascended during childhood (Hack et al., 2003; Wohlfahrt-Veje et al., 2009). Despite the difficulties with diagnosis, over the last few decades there is clear evidence that the incidence of cryptorchidism has increased in Denmark (Bueman et al., 1961; Boisen et al., 2004) and the UK (Scorer, 1964; Group, 1992; Acerini et al., 2009). In a joint Danish-Finnish cohort study, Denmark showed a 3-4 fold higher birth rate of cryptorchidism than Finland (Boisen et al., 2004). The reasons for these trends are not known, although it is apparent that environmental factors play an important role. **Table 2.3** includes incidence data from prospective cohort studies on congenital cryptorchidism and **Figure 2.5** highlights the increasing trends.

Hormonal mechanisms underlying cryptorchidism

Testicular descent is regulated by two hormones, testosterone and insulin-like peptide 3 (INSL3), that are secreted by the Leydig cells in the testis. INSL3 stimulates development of the gubernaculum that attaches the testis close to the inner opening of the inguinal canal. During late gestation the testes migrate through the inguinal canals to the scrota. This is critically dependent on normal androgen action. When there is a lack of androgen action (insensitivity or defective androgen production), the gonads remain either in the abdomen or in the inguinal canals. Therefore disruption of either INSL3 or testosterone production or action can cause cryptorchidism.

Mutations in the androgen receptor gene, steroidogenic enzymes needed for androgen production, or hypothalamic-pituitary regulators needed for testicular stimulation are rare reasons for cryptorchidism (Virtanen et al., 2007; Barthold, 2008). Mutations in *INSL3* and its receptor, *RXFP2,* have been

Table 2.3 Rate of congenital cryptorchidism in prospective clinical studies using clearly defined criteria of cryptorchidism.

Country	Reference	Number of subjects	Diagnosis based on	Rate of cryptorchidism at birth
USA, Rochester, Minnesota, St. Mary's Hospital	(Harris & Steinberg, 1954)	n=4474	position (testis cannot be manipulated into the scrotum)*	1.3% (BW>2500g), 1.5% of all boys
Denmark, Copenhagen, Rigshospitalet	(Buemann et al., 1961)	n=2701	position	1.8% (BW>2500g), 4.1% of all boys
UK, West London, Hillingdon Hospital	(Scorer, 1964)	n=3612	distance measurement	2.7% (BW>2500g), 4.2% of all boys
India, Kanpur, Dufferin Hospital and U.I.S.E Maternity Hospital	(Mital & Garg, 1972)	n=2850	distance measurement	1.6% of full-term single born boys
Taiwan, Provincial Tao-Yuan Hospital	(Hsieh & Huang, 1985)	n=1208	position (presence or absence of testes in the scrotum)*	4.1% in preterm boys, 1.4% in mature boys
Korea, 38 hospitals	(Choi et al., 1989)	n=7990	position	0.7% of all boys
UK, Oxford, John Radcliffe Hospital	(Group, 1992)	n=7400	position distance measurement	3.8% (BW≥2500g), 4.9% of all boys (excluding boys with severe congenital malformations) 4.1% (BW≥2500g), 5.0% of all boys (excluding boys with severe congenital malformations)
USA, New York, Mount Sinai Hospital	(Berkowitz et al., 1993)	n=6935	distance measurement	2.2% (BW≥2500g), 3.7% of all boys
Malaysia, Kuala Lumpur, University Hospital	(Thong, Lim & Fatimah, 1998)	n=1002	position	2.4% (BW≥2500g), 4.8% of all boys
Italy, Pisa, S. Chiara Hospital and Division of Neonatology at the University of Pisa	(Ghirri et al., 2002)	n=10730	position	3.5% (BW>2500g), 6.9% of all boys
Denmark, Copenhagen, Rigshospitalet	(Boisen et al., 2004)	n=1046	position	8.4% (BW>2500g), 9.0% of all boys
Finland, Turku, Turku University Hospital	(Boisen et al., 2004)	n=1455	position	2.1% (BW>2500g), 2.4% of all boys
Lithuania, Panavêžys City Hospital	(Preiksa et al., 2005)	n=1204	position	4.6% (BW>2500g), 5.7% of all boys
UK, Cambridge Baby Growth Study	(Acerini et al., 2009)	n=742	position	5% (BW>2500g), 5.9% of all boys

*Does not seem to include high scrotal testis as cryptorchid testis

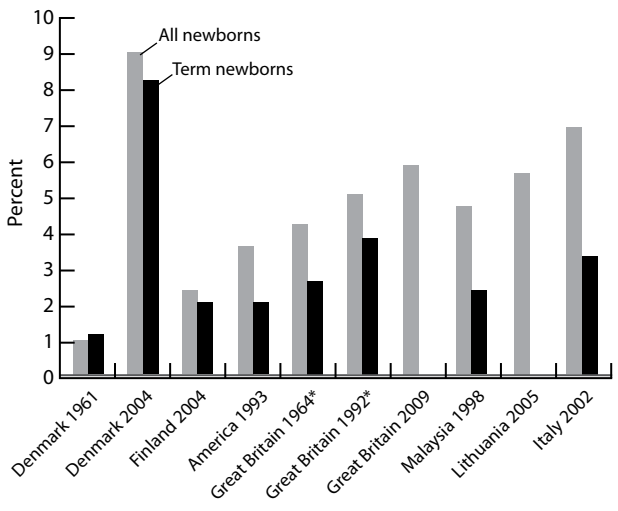

Figure 2.5. Increasing trends in cryptorchidism in newborn children in several European countries in various years.

reported in cryptorchid boys (Ferlin et al., 2003; Foresta et al., 2008), but rather these may be polymorphisms, because they were also frequently found in the normal population (El Houate et al., 2008; Nuti et al., 2008). In Finnish patients, no mutations either in *INSL3* or in *RXFP2* were found (Koskimies et al., 2000; Roh et al., 2003). However, down-regulation of these genes might lead to cryptorchidism. There are several other gene defects that cause cryptorchidism in gene-modified mice, e.g. *Hoxa10*, *Hoxa11* (Hsieh-Li et al., 1995; Rijli et al., 1995; Satokata, Benson & Maas, 1995; Overbeek et al., 2001; Daftary & Taylor, 2006), but there is hardly any evidence for their role in humans. Several syndromes include cryptorchidism as a part, and some of these are caused by known gene defects (Virtanen et al., 2007). Most often cryptorchidism, however, occurs as a single disorder. A single study reports lower cord blood levels of INSL3 in cryptorchid boys as compared to controls, suggesting that low INSL3 production may have affected testicular descent (Bay et al., 2007).

Laboratory evidence for EDCs causing cryptorchidism in rodent models of humans

In laboratory rodents, estrogens can down-regulate *INSL3* expression (Emmen et al., 2000; Nef, Shipman & Parada, 2000). Mice lacking INSL3 or its receptor (or with mutations in INSL3 or anti-Müllerian hormone) also exhibit bilateral intra-abdominal cryptorchidism with testes moving freely within the abdominal cavity. Over-expression of INSL3 or of

its receptor in females causes descent of the ovaries into the scrotal position. Prenatal exposure of laboratory rats or mice to 17-β estradiol or the non steroidal estrogen DES (see **Table 2.1**, section 2.1) disturbs the balance between androgens and estrogens and causes demasculinizing and feminizing effects in male embryos, including cryptorchidism. Moreover the estrogen receptor esr-1 (ERα) clearly plays a key role in the mechanism of this disorder, as mice lacking esr-1 but not esr-2 (ERβ) do not become cryptorchid when exposed to estrogens. Moreover, estrogen exposure down-regulates around 63 genes in the mouse fetal testes and up regulates 175, more than half of which are mediated by esr-1.

Epidemiological evidence that EDCs are linked to cryptorchidism in humans

There have been considerable efforts in the human health arena to link maternal exposure to particular chemicals, or groups of chemicals, with the incidence and severity of cryptorchidism. However, a fundamental problem with this approach is the difficulty in capturing exposures that have occurred during pregnancy. As such, the evidence from these studies is mixed: whilst the data linking cryptorchidism to EDC exposure in occupational settings are generally quite convincing (e.g. in agricultural workers; Pierik et al., 2004; Andersen et al., 2008; Weidner et al., 1998; Kristensen et al., 1997), studies that consider effects on the population in general are hampered by difficulties in deciding what chemical, or suite of chemicals, are of significance. All currently published studies are, however, employing a single chemical or group of chemicals approach. Studies that focus on the effects of each chemical in isolation appear to be less informative than those that deal with mixtures. For example, there is currently no evidence for an association between cryptorchidism and exposure to any of several organochlorine pesticides (Damgaard et al., 2006; Longnecker et al., 2002) or with concentrations of any of the individual congeners of polybrominated diphenyl ethers (PBDEs) found in mothers' milk (Main et al., 2007; Carmichael et al., 2010). When the sum of individual congeners was compared, however, Main et al. (2007) and Carmichael et al. (2010) reported significantly higher levels of PBDEs in the mothers' milk of boys with undescended testicles than in those with normal testicular development. Similarly, Damgaard et al. (2006) also detected a significant association between cryptorchidism and exposure to the eight most prevalent organochlorine pesticides. Moreover, analysis showed that the exposure of mothers had occurred long ago rather than recently (Shen et al., 2006). Fernandez et al. (2007) also found that the combined estrogenicity of placenta extracts was strongly related to rates of cryptorchidism. These observations echo the "something from nothing" phenomenon that has emerged from experimental studies with endocrine disruptor mixtures in which EDCs have been shown to act together in combination, even at low and individually ineffective concentrations (Silva, Rajapakse & Kortenkamp, 2002). Only a single study has identified differences in the levels of individual compounds between cryptorchid and normal boys, e.g. higher levels of heptachloroepoxide and hexachlorobenzene in fat samples of cryptorchid boys than in controls (Hosie et al., 2000). This was a small study and has not been repeated. Because the effects of EDCs on the same endpoint are expected to be additive, it would make more sense to use novel bioinformatics tools to integrate and analyze all exposures in order to elucidate whether distinct chemical signatures for different populations exist, e.g. in Denmark and Finland where rates of cryptorchidism differ (Krysiak-Baltyn et al., 2010).

Organochlorine pesticides and PBDEs are rather persistent, and some of them are anti-androgenic (Stoker et al., 2005). Although many of them have been banned after their initial introduction and by countries adhering to the Stockholm and Rotterdam Conventions (web sites www.pops.int and www.pic.int) they still persist in the environment as POPs and continue to add to the contaminant burden of the children (Darnerud et al., 2001; Betts, 2002; see Chapter 3.2). Apart from these well studied chemicals, phthalate plasticizer levels in mothers' urine have been associated with an anti-androgenic effect on the anogenital index of their sons (Swan et al., 2005), and with reduced testosterone and reduced sperm counts. Phthalate levels in breast milk were not, however, associated with the risk of cryptorchidism in these sons, but they were positively correlated with an increased luteinizing hormone/testosterone ratio, a sign of an anti-androgenic effect (Main et al., 2006b).

Several recent studies have addressed the question as to whether painkillers that inhibit prostaglandin synthesis contribute to the risk of developing cryptorchidisms: a study involving the Danish national birth cohort found an association between the use of paracetamol (an inhibitor of prostaglandin synthesis) in weeks 8-14 of pregnancy and a moderate increase in the occurrence of cryptorchidism (Jensen et al., 2010). For a second Danish cohort, Kristensen et al. (2011) reported that intake of paracetamol during the first and second trimester of pregnancy, and for longer than 2 weeks, increased the risk of giving birth to boys with cryptorchidism. The risk was even higher for mothers who had taken more than one compound, such as aspirin and ibuprofen, simultaneously. In the same paper, an association between the use of analgesics during pregnancy and cryptorchidism was not found for a Finnish cohort. Very recently, associations between paracetamol use during weeks 14 -22 of pregnancy and cryptorchidisms were also observed in a Dutch cohort (Snijder et al., 2012). In all these studies, drug use was established by questionnaire.

Low birth weight, being small for gestational age, prematurity and having other genital malformations are well-known risk factors for cryptorchidism (Hjertkvist, Damber & Borg, 1989; Group, 1992; Berkowitz et al., 1993; Berkowitz et al., 1995; Jones et al., 1998; Thong, Lim & Fatima, 1998; Akre et al., 1999; Weidner et al., 1998; Ghirri et al., 2002; Boisen et al., 2004; Preiksa et al., 2005). Mothers' smoking and alcohol consumption may also be risk factors, although studies on this are somewhat controversial. In a prospective cohort study, mothers' alcohol consumption was associated with an increased risk of cryptorchidism (Damgaard et al., 2007), whereas in registry- and interview-based studies including

severe cases, only binge drinking during early gestation was associated with a small increased risk (Jensen, Bonde & Olsen, 2007; Mongraw-Chaffin et al., 2008; Strandberg-Larsen et al., 2009). Many studies have not shown any association of mothers' smoking with cryptorchidism (Mongraw-Chaffin et al., 2008; Damgaard et al., 2008), whereas the use of nicotine substitutes was associated with an increased risk (Damgaard et al., 2008). However, heavy smoking was shown to be associated with an increased risk of bilateral cryptorchidism (Thorup, Cortes & Peterson, 2006), as was gestational diabetes (Virtanen et al., 2006).

2.3.2.3 Hypospadias

The condition in which the urethra opens on the ventral side of the penis or in the perineum instead of the tip is called hypospadias. It results from an incomplete closure of the urethral folds, leaving a split on the penis. (Källen et al., 1986; Kalfa et al., 2011). When the urethra opens to the glans or corona of the penis, it is called distal, whereas opening to the shaft or penoscrotal area defines hypospadias as proximal. Distal hypospadias is often left untreated and therefore is not registered in many malformation registries. Physiological phimosis may hide distal forms of hypospadias at birth, and these may become visible only later when the foreskin can be retracted behind the glans, which explains why the prevalence may differ when boys or men are evaluated at different ages. For example, in Denmark the birth rate of hypospadias was 1% and the cumulative incidence at 3 years was 4.6% due to appearance of distal hypospadias after loosening of the foreskin (Boisen et al., 2005).

Registry-based studies tend to underestimate rates of hypospadias (Toppari, Kaleva & Virtanen, 2001). This is caused by problems in clinical ascertainment, variable reporting to the registry, and differences of registry policies in recording distal cases. Many malformation registries do not register distal hypospadias, although these are most common in population-based clinical studies (Virtanen et al., 2001; Pierik et al., 2002; Boisen et al., 2005). The prevalence of hypospadias used to be between 0.4 and 2.4 per 1000 total births, but these estimates were recently found to be underestimates (Dolk et al., 2004) and there are clear time trends and regional variation (**Figure 2.6**). Increasing trends have been reported in several regions of Australia, Europe, and USA (Källen et al., 1986; Paulozzi, 1999; Toppari, Kaleva & Virtanen, 2001; Nassar, Bower & Barker, 2007; Lund et al., 2009).

Some of the controversies on trend analyses may have been influenced by the policy changes of many malformation registries that started to search for hypospadias more actively than previously, i.e. they did not wait for reporting by the clinicians but checked the diagnoses through the hospital discharge registries revealing many non-reported cases (Hemminki, Merilainen & Teperi, 1993). These changes have to be considered when assessing conflicting reports on the incidence data (Aho et al., 2000; Carmichael et al., 2003; Dolk et al., 2004; Porter et al., 2005; Fisch et al., 2009). Incidence data from prospective and cross-sectional clinical studies of hypospadias are presented in **Table 2.4**.

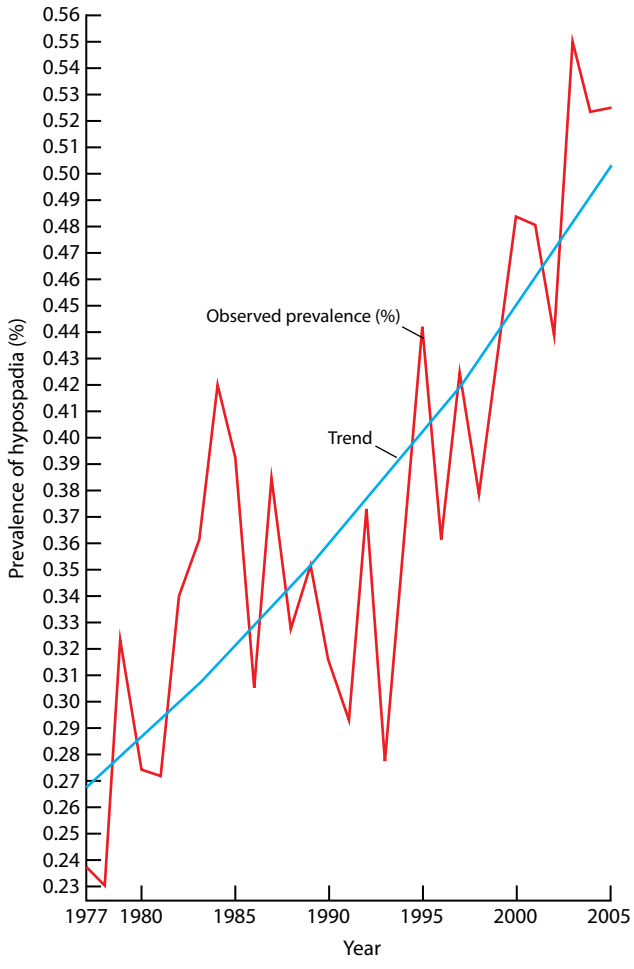

Figure 2.6. Observed prevalence of hypospadias among newborn Danish boys, 1977-2005 (Adopted from Lund et al., 2009).

Hormonal mechanism of hypospadias

Androgens regulate masculinization of external genitalia. Therefore any defects in androgen biosynthesis, metabolism or action during development can cause hypospadias. Gene defects causing disorders of testicular differentiation, conversion of testosterone to dihydrotestosterone or mutations in the androgen receptor can also result in hypospadias (Kalfa et al., 2008). In about 20% of patients with isolated hypospadias there are signs of endocrine abnormalities by the time of diagnosis (Rey et al., 2005).

Several genes that are involved in the penile development from the genital tubercle have been studied for their possible link to hypospadias, but few associations have been found (Kalfa et al., 2008; Wang & Baskin, 2008). The genes include *HOXA13, FGF 10,* and *FGF receptor 2* (Mortlock & Innis, 1997; Frisen et al., 2003; Beleza-Meireles et al., 2007). *Sonic Hedgehog* (*Shh*) is necessary for normal genital development in the mouse (Haraguchi et al., 2001; Perriton et al., 2002; Yucel et al., 2004), but no human mutations have been reported.

Table 2.4 Rate of hypospadias in boys in prospective or cross-sectional clinical (non-register based) studies (adopted from WHO, 2012).

Country	Reference	Study type	Rate of hypospadias
USA, Rochester, Minnesota, St. Mary's Hospital	(Harris & Steinberg, 1954)	Prospective study (n=4474)	0.70% (BW>2500g), 0.76% of all live-born boys
USA, ante partum clinic of the Sloane Hospital, New York City	(McIntosh et al., 1954)	prospective study on pregnant women and infants (n=2793 live-born males)	0.54% of live-born boys
USA, Collaborative perinatal project	(Myrianthopoulos & Chung, 1974)	prospective study (n=53394 consecutive single births (boys and girls))	0.80% of single-born boys (76% of cases detected at birth)
Korea, 38 hospitals	(Choi et al., 1989)	prospective study (n=7990)	0.21% of newborn boys
Southern Jordan	(al-Abbadi & Smadi, 2000)	Clinical study of 1748 boys (aged 6 to 12 years)	0.74% of boys
Finland, Turku, Turku University Hospital	(Virtanen et al., 2001)	Prospective cohort study (n=1505) Total hospital cohort (n=5798)	0.27% of live-born boys 0.33% of live-born boys
Netherlands, Rotterdam	(Pierik et al., 2002)	Prospective study (n=7292)	0.73% of newborn boys
Denmark, Copenhagen, Rigshospitalet	(Boisen et al., 2005)	Prospective cohort study (n=1072)	1.03% of live-born boys (at 3 years: 4.64% of boys (including also milder cases detected when physiological phimosis dissolved))
Bulgaria, 5 regions	(Kumanov et al., 2007)	Cross-sectional clinical study (n=6200 boys aged 0 to 19 years)	0.29% of boys

Activating transcription factor (ATF) 3 may also play a role on the basis of its expression levels locally in the foreskin (Liu et al., 2005). ATF3 is influenced by estrogens, suggesting that this could partly explain why estrogens increase the risk of hypospadias (Liu et al., 2006; Willingham & Baskin, 2007). Hypospadias is often a component in multi-organ syndromes.

Mutations in the gene *MAMLD1* (or *CXORF6*) lead to hypospadias (Fukami et al., 2006), but they are very rare (Ogata, Wada & Fukami, 2008; Ogata, Laporte & Fukami, 2009). The gene has a *NR5*/*SF1* target sequence and the defect affects androgen production (Fukami et al., 2008). Similarly, defects in *NR5*/*SF1* cause testicular dysgenesis (Bashamboo et al., 2010). This gene is one target for endocrine disruptors (Suzawa & Ingraham, 2008).

Genetic polymorphisms in androgen and estrogen receptors have been associated with the risk of hypospadias (Aschim et al., 2004b; Yoshida et al., 2005; Beleza-Meireles et al., 2006; Watanabe et al., 2007). Some of the studies have not been replicated successfully and further analyses in large populations are needed (van der Zanden et al., 2010, 2011; Wang et al., 2008).

Epidemiological evidence that EDCs cause hypospadias

Cryptorchidism and hypospadias have similar risk factors, e.g. being small-for-gestational age (Akre et al., 1999; Aschim et al., 2004a; Pierik et al., 2004; Akre et al., 2008). Estrogens and anti-androgens can cause both conditions, as evident in epidemiological studies following the children exposed to diethylstilbestrol (DES) during pregnancy (for review see Toppari et al., 1996). In addition, the sons of women who were exposed to DES in utero have a higher prevalence of hypospadias than other men, suggesting possible transgenerational effects via epigenetic mechanisms (Klip et al., 2002; Brouwers et al., 2006; Kalfa et al., 2008). All DES effects found in humans had been previously reported in experimental animals exposed to DES (McLachlan et al., 2001).

Association of exposure to pesticides with the risk of hypospadias has been analysed in several studies. A meta-analysis of nine studies showed an elevated, marginally significant risk associated with maternal occupational exposure, whereas paternal occupational exposure was not statistically significant (Rocheleau, Romitti & Dennis, 2009). In the Avon Longitudinal Study of Parents and Children (ALSPAC; a large British child cohort), vegetarian diets of mothers were associated with an increased risk of hypospadias (North & Golding, 2000), while a Swedish study showed a decreased risk for sons of mothers who had fish or meat in their diet during pregnancy (Akre et al., 2008). Whether vegetarians were exposed to more pesticides than omnivorous women is not known. Sub fertility and the use of assisted reproductive techniques increase the risk of hypospadias (Sweet et al., 1974; Czeizel 1985; Wennerholm et al., 2000; Klemetti et al., 2005; Källen et al., 2005). The risks posed by pharmaceutical sex steroids other than DES are controversial. In the past, the use of progestins was associated with an increased risk of hypospadias (Czeizel, Toth & Erodi, 1979; Calzolari et al., 1986), but a more recent meta-analysis of fourteen studies did not find any association between exposure to sex steroids (excluding DES) during the first trimester and external genital malformations (Raman-Wilms et al., 1995). Progestins have been recently introduced again to the market in USA as preventive medicines against threatening miscarriage without evidence of efficacy or lack of untoward effects to date (Wahabi et al., 2011).

2.3.2.4 Reduced semen quality

A meta-analysis from 1992, with results from 14,947 men, included in a total of 61 papers published between 1938 and 1991, indicated that there had been a decline in semen quality

during a period of half a century (Carlsen et al., 1992). The paper was followed up by several studies in which people used available databases in search for trends. Although the results caused controversy (Jouannet et al., 2001), a new meta-analysis with expansion of the data to 101 studies gave similar results (Swan et al., 2000). However, the question of declining sperm counts continues to cause controversy, even today (Jouannet et al., 2001; Skakkebæk et al., 2011). The reason for the controversy may partly be explained by geographical differences in semen quality (Jørgensen et al., 2001), and partly by differences in methods for semen analysis and variation in results within individuals (Jørgensen et al., 1997). Furthermore, all the studies on which the meta-analyses were based were retrospective. To test the hypothesis that semen quality might have deteriorated, Nordic, Baltic, German, Spanish and Japanese investigators have since carried out prospective studies on men from the general population (Jørgensen et al., 2011; 2013). The common finding has been that a significant proportion of young men have semen quality below what is considered to be compatible with good fecundity, although geographical variation exists between countries and between different parts of the same country. Several studies have shown that a sperm concentration below 40 mill/mL is associated with reduced fecundity (Bonde et al., 1998; Guzick et al., 2001; Skakkebæk et al., 2010). 20-40% of young men have a sperm concentration below this level. The problems with human semen quality were recently confirmed in a large French study; decline in semen concentration and morphology in a sample of 26 609 men close to general population between 1989 and 2005 in France (Rolland et al., 2012).

Since 1980, WHO has published five editions of guidelines for semen analysis (WHO, 1980; 1987; 1992; 1999; 2010). The guidelines have been of great value for world wide standardisation of the analysis. WHO has also included reference ranges for semen. In the most recent edition of the guidelines, the reference ranges were changed to reflect the distribution of men who have sired children. The lower cut-off level was estimated to be 14 mill/mL (WHO, 2010). However, as some authors have suggested that no clear borderline exists between the sperm counts of sterile and fertile men, caution should be taken to categorize all men with sperm counts above 15 mill/mL as normally fertile (Skakkebæk et al., 2010; Björndahl, 2011).

It is well known from animal studies that males can sire normal numbers of children in spite of severe defects in spermatogenesis. However, at some tipping point impaired spermatogenesis will result in lower fertility (Andersson et al., 2008; Slama et al., 2002). The question is whether some human populations have reached that point. Besides the low number of sperms in a proportion of men, the average young Scandinavian man has more than 90% abnormal spermatozoa (Jørgensen et al., 2006).

It is still crucial to know whether the increasing use of assisted reproductive techniques (Nyboe Andersen & Erb, 2006) and widespread low fertility rates seen in many industrialized countries (Hvistendahl, 2011) are caused by social factors alone or are also related to male sub fertility (Jensen et al., 2008). Importantly, low sperm counts may be related to poor Leydig cell function (Andersson et al., 2004) and, in some cases, they may be a symptom of TDS (Skakkebæk, Rajpert-De Meyts & Main, 2001). Poor semen quality is clearly also linked to TGC (Petersen et al., 1999) and undescended testis and some cases of hypospadias (Giwercman & Giwercman, 2011). However, most cases of poor semen quality in infertile men are not linked to cryptorchidism and hypospadias. In spite of this, recent evidence from studies of anogenital distance (AGD) in men indicate that poorer semen quality was associated with a shorter AGD, indicating that the low sperm count in some cases could have a prenatal origin, *even* if it is not accompanied by undescended testis and/or hypospadias (Mendiola et al., 2011; Eisenberg et al., 2011).

Evidence of a role of endocrine disruptors in causing low semen quality

Although genetic factors play important roles in causing poor semen quality in some men (Krausz, 2011), most cases of poor semen quality have no known etiology. Smoking and particularly exposure to maternal smoking in utero have been associated with reduced sperm counts (Jensen et al., 2004; Ramlau-Hansen et al., 2007; Ravnborg et al., 2011). A role of EDCs has been hypothesized, but to date there are no clear data except for some rare cases of environmental or occupational accidents where men were exposed to toxic agents like DBCP, which caused azoospermia in workers producing or using the pesticide (Whorton et al., 1979), or dioxin (Mocarelli et al., 2011), which reduced semen quality. Interestingly, in both the DBCP and dioxin cases, the sex ratio was skewed towards an excess of girls fathered by those men during recovery from the exposure. Ongoing preliminary studies in many countries are focussing on possible effects of POPs (Meeker & Hauser, 2010), PFA (Joensen et al., 2009), and non-persistent chemicals (including phthalates (Hauser, 2008), bisphenol A (Mendiola et al., 2010, Li et al., 2011) and DDT/DDE (reviewed in IPCS, 2011) on reproductive functions. The work to unravel the possible effects is cumbersome, as some effects may be prenatal (as the AGD studies suggest) and other effects may be postnatal or perhaps most likely a combination of several types of effects. Considering that the total exposome covers the whole life time and perhaps hundreds of exposures in varying concentrations, links to specific chemicals – if they exist - may be very difficult to establish.

Whilst epidemiological studies of male genital malformations have correlated effects with exposures occurring in utero, studies on semen quality have, almost exclusively, focused on the influence of exposures experienced in adulthood. One of the few exceptions involved an investigation into the consequences of PCB exposure in fetal life for semen quality in later life and was conducted among victims of the Yuscheng incident in Taiwan. Between 1978 and 1979, large quantities of cooking oil contaminated with PCBs were consumed by the Taiwanese people. Guo et al. (2000) examined semen quality among boys whose mothers consumed the oil during pregnancy and found that the boys exposed in utero had sperm with abnormal morphology and reduced motility. Similar effects were observed in men who consumed the cooking oil in adulthood

(Hsu et al., 2003). These men had higher numbers of sperm with abnormal morphology than unexposed men. However, other determinants of semen quality were similar between the two groups. In contrast, the comparison of blood PCB levels in men with poor and normal semen quality did not differ significantly, although an inverse relationship between sperm counts and PCB levels was found among men with normal semen quality (Dallinga et al., 2002).

2.3.2.5 Decreased testosterone

There are two population-based studies indicating a decline of testosterone levels in a birth-cohort dependent manner, i.e. younger generations have lower testosterone levels than the older ones at the same age (Andersson et al., 2007; Travison et al., 2007). Obesity contributes to a decreased testosterone concentration, but it does not explain the adverse trends observed in the above-mentioned studies. Similar findings have been repeated also in Finland (Perheentupa et al., in press). No exposure associations are available.

2.3.2.6 Testicular dysgenesis syndrome

As already stated, hypospadias, cryptorchidism and TGC may, in fact, be related components of a single underlying condition, termed 'testicular dysgenesis syndrome' (TDS), originating during fetal development. Consequently, evidence to support chemical causation of any one of these disorders also adds credence to the hypothesis of chemical causation of the associated disorders. TGC is associated with other reproductive disorders such as cryptorchidism, lower testosterone levels and intersex conditions with hypospadias, in line with a hypothesis of a common origin of these testicular problems (Skakkebæk et al., 2007; Møller & Skakkebæk, 1999). In Finland a recent increasing trend in TGC has coincided with a declining trend in semen quality (Jørgensen et al., 2011), whereas the increase in Denmark seems to have leveled off, at least with regard to TGC (Schmiedel et al., 2010)

Cryptorchidism is a risk factor for testicular cancer. Men with a history of cryptorchidism have a 4-6 fold higher risk of developing testicular cancer than men without cryptorchidism (Dieckmann & Pichlmeier, 2004; Schnack et al., 2010a). However, only about ten percent of men with testicular cancer have been cryptorchid. Bringing the cryptorchid testis down to the scrotum (orchidopexy) does not affect the cancer risk much. Thus, these two disorders share etiological factors rather than having a direct causal relationship with each other, i.e. cryptorchidism is not the cause of testicular cancer. Due to shared etiological factors, a high incidence of cryptorchidism is accompanied with a high rate of testicular cancer, which is apparent in Denmark and Finland; these countries have high and low incidence rates, respectively (Boisen et al., 2004; Jacobsen et al., 2006). Therefore any causal relationship of cryptorchidism with environmental effects can also be considered a putative risk factor for testicular cancer.

Semen quality and fertility are closely related to cryptorchidism (Lee & Coughlin 2001; Virtanen et al., 2007). This connection is reflected also by the differences in semen quality between Finland and Denmark. Sperm counts are significantly lower in Denmark than in Finland (Jørgensen et al., 2001; Jørgensen et al., 2002). The Danish-Finnish cohort study of cryptorchidism, where the testes were measured by ultrasound and reproductive hormones were analysed at the age of three months, showed differences in genital size in early childhood (Boisen et al., 2004; Main et al., 2006a). In Denmark, the testes were smaller and grew slower than in Finland (Main et al., 2006a). Inhibin B levels, reflecting testicular volume, were also lower in Danish boys than in Finnish boys.

The incidence of hypospadias is much lower than that of cryptorchidism, but these disorders are also linked (Toppari, Kaleva & Virtanen, 2001; Schnack et al., 2010b). The incidence of hypospadias differs between Denmark and Finland similar to testicular cancer and cryptorchidism (Suomi et al., 2006; Virtanen et al., 2001; Boisen et al., 2005). All these disorders and spermatogenic problems are linked to androgen action and hormonal regulation during development (Sharpe & Skakkebaek, 2008).

Laboratory evidence for EDCs causing testis dysgenesis syndrome in rodent models of humans

Much of the laboratory-based research into the reproductive implications of EDC exposure in men has been carried out using the rat as a model. This is because, whilst male rats differ from men to some extent with regard to steroidogenesis (discussed by Scott, Mason & Sharpe, 2009), in general the processes underlying their testicular development are thought to be remarkably similar. The assessment of reproductive toxicity in rats comprises a test in which chemicals are administered to dams during gestation. This method has proven to be extremely informative, not only in helping to identify chemicals that interfere with male reproductive development, but also in aiding the discovery of the male programming window and in demonstrating the irreversible nature of the ensuing events. The discovery of the male programming window has been of particular significance in helping to identify the reproductive toxicity associated with certain phthalates and other types of EDCs that only elicit an effect if dosing occurs during a particular window of gestation. Moreover, the analysis of the male offspring produced by these studies has demonstrated that all of the constituent elements of TDS can be recapitulated in the rat, as in men, with the exception of TGC. Male rats exposed to certain phthalates and other chemicals that block the actions of androgen exhibit a range of symptoms, including non-descent of testes, malformations of the external genitalia (similar to hypospadias), poor semen quality and malformations of other sex organs (Foster, 2005; 2006). Evidence of the induction of this so-called "phthalate syndrome" in rats, which closely mirrors TDS in men, suggests that EDCs may be involved in the causation of male reproductive dysgenesis in the human population.

Mechanistically, phthalate syndrome appears to result from lowered fetal testosterone and malformations of the internal genitalia as a consequence. As the development of the male

reproductive tract, prostate and external genitalia also depends on dihydrotestosterone (DHT), a more potent androgen derived from testosterone, lower testosterone concentrations can also cause malformations such as hypospadias. Male rats also require DHT for the regression of nipple anlagen and for the growth of the perineum to produce the normal male AGD. Reduced DHT levels in the wake of suppressed testosterone synthesis also leads to retained nipples and feminized AGDs. These additional signs are useful endpoints for laboratory studies and, as such, the rodent model has been widely used to explore a range of pertinent issues in the EDC field. The data generated have been crucial, for example, in contributing to the controversy regarding bisphenol A and its effects at low doses (see Vanderberg et al., 2012), demonstrating the capacity for mixtures of antiandrogens to act in combination (Rider et al., 2008), and aiding in the identification of new and emerging EDCs, such as inhibitors of prostaglandin synthesis (Kristensen et al., 2010).

There is a growing body of experimental evidence showing that, apart from phthalates, other chemicals that inhibit androgen production or action (anti-androgens) can disturb testicular descent (e.g. Hotchkiss et al., 2010; Rider et al., 2010), and cause hypospadias (e.g. Wilson et al., 2008, but see also **Table 2.1** and section 2.3.5), lowered testosterone and decreased sperm counts. The central role of androgens in both penile development and testicular descent is an important physiological link between cryptorchidism and hypospadias, suggesting a common etiology for these conditions.

Several chemicals can act as androgen receptor antagonists in rodents, and this mode of action is easy to assess also in vitro and by using QSAR methods. The fungicide procymidone and the insecticide metabolite p,p´-DDE are examples of such anti-androgens acting at the receptor level (Wilson et al., 2008). Vinclozolin is not an anti-androgen by itself, but its two metabolites are androgen receptor (AR) antagonists (Kelce et al., 1995). Diethylhexyl (DEHP) phthalate, benzyl butyl phthalate and dibutyl phthalate are compounds that exert anti-androgenic action by inhibition of testosterone production, without affecting AR (Wilson et al., 2008). Some chemicals have both of these anti-androgenic properties, i.e. they inhibit testosterone synthesis and block its action on the receptor. The herbicide linuron and fungicide prochloraz are examples of this (Wilson et al., 2008). When any of these compounds are combined in mixtures, they show dose-additive effects rendering adverse effects even when each of them is in the mixture below its individual NOAEL (Rider et al., 2008; Christiansen et al., 2008).

Anogenital distance (AGD)

The effects of EDCs reported in the rats and mice have also been used to inform human epidemiology studies. In this respect, AGD in baby boys, as in rodents, is a valuable biological marker of disruption of androgen action in fetal life and is inversely related to the risk of cryptorchidism and hypospadias in baby boys (Swan et al., 2005). The study by Swan et al. (2005) investigated changes in AGD relative to maternal levels of urinary phthalate metabolites and found significant relationships between the highest levels of maternal phthalates and shortened (i.e. feminised) AGD in young boys. The patterns reported mirror experimental evidence from rats maintained under controlled laboratory conditions and provide strong evidence that developmental phthalate exposures contribute to disruptions of androgen action in the human population. An expansion of the Swan study, which incorporated a larger number of mother-infant pairs, has confirmed the earlier results (Swan, 2008) and correlations between AGD in boys and maternal exposure to phthalates were also reported in a study of Mexican women (Bustamante-Montes et al., 2008). Moreover, reduced AGD was also found in adult men with corrected hypospadias or cryptorchidism (Hsieh et al., 2008). Poor fertility and impaired semen quality have also been associated with short AGD (Eisenberg et al. 2011; Mendiola et al., 2011).

Although the processes of steroidogenesis and hormone action are essentially the same across most mammalian species, studies suggest that the detailed regulation of testosterone synthesis and pathways through which chemicals act obviously show species differences that will be important for risk assessment of some chemicals. With phthalates, for example, when the rat model of in utero phthalate exposure was extended to the mouse, no suppression of testicular testosterone or its biosynthetic genes was observed (Gaido et al., 2007; Johnson et al., 2011), thus raising the question of whether human health effects of phthalates are better predicted by the rat or the mouse? Moving closer to humans, monobutyl phthalate (MBP) was shown to suppress testosterone levels acutely in the neonatal marmoset, although this suppression was rapidly compensated for, presumably via elevation of luteinizing hormone (LH) levels. In human cells, studies using fetal human testis explants failed to find any effect of MBP on testosterone production (Hallmark et al., 2007; Lambrot et al., 2008) and human fetal testis xenografts exposed to DBP showed no decrease in expression of the steroidogenic genes responsible for fetal testosterone biosynthesis or a reduction in testosterone either (Mitchell et al., 2012; Heger et al., 2012).

Heger et al. (2012), however, found that multinucleated germ cells were induced by phthalates in all three species, human, mouse and rat, showing clearly that concordance across species can be present for some responses but absent for others. Multinucleated spermatogonia have been observed in the testes of both juvenile cryptorchid boys (Cortes et al., 2003) and adult men (Nistal et al., 2006) presenting with carcinoma in situ of the testis. The long-term effects of these dysgenetic germ cells remain unclear.

Other factors may also play critical roles in TDS. Very recently, van den Drische et al. (2012) showed a potentially important role for Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII) in Leydig cell (LC) steroidogenesis that may partly explain phthalate syndrome seen in rats. Exposure of fetuses to DBP dose-dependently prevented the age-related decrease in Leydig cell COUP-TFII expression and the normal increases in Leydig cell size and intratesticular testosterone, thus revealing a further aspect to the mechanisms through which phthalates may act which may or may not be preserved in humans.

The answer to the question of whether phthalates affect steroidogenesis in humans as they do in the rat may mean that concerns about current exposures to phthalates are redundant and that we should focus on chemicals that act via other mechanisms, such as anti-androgenic pesticides and fungicides. Regardless of the conclusion reached, it seems likely that a risk assessment for TDS will have to take into account coexposures to environmental chemicals that are "antiandrogenic" via one or more mechanisms.

2.3.3 Evidence for endocrine disruption of the male reproductive system in wildlife

2.3.3.1 Wild mammals

Some wildlife populations appear to exhibit a suite of demasculinizing and/or feminizing disorders consistent with the symptoms seen in human populations, indicating that the human and wildlife evidence should be considered in parallel when assessing whether EDCs contribute to the etiology of male reproductive disorders. In addition, there is a wealth of data from other types of animal studies that may also be considered as evidence. For example, cryptorchidism has been reported across many farmed and domestic animals, including horses, pigs, rams, rabbits and cattle, as well as cats and dogs (e.g. it was reported with a frequency of up to 6.6% in the case of a sample of more than 300 stray dogs sampled in the tropics; Ortega-Pacheco, 2006). These studies may present opportunities to learn more about its etiology and also serve to highlight the similarities between effects occurring in humans and in animal species. Indeed, a recent study by Bellingham et al. (2012) showed that exposure of sheep to chemicals associated with sewage sludge amended pastures showed that 5 of 12 sludge-exposed rams exhibited major spermatogenic abnormalities, including major reductions in germ cell numbers per testis or per Sertoli cell and more Sertoli cell-only tubules, when compared with controls, which did not show any such changes. Hormone profiles and liver concentrations of a suite of chemicals were not measurably affected by exposure. Such effects seen in real-world exposures could have adverse consequences for sperm counts and fertility in some of the exposed males.

The Florida panther attracted attention when reports indicated a sharp rise in the incidence of cryptorchidism over a thirty year period, beginning in the early 1970s. By 2001, this meant that over half of the population was cryptorchid (mostly unilaterally) and, in addition, almost a quarter of the juveniles exhibited signs of delayed testicular descent (Buergelt, 2002; Mansfield & Land, 2002; Buerguelt, Homer & Spalding, 2002). Coincident with cryptorchidism, these individuals also exhibited reduced testicular volume, low sperm motility and density and semen volume, as well as higher numbers of morphologically abnormal sperm (flaws in the acrosome and mitochondrial sheaths) in comparison with other populations with much lower levels of cryptorchidism. Although this panther population is known to be severely inbred due to its small size, an analysis by Facemire, Gross & Guillette (1995) suggested that genetic composition does not fully explain the observed reproductive abnormalities and proposed dietary exposure to EDCs as a causal factor in the etiology of this apparent male reproductive syndrome. This was refuted by Mansfield & Land (2002), who reported drastically reduced cryptorchidism rates in the progeny of the Florida panther during a genetic restoration plan in which eight female puma from Texas were released into the Florida panther population in 1995. None of the progeny resulting from genetic restoration efforts have been cryptorchid, thus suggesting that cryptorchidism in panthers is genetically rather than environmentally based.

Both uni- and bilateral cryptorchidism, along with many of the other symptoms associated with TDS in humans, have been reported in large numbers (68% in some populations) of Sitka black-tailed deer on Kodiak Island, Alaska. Bubenik & Jacobson (2002) obtained cryptorchid testes from affected individuals and found that these contained malformed or degenerated seminiferous tubules, which contained Sertoli cells but lacked spermatogenic activity. Other abnormalities were evident, such as carcinoma in situ-like cells (possible precursors of seminoma) and microlithiasis, a condition also observed in men with TDS (Skakkebæk, 2004). In addition to these signs of testicular dysgenesis, the affected population also suffered from abnormal antler development. Although genetic causes cannot be ruled out completely, a recent critical analysis favours exposure to environmental contaminants as a likely causal factor (Latch et al., 2008). Such exposure may transform the spermatogenic cells, affect the development of primordial antler pedicles, and also block transabdominal descent of fetal testes, thereby resulting in testis-antler dysgenesis in the affected population (Veeramachaneni, Amann & Jacobson, 2006; **Figure 2.7**).

Similar abnormalities including undersized and mispositioned scrota and ectopic testes were also reported in 67% of 254 male white-tailed deer sampled in the Bitterroot Valley of west-central Montana, USA, between 1996-2000 (Hoy et al., 2002). A high incidence of cryptorchidism in wildlife populations could contribute to population declines and low genetic diversity. In the Sitka deer, however, despite a low proportion of potentially fertile male (only 32% in one population of the Sitka-tailed black deer in Alaska), population growth rates and levels of neutral genetic diversity remain high due to a reproductive strategy whereby few males impregnate many females.

Male polar bears have also shown a multitude of reproductive disorders in recent years that may be linked to their accumulation of high concentrations of persistent organochlorine pollutants such as PCBs (see Chapter 3.2.1). In this respect, the analysis of the reproductive organs of 55 male bears from East Greenland by Sonne et al. (2006) revealed negative correlations between testes size and baculum (penis bone) length and weight in relation to their tissue concentrations of organohalogen pollutants. There was also evidence of testicular fibrosis, atrophy, and inflammation in 20 of the bears analysed. These symptoms may pose a threat to

the reproductive capacity of contaminated populations via their effects on sperm quality and quantity and by compromising their ability to perform coitus, particularly in view of the fact that females of this species are induced ovulators. However, there is no evidence for this as yet. In addition, an inverse relationship between organochlorine contaminant exposure and blood testosterone levels has been reported in polar bears from the Svalbard region (Norway), in which body burdens of organochlorines are particularly high (Oskam et al., 2003). Low fertility and rates of reproduction have also been reported for this population relative to others (Derocher et al., 2003), although whether this is linked to chemical exposure or to differing population age structure and harvesting pressure is the source of some debate (Haave et al., 2003).

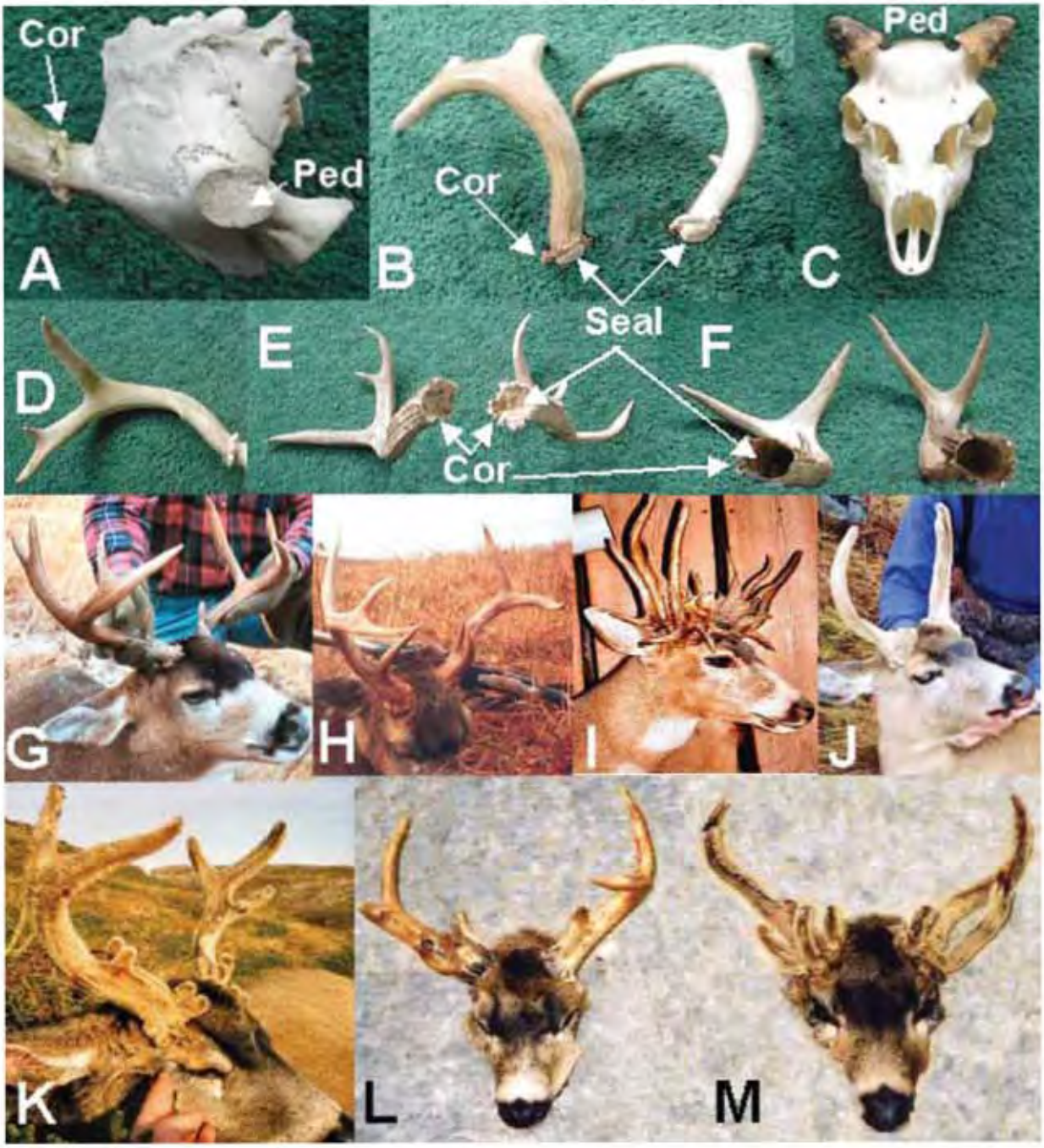

Figure 2.7. Antler dysgenesis in Sitka black-tailed deer. A: Normal skull with coronet (Cor) and concave antler pedicle (Ped) designated. B: Pair of normal antlers each with a convex seal designated. C: Skull with atypical and extremely abnormal, convex antler pedicles (Ped) from a BCO deer. D: Normal antler partially shown in A. E: Pair of antlers each with a thickened base and concave seal (compare with B). F:Pair of antlers each with a thickened base, including extra points, and an extremely concave seal; associated with skull in C. G: Normal antlers. H: Abnormal antlers, typifying those classified as "polished with sharp tips" (compare with G). Also compare tips in B, D, and G (normal) with those in E, F, and H (polished with sharp tips). I through M are abnormal antlers. I: Extraordinary antlers of deer shot in 1967 by N Sutliff. J: Deer with spike antlers, despite age and size, still in velvet and arising from a thick base. K: Deer with unusual antlers bearing extra "points" or " nubs" still covered with velvet. L: Deer with odd antlers and retaining some velvet. M: Deer with bizarre antlers still covered with velvet. From Veeramachaneni, Amann & Jacobson, 2006, supplemental material (Figure used with publisher's permission).

Despite the examples outlined above, which are well known and widely studied, there are a lower number of documented examples of reproductive dysfunction in wild male mammals than one would expect. It is unclear whether this is because the phenomenon is relatively rare, or whether this paucity of data is simply a reflection of the lack of studies that have been done. The paucity of literature suggests that further studies of male reproductive system disorders in mammalian wildlife are needed and that they should focus on predators, which are expected to have a greater EDC burden (as a consequence of biomagnification; see Chapter 3.2.1). In this respect, particularly high concentrations of hydrophobic contaminants (PCBs and brominated flame retardants) have been measured in marine mammals, which, with the exception of plankton-feeding whales, are top predators (see Aguilar et al., 2002; Hansen et al., 2004; Lie et al., 2004; Ross et al., 2000; Noel et al., 2009). Such mammals may be more plastic in terms of their sexual development than humans, potentially making them more sensitive to the effects of EDCs. For example, with regard to cetaceans, whilst both pseudo and true hermaphroditism have been reported (with the external phenotype appearing to be female, but with the internal reproductive organs, or elements thereof, appearing male), there is also evidence of more subtle abnormalities, such as the development of ovotestes (Murphy et al., 2010). This indicates that the suite of symptoms that reflect testicular dysgenesis may be greater in some mammalian species than in others, and that their sensitivities to EDCs may vary. Although it is very difficult to establish causation in such cases, the possible implications of these isolated observations for these species can also not be ignored.

2.3.3.2 Non-mammalian vertebrates

Examples of male reproductive system disorders, occurring concomitantly with chemical exposure, can be provided for all non-mammalian vertebrate classes. Perhaps one of the best documented cases is that of a reptile exposed to high concentrations of pesticides (dicofol, DDD, DDE and DDT) following an accidental spill into a tributary of Lake Apopka in 1980. This had a profound effect on the resident American alligator population. Alongside a dramatic decline in juvenile recruitment, Guillette and colleagues reported various malformations of the male genitalia, including reduced phallus size and histological changes in structure of the testes, along with depressed plasma testosterone concentrations (reviewed by Guillette et al., 2000). However, subsequent studies of alligators from other contaminated lakes yielded inconsistent findings: plasma testosterone concentrations were not reduced at contaminated sites relative to those measured at a control site (Milnes et al., 2002; Gunderson et al., 2004). Furthermore, there was no evidence of a correlation between plasma testosterone, phallus size and contamination status, though a correlation was anticipated based on the Lake Apopka data (Gunderson et al., 2004). The reason for this lack of consistency cannot be ascertained using currently available data. However, it is clear that contaminant exposure can profoundly affect the reproductive development of male alligators, potentially compromising their reproductive capabilities, and that reptiles can exhibit a suite of symptoms in response to chemical exposure which are not dissimilar to those reported in humans and other mammalian species.

Reproductive endocrine disruption has also been reported across a range of bird species since the 1950s. Historically, investigations centred on the issue of eggshell thinning in predatory birds in relation to organochlorine exposure, which ultimately prompted the ban on the use of DDT in North America and Europe. This led to a reduction in body burdens in birds and an improvement in eggshell thickness, with the subsequent recovery of the affected populations (reviewed by Cheek, 2006). However, various other abnormalities have been reported, some of which appear to be consistent with the feminization and/or demasculinization of the male reproductive system. In this respect, the retention of ovarian tissue in the testes of male terns was reported in a breeding colony on Bird Island, Massachusetts (USA) and the prevalence (although not the severity) of this abnormality was associated with PCB and dioxin levels in the developing eggs (Hart et al., 1998). Approximately half of the newly-hatched male chicks had primordial germ cells, which were arranged in a female-like pattern, but no oviducts. A subsequent study of the same colony, however, revealed that these intersex characteristics were no longer apparent by the time the chicks were 21 days old and thus were considered unlikely to influence fertility (Hart et al., 2003). The capacity for EDCs to cause testicular dysgenesis in avian species is supported by laboratory evidence of the effects of chemical exposure on testicular structure and size, seminiferous tubule diameter, delayed germ cell differentiation and sperm quantity (reviewed in Edwards, Moore & Guilette, 2006). In addition, there is evidence of a relationship between high EDC burden and lowered testosterone levels in the eggs of wild birds (Arctic-breeding glaucous gulls; Verboven et al., 2008). Contaminant-induced changes in the ratios of estrogen to testosterone were also reported, indicating that testicular dysgenesis in birds has a similar aetiology to that reported in other vertebrate classes.

Male amphibians also appear to be vulnerable to EDC exposure; intersex has been reported in a range of Anuran species (i.e. frogs and toads) exposed to contaminants in the field. In this respect, Reeder et al. (2005) used historical specimens of the cricket frog, which were collected from 1830-1996, to analyse both temporal and spatial trends in the rates of intersex across the state of Illinois, USA. The authors found that that incidence was low pre-1930 (1.2% from 1852-1929), increased during the period of industrial growth (7.5% from 1930-1945), and was highest during the period of industrialisation and use of organochlorines (11.1% from 1946-1959). It then decreased when sales of DDT were restricted (6.3% from 1960-1979) and continued to decrease in more recent years (2.7% from 1980-1996). Over the total period, the incidence was highest in industrialised areas (10.9%), intermediate in agricultural areas (4.9%) and lowest in less intensively-managed areas (2.6%). Further evidence that testicular maldevelopment is

linked to EDC exposure is provided by a study on leopard frogs by Hayes et al. (2003), which revealed that, out of eight test sites, the only site that had no detectable atrazine was also the only site at which there were no intersex frogs. In addition, gonadal dysgenesis (underdeveloped testes with poorly structured, closed lobules and low to absent germ cells) were observed in frogs at one of the sites that had relatively high levels of atrazine. However, robust evidence that there was a relationship between the rate of reproductive abnormalities and atrazine exposure was lacking. This was also the case in a subsequent study involving leopard frogs, in which the incidence of intersex did not correlate with atrazine exposure on its own; however, there was a positive association between intersex incidence and the total concentrations of all analysed pesticides (McDaniel et al., 2008). Reduced testosterone levels in males from agricultural sites were also reported, a finding that was mirrored in a field study of cane toads, which revealed that intersex correlated with agricultural land use and that intersex toads had lower levels of testosterone (McCoy et al., 2008). Further signs of feminization, including changes in colour, forelimb size and number of nuptial pads, were also reported in toads at the affected sites. Thus, although research into the effects of EDCs on amphibians has been hampered by a range of factors (e.g. low sample size, lack of data on chemical exposures), and the issue concerning atrazine, in particular, remains contentious, it would appear that endocrine disrupting effects on amphibians are manifested as a suite of symptoms, which may be similar to testicular dysgenesis syndrome seen in men.

The feminization of male fish living near to the outfalls from sewage treatment works (STW) plants is consistent across investigators, geographical regions, species and habitats (Cheek, 2006). Various conditions have been reported in wild caught male fish, including the abnormal induction of egg yolk protein (vitellogenin; VTG), as well as a range of abnormalities of the reproductive system, such as altered spermatogenesis, intersex and the feminization of ducts. There have been considerable efforts to determine the functional significance of these abnormalities. In this respect, studies by Jobling and colleagues on roach inhabiting UK water courses have been most informative in terms of linking intersex and vitellogenin (egg yolk protein) induction in male fish to their reproductive capability. For example, Jobling et al. (2002b) reported that all of the phenotypically male fish from polluted sites were intersex and contained female-like ducts and, in addition, exhibited delayed spermatogenesis compared to either intersex or normal fish from reference populations. Intersex roach from polluted sites also had a reduced percentage of spermiating males and lower milt volume, though sperm density was adversely affected at only one polluted site (the Aire), as well as altered testosterone levels compared to normal males. In a related study by Jobling et al. (2002b), intersex fish were again observed to have a lower percentage of spermiating individuals, although milt volume was not affected. Sperm motility and velocity were also low and these characteristics correlated with the severity of intersex. Furthermore, the fertilisation rates of intersex fish from polluted sites was reduced from 93 to 68%, although this difference was only observed in one out of two of the years studied. More recently, competitive breeding experiments with wild roach revealed that reproductive performance was negatively correlated with the degree of intersex, with a 76% reduction in the number of offspring parented by the most feminized individuals (Harris et al., 2011). In some species of fish, evidence of feminization of male urogenital papillae, a condition denoted as morphologically intermediate papilla syndrome (MIPS), has also been seen in wild populations and was more prevalent at sites contaminated with estrogens.

Although most fish species do not have sex chromosomes, they do share other sex differentiation gene products with humans and other mammals (such as DAX-1, DMRT-1, cytochrome P450 ovarian form, cyp19a1, etc.) that have been found to change according to the sexual phenotype of the developing gonads. In the genetically male medaka fish, for example, exposure of male embryos to exogenous estrogen during the process of sex differentiation up-regulates ESR-1, inhibits the expression of enzymes involved in androgen biosynthesis (CYP17, 11 beta-HSD and 17β-hydroxysteroid dehydrogenase [17 beta-HSD]) and testis differentiation (anti-Müllerian hormone [AMH] and doublesex and mab-3 related transcription factor 1 [DMRT1]), and induces proliferation of the germ cells that then redirect the already committed male somatic cells toward female development causing full sex reversal or intersex which is also seen in a variety of other fish species exposed to estrogen. A few laboratory studies have also examined the reversibility of some of the induced effects, such as those on VTG production in males and on intersex induction. These studies seem to suggest that feminization of the germ cells is, in some cases, reversible when exposed subjects are transferred to clean water, but that the overall fertility of these "reversed males" never reaches that of the true males and that courtship behaviour is incomplete.

Although few studies have examined the endocrine control of copulatory organ development and growth in nonmammalian species, what is known suggests that the development and growth of the phallus of reptiles and the gonopodium/genital papillae of fish is androgen-dependent (e.g. for reptiles see Raynaud & Pieau, 1985; fish, see van Tienhoven, 1983). Thus, if these species are exposed to EDCs with antiandrogenic activity, the androgen-dependent phallus would be developmentally altered and feminized. This could occur not only through the actions of environmental anti-androgens, but also through the actions of estrogens as well. In fish, for example, juvenile goby experimentally exposed to 17β-estradiol for 11 to 32 weeks exhibited signs of feminization of the genital papilla, showing that it was inducible by estrogenic exposure and could therefore be a form of estrogenic endocrine disruption. The estuaries where this condition was most prevalent (>50% at certain sites) were also those where estrogenic contamination was the most prominent.

The extensive literature on fish has revealed that the extent and severity of effects varies considerably between species, indicating a species-specific sensitivity to EDC

exposure. In this respect, a study of the incidence of intersex in the lower Great Lakes region, revealed that certain fish species sampled from the same site did not show any gonadal abnormalities (e.g. goldfish, carp, gizzard shad, brown bullhead, pumpkinseed and bluegill), whilst up to 45% of white perch were affected (Kavanagh et al., 2004). This pattern was also borne out during a whole lake study in Canada. Kidd et al. (2007) dosed an experimental lake with the steroid estrogen, ethinylestradiol (EE2; at a concentration of 5 ng/L), to assess the effects on VTG and gonad histology and, subsequently, population sustainability. The data revealed that, whilst VTG concentrations in male fathead minnow, pearl dace and lake trout were dramatically increased (by 1 900-24 000-fold), the effects were much less marked in male white sucker (by up to 118-fold; Palace et al., 2009). Although this scenario is not completely representative of a natural system in which fish are continuously exposed to a mixture of EDCs at low effect levels, the data provide very strong evidence that chemical exposure is associated with a suite of male reproductive abnormalities (intersex and abnormal spermatogenesis), compromising their reproductive capabilities and ultimately leading to the collapse of a "wild" population (Kidd et al., 2007).

2.3.3.3 Invertebrates

Compared with vertebrates, little is known about the manifestation of endocrine disrupting effects on the reproductive system of male invertebrates. However, there are some historical reports in which populations have exhibited signs of feminization, apparently in association with exposure to EDCs. For example, copepods, a type of minute crustacean, living near a long-sea sewage outfall in Inverkeithing, Scotland, were found to have higher than expected rates of intersex, a phenomenon which persisted up to 10 miles (16 km) from the outfall. No evidence was found of disease or parasitism that could have accounted for this phenomenon (Moore & Stevenson, 1991). Intersex has also been reported in lobsters living near sewage outfalls (Sangalang, 1997). More recently, intersex has been reported in molluscan species; following the oil spill from the *Prestige* oil tanker in 2002, populations of Mediterranean mussels in the estuary of the Oka River, in the Bay of Biscay, were found to have a high prevalence of intersex (26%). The area is also subject to pollution from industrial activity (metallurgic industry, ship building, foundries and cutlery making amongst other things). Intersex was absent in mussel populations from a nearby unpolluted area unaffected by the oil spill (Ortiz-Zarragoitia & Cajaraville, 2010). Intersex has also been reported in the estuarine bivalve, *Scrobicularia plana*, in UK estuaries; varying degrees of intersexuality were reported in over 20% of individuals sampled from 17 out of 23 populations and this was putatively linked to exposure to EDCs (Chesman & Langston, 2006). However, as a whole, field-based evidence of endocrine-mediated reproductive disorders in invertebrate males is scarce and solely concerns aquatic Crustacea and molluscs. Much more data are needed on other phyla and on terrestrial species. As a result, it is not yet possible to draw parallels with vertebrates with regards to the likelihood of a testis dysgenesis syndrome, although this remains a possibility.

2.3.4 Evidence for a common cause of male reproductive endocrine disruption in wildlife and humans

Taking all of the evidence together, it is clear that the patterns disturbance of male reproductive health in humans have clear analogies in wildlife: male alligators living in Lake Apopka, which exhibited a similar suite of male reproductive abnormalities following exposure to pesticides from a chemical spill (see section 2.4.1.2) to those seen in similarly exposed laboratory rodents, provide a notable example. Moreover, there is evidence that with accidental and occupational exposures in both humans and wildlife, reproductive dysgenesis occurs in response to high environmental exposure levels. However, there is still a paucity of evidence as to whether the lower levels widely encountered in human populations pose a risk to male reproductive development, although in wildlife species there are examples of widespread dysgenetic male reproductive development linked to EDC exposure, for example in male fish throughout UK rivers (Tyler, Jobling & Sumpter, 1998).

In addition to the rodent studies already described, there is also laboratory-based evidence to support the assertion that EDCs are involved in the causation of male reproductive disorders in wildlife species; vast numbers of exposure experiments, involving the analysis of a wide range of species, have been reported in the literature over the past twenty years. In reviewing this evidence, it becomes apparent that it is difficult to make generalisations about the effects of a particular chemical, or group of chemicals, in terms of their mode(s) of action and/or potency. This is largely due to differences in the ways different species respond to EDCs, but is also confounded by experimental variability in factors such as diet, exposure regime and duration of exposure, as well as differences in the effect level and endpoint under investigation and the technical approaches employed. For example, differences in potency of up to 3-4 orders of magnitude have been reported in fish, depending on the species under investigation and the experimental methodology (see Brian et al., 2005). As a result of their influence on experimental outcomes, these factors can confound the risk assessment of the chemical(s) in question and ultimately make it difficult to decide upon the hazards that these chemicals pose. However, there is a strong consensus that many EDCs have the capacity to derail male reproductive development in the various wildlife species in which endocrine disruption has been studied, leading to the feminization and/or demasculinization of the male form.

Some of the most convincing evidence stems from the analysis of EDC impacts on fish, through which it has been possible to explore and replicate sexual disruption in wild populations under controlled conditions in the laboratory. Since the first discovery that caged male fish placed downstream of sewage treatment works in British rivers exhibited elevated

VTG levels and intersex (Purdom et al., 1994), there have been intensive efforts to identify the chemical(s) responsible. This research has focused on the steroid estrogens and there is now unequivocal evidence from a wealth of laboratory-based studies demonstrating their capacity to feminize fish, with effects reported at all levels of biological organisation, from the molecular level through to impacts on reproductive capacity and population dynamics. However, many other types of chemicals that are also present in STW effluents have also been found to mimic the actions of the steroid estrogens, thus contributing to sexual disruption in fish. Although most of these chemicals, such as the alkylphenols (e.g. nonylphenol), are only weakly estrogenic and are generally found in the environment at low and individually ineffective concentrations, there is now convincing evidence that they can act together in combination with other chemicals that have the same mechanism of action (Thorpe et al., 2003; Brian et al., 2005; 2007). Moreover, it has recently been suggested that sexual disruption in wild male fish may not occur exclusively in response to estrogens and is, instead, a function of combined exposure to chemicals that act via the androgen receptor as well as those with estrogenic properties. A statistical modelling approach has been used to demonstrate that feminizing effects in wild fish can be best modelled by taking account of their predicted exposure to both antiandrogens and estrogens in STW effluents, as opposed to estrogens alone (Jobling et al., 2009). Although this theory is plausible, there are currently a paucity of laboratory-based data on the influence of anti-androgens on the reproductive development of fish and in view of the prevalence of anti-androgenic chemicals in the environment and their reported effects on laboratory rodents, further data addressing this issue in fish are needed.

Thus, it is clear that laboratory-based studies have contributed greatly to the evidence that EDCs are involved in the causation of male reproductive disorders in humans and wildlife. Although differences in sensitivity have been reported, in general, it would appear that the same chemicals, or groups of chemicals, elicit similar response patterns, regardless of the species in question and the test system used. Laboratory-based studies using rodents and non-mammalian species, and most notably fish, have been invaluable in demonstrating the capacity for EDCs to affect reproductive development at low and environmentally-relevant concentrations and in helping to identify critical periods of exposure during development. The data generated support the theory concerning the involvement of EDCs in the causation of male reproductive disorders in wildlife and, in many cases, mirror the evidence concerning the etiology of TDS in the humans. However, until recently, there has been an anomaly in the evidence in support of the hypothesis that effects in humans and wildlife have a common causation; the symptoms of TDS are most easily reproduced in rodent models by exposure to mixtures of antiandrogenic chemicals, whereas the feminization of wild male fish has been attributed mainly to exposure to steroidal estrogens. This casts some doubt on the concept of a shared etiology. However, recent studies indicating widespread anti-androgen presence in rivers and estuaries add credence to the hypothesis that the effects seen in both wild fish and humans may be caused by similar combinations of endocrine-disrupting chemical cocktails to which they are exposed (Jobling et al., 2009). If supporting laboratory-based data can be produced, this gap between the human and fish literature will ultimately be bridged.

It is important to recognise that some components of testis dysgenesis syndrome are not comparable across all species. Using cryptorchidism as an example, it is clear that, whilst widely reported in humans and many other mammals, this condition is not universally applicable to all mammalian species (e.g. elephants and marine mammals do not develop a scrotum and the testes are either held in an abdominal or inguinal location), or indeed to any species of reptile, fish, bird or amphibian. Within these classes, the testes are maintained within the body wall and, thus, do not exhibit testicular descent. However, situations such as this, in which there are clear differences in the structure of the reproductive system across classes and taxonomic groups, it is possible that the symptoms of endocrine disruption may still occur, but in a form that does not, at first, appear to be comparable with previously reported effects. For example, whilst the feminization of the males external genitalia may be characterized by hypospadias in mammals, in some species of fish, it may be that the same phenomenon is manifested by the abnormal development of the urogenital papillae (UGP), a structure that is normally well developed in males (like a penis), but less so in females. Evidence of feminized UPG (known as morphologically intermediate papilla syndrome; MIPS) has been reported in wild-caught sand gobies from contaminated sites around the coast of the UK (Kirby et al., 2003), as well as in sharp tooth catfish inhabiting an estrogen polluted freshwater source in South Africa (Barnhoorn et al., 2004). This provides an interesting parallel with hypospadias, despite the fundamental differences between the genital structure and morphology of mammals and fish. Alternatively, it is important to note that, due to the greater plasticity observed in wildlife, additional endpoints may be affected that have no clear analogy in humans. However, these symptoms could still form part of the same underlying syndrome and, thus, have the capacity to inform the overall understanding of testicular dysgenesis in other forms of wildlife, as well as in the human population.

From the above, it is apparent that most of the symptoms associated with TDS in humans, namely genital malformations and poor semen quality (along with depressed sex hormone concentrations), have also been reported in wildlife, although the wildlife literature clearly encompasses a much larger and more diverse range of symptoms. However, there is a major exception: testis germ cell cancer. Whilst this disease forms an important component of TDS in men, this symptom has not been associated with contaminant-induced endocrine disruption in any wildlife study to date. This may be due, at least in part, to the logistical difficulties in detecting this disorder in wild animals (Edwards, Moore & Guillette, 2006), combined with the likelihood of a low rate of occurrence (its incidence in the

human population is only around 1%). That is not to say that TGC cannot occur in any other species besides humans. Indeed, there are some highly suggestive cases in which atypical germ cells resembling the CIS cells found in human testes have been reported in domestic and laboratory animals, including horses (Veeramachaneni & Sawyer, 1998) and rabbits (Veeramachaneni & Vandewoulde, 1999). CIS is a pre-invasive precursor of TGC, the most common cancer type of human male adolescents and young adults (Rajpert-De Meyts, 2006). There is also growing evidence of abnormal testicular development in wild mammals. In this respect, a variety of testicular tumours, along with microlithiasis and CIS, have been detected in Sitka black-tailed deer on Kodiak Island, Alaska, which were suspected to have been developmentally exposed to estrogenic chemicals (Veeramachaneni, Amann & Jacobson, 2006). In addition, atypical germ cells were encountered in the testes of wild eland in South Africa, although detailed morphological examination for CIS was not possible (Bornman et al., 2010). Testicular microlithiasis and neoplastic lesions were also reported in these animals, which was coincident with high body burdens of environmental pollutants, in particular, alkylphenols.

Data from laboratory-based studies also support the chemical causation of these testicular abnormalities. For example, there is evidence that the developmental exposure of rabbits to a range of chemicals, including those with endocrine disrupting properties, produces symptoms of testicular dysgenesis in the form of atypical germ cells with features characteristic of CIS (Veeramachaneni, 2008). These findings indicate that a parallel for TGC does exist in other species of mammal and, furthermore, add credence to the hypothesis that EDCs are a factor in the increasing rate of TGC in the human population. In contrast, however, it may be that, for some classes of non-mammalian wildlife, TGC cannot occur due to absence of a similar mechanistic pathway.

2.3.5 Main messages

- Recent prospective studies indicate that chances of pregnancy decrease when sperm concentrations decrease below 40-50 million per mL and/or percentage of morphologically normal sperm declines below 9%.
- In a few countries (Denmark, Finland, Germany, Norway, and Sweden) where studies on semen quality in the general population have been systematically done, approximately 20-40% of men have suboptimal sperm concentrations and half of the men have less than 9% morphologically normal sperm. This most likely reflects recent declines in semen quality.
- These decreases in semen quality parallel increases in both the incidence of genital abnormalities in babies and the incidence of testis germ cell cancer in men in the same areas over the last 60 years. The occurrence of cryptorchidism at birth is associated with five-fold increased risk of testicular cancer, impaired semen quality and sub-fecundity.
- Increases in incidences of TGC, cryptorchidism and hypospadias and wide spread poor semen quality are most likely due to environmental factors. Exposures which interfere with the developing testis, including androgen action and/or production during fetal life, are likely to be crucial in the pathogenesis of TDS disorders. Other causes for poor semen quality are also known, such as genetic defects in sex chromosomes.
- Some epidemiological studies show weak associations between exposures to EDCs and the risk of cryptorchidism, hypospadias and decreased sperm production (occupational studies on greenhouse workers; chlorinated pesticides, PBDEs and dioxins).
- Exposures to several anti-androgenic endocrine disruptors have been shown to induce cryptorchidism, hypospadias and reduced semen quality in rodent experiments, often also linked to shortened anogenital distance.
- Wildlife are important sentinels for human male reproductive health as they are more easily sampled and live in direct contact with similar/the same complex mixtures of anthropogenic environmental contaminants to which humans are exposed. However, with the exception of fish, there are limited studies on reproductive abnormalities in males of other wildlife species.
- Symptoms of androgen deficiency and/or estrogen exposure also occur in a variety of wildlife species in both urban and rural environments and have been linked to exposure to contaminants in some cases.
- The symptoms of androgen insufficiency/androgen: estrogen imbalance are sometimes more severe than those seen in humans (i.e. developing eggs within the male testis of fish) because some non-mammalian species exhibit greater innate reproductive plasticity, and are thus more easily feminized.

2.3.6 Scientific Progress Since 2002

Since IPCS (2002), major advancements in our knowledge of endocrine disruption in males have occurred. These include:

- Testicular germ cell cancer has further increased increased in almost all countries in which it has been studied.
- Semen quality among 20-40% of young men from general populations in several European countries is in the sub-fertile range.
- An animal model for Testicular Dysgenesis Syndrome has been established in the rat, showing an inter-relationship between testicular dysgenesis and exposure to some EDCs during the fetal male programming window. There is now a mechanism via which irreversible disorders of the male reproductive tract can be caused.
- Various animal studies have confirmed that the fetus and the pre-pubertal animal are particularly sensitive to EDCs.

- Effects of estrogens in effluents from sewage treatment works on male fish have been seen in many countries and in several species of fish, indicating that this is a widespread phenomenon.
- The feminizing effects of estrogenic chemicals from sewage effluents on male fish, first reported in the 1990s, have now been seen in many countries and in several species of fish, indicating that this is a widespread phenomenon. Feminized (intersex) male fish have reduced sperm production and reduced reproductive success.

2.3.7 Strength of evidence.

There is sufficient evidence that male reproductive disorders, originating during fetal life, are increasing in the human populations in which they have been studied, and that this is partially related to environmental exposures. These diseases include cryptorchidism (testicular non-descent), hypospadias and testis germ cell cancer. There is also limited evidence linking these diseases and disorders with specific occupations and with exposures to chemicals with endocrine disrupting properties, particularly agricultural workers (pesticides and fungicides), PBDE flame retardants and phthalate plasticizers.

Prospective studies show that the occurrence of cryptorchidism at birth is associated with increased risk of testicular cancer, and impaired semen quality and sub-fecundity later in life, and there is limited evidence to suggest that this suite of male reproductive disorders (poor semen quality, TGC, cryptorchidism and hypospadias) are related components of a single underlying condition with a common etiology, termed testicular dysgenesis syndrome, originating during fetal development. There is, however, very little direct evidence for a role of endocrine disrupting chemicals in causing low semen quality in men following developmental exposures. Datasets that include both fetal exposures and adult measures of semen quality are rare.

There is evidence of suboptimal or poor semen quality in large proportions (20-40%) of men in countries in which this has been studied. There is even some evidence for a declining semen quality in these countries.

There is sufficient experimental evidence (from rodent models) supporting the hypothesis that androgen insufficiency during fetal/embryonic development could cause these male reproductive disorders and that EDCs that occur in our environment can contribute to the causation of these disorders.

With the exception of testis cancer (where the evidence is lacking), there is sufficient evidence in rodent models to support the hypothesis that phthalate plasticizers are causal factors in the manifestation of TDS but inadequate evidence to implicate these chemicals as the cause of TDS (or its separate entities) in humans or in any non-mammalian vertebrate.

The strength of evidence that EDCs occur in tissues at concentrations likely to cause endocrine disrupting effects as seen in the laboratory ranges from insufficient to sufficient (dependent on the case studied).

In some wildlife populations, there is sufficient evidence for developmental reproductive disorders and low semen quality in a proportion of the male animals, particularly in areas contaminated by anthropogenic contaminants with endocrine disrupting properties (primarily organochlorines, PBDEs and steroid estrogens). In non-mammalian vertebrate populations studied, the evidence for endocrine disruption is sufficient.

In wildlife also, a suite of effects often occur in concert and can be reproduced in laboratory studies by exposures to EDCs during early development. Taking the wildlife and human evidence together, there is a possibility that exposure to EDCs during fetal life and/ or during puberty plays a role in the causation of male reproductive health problems in humans, in some populations.

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2.4 Endocrine disrupting chemicals and sex ratio in humans and wildlife

2.4.1 Overview of endocrine disrupting effects on sex ratios in humans and wildlife

In humans, the sex ratio (numbers of boys divided by the numbers of girls born) is slightly greater than one, typically resulting in 51 to 52% boys being born (Allan et al., 1997; Astolfi & Zonta, 1999; Davis et al., 2007; Gutierrez-Adan, Pintado & De la Fuente, 2000; Moller, 1996; van der Pal-de Bruin, Veloove Vanhorick & Roeleveld, 1997). In vertebrate wildlife, however, the "normal" sex ratio may vary from one according to the life history of the animal; for example some fish species are first male and then female (or vice versa) and still other species are simultaneous or sequential hermaphrodites. Imbalances in sex ratios induced by exposure to chemicals may be the result of several processes: 1) the chemicals interfere directly with sex determination due to their endocrine properties inducing an increase in the number of individuals of a specific phenotypic sex, 2) the sperm cells or fetal stages of one of the sexes are more susceptible to the effects of a particular chemical than the other, resulting in lower conception or early death and thereby reduction in the number of individuals with the most vulnerable sex and subsequent gender imbalances, or 3) gender imbalances in laboratory experiments may be seen if exposure to a specific chemical promotes sexual maturation of one of the sexes. Given that estrogens and androgens participate in the phenotypic manifestation of genotypic sex, it is reasonable to question whether exposures to endocrine disrupting chemicals are influencing sex ratio in humans and wildlife. Available data suggest the following:

- Several researchers have reported recent small declines in the proportion of human male births in the USA, Canada, Denmark and the Netherlands.
- In addition, there are several specific cases of sharp alterations in the sex ratio of newborns associated with parental exposure to chemicals in industrial accidents or through their occupation.
- Feminized sex ratios induced by exposure to endocrine disrupting chemicals have been observed in a number of wild fish species exposed to the discharge of estrogenic wastewater effluents, whilst masculinized sex ratios are also observed below paper mill effluents and maybe in tributyltin-contaminated marinas. Laboratory studies corroborate these findings.
- There is also some evidence of changes in the sex ratio of some wild bird species, but this appears to be related to poor parental body condition. Similarly, some invertebrates are known to switch between parthenogenetic and sexual modes of reproduction when environmental quality declines.

2.4.2 Evidence for endocrine disruption of sex ratios in humans and in mammalian models of humans (rodents and primates)

Altered Sex Ratio Trends in Humans

During recent decades reduced proportions of male births have been reported in the populations or subpopulations of a number of countries, i.e. USA and Japan (Davis et al., 2007), Canada (Allan et al., 1997), The Netherlands (van der Pal-de Bruin, Veloove Vanhorick & Roeleveld, 1997), Spain (Gutierrez-Adan, Pintado & De la Fuente, 2000), Denmark (Moller, 1996) and in metropolitan areas in Italy (Astolfi & Zonta, 1999).

Mechanisms underlying sex determination in humans

The determination of sex is under stricter genetic control in mammals than in many other taxonomic groups. In an average population, slightly more boys (51-52%) than girls are born. The exact mechanism underlying this phenomenon is unknown.

The sex ratio in a population may vary in response to changes in several socio-economic factors, such as the nutritional status and the age of the mothers giving birth and the number of adult males in the population: thinner/malnourished and older mothers are less likely to have sons, whereas a low proportion of adult men in the population increases the probability of having sons (reviewed by Rosenfeld & Roberts, 2004).

Epidemiological evidence of a role for chemicals in causing alterations in sex ratio

Although the sex ratio is reported to be correlated with the age of mothers in some countries (e.g. Gutierrez-Adan, Pintado & De la Fuente, 2000), exposure to chemicals has also been suggested as a potential causative factor in declines in male births (van Larebeke et al., 2008). High occupational or accidental exposures to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in Seveso (Mocarelli et al., 1996; 2000), to contaminated trichlorophenate in the saw-mill industry (Dimich-Ward et al., 1996), to the pesticide dibromochloropropane (reviewed by Goldsmith, 1997), or to PCBs (Hertz-Picciotto et al., 2008; Weisskopf, Anderson & Hanrahan, 2003) have all been associated with changes in the sex ratio of human populations. Moreover, lower sex ratios have also been reported for populations living in areas affected by air pollution from incinerators (Williams, Lawson & Lloyd, 1992) or industries (Williams, Ogston & Lloyd, 1995) and in a population potentially affected by the petroleum industry (Mackenzie, Lockridge & Keith, 2005). Overall, the results do not allow a robust conclusion regarding EDC influences on sex ratio in human populations, although the associations between chemical exposure and sex ratio are suggestive of this possibility.

Evidence for EDC effects on sex ratio in animal models

Studies on laboratory animals can shed light on sex ratio changes in the human population, such as the reduction in the percentage of boys (to 40%) born to mothers exposed to TCDD after the Seveso accident (Mocarelli et al., 2000). These studies reveal

that sex ratios in experimental mammals can be affected by a multitude of factors such as food availability, composition of the diet, and exposure to chemicals (reviewed by Rosenfeld & Roberts, 2004). In the Seveso case, whilst a three generation study of TCDD in rats found no effect on sex ratio in any generation of the treated animals (Rowlands et al., 2006), an experiment aimed at obtaining an exposure equivalent to the exposure following the Seveso accident found a decrease in the proportion of male offspring sired by exposed male mice (Ishihara et al., 2007). In a subsequent investigation, Ishihara et al. (2010) found no decrease in the Y-bearing/X-bearing sperm ratio, but the sex ratio of the 2-cell embryos of the TCDD exposed group was lower than that of the control group, indicating that the sex ratio of the offspring was decreased at fertilization, rather than at the spermatozoa stage.

2.4.3 Evidence for endocrine disruption of sex ratios in wildlife

2.4.3.1 Mammals

There are no documented examples showing changes in sex ratios related to exposure to EDCs in non-human mammals.

2.4.3.2 Non-mammalian vertebrates

Sexual determination and differentiation is generally more labile in non-mammalian vertebrates than in mammals. This is especially true in fish, amphibians and reptiles where a multitude of ambient factors (i.e. temperature, pH, population density, food availability, growth rate) may affect sex determination and differentiation. The exact genetic background for sex determination is not known for all species.

Skewed sex ratios induced by exposure to endocrine disrupting chemicals have been observed in a number of fish species in the field. The sex ratio in roach does not deviate from 1:1 in an uncontaminated environment (Allner et al., 2010; Geraudie et al., 2010), but controlled exposure to estrogenic wastewater effluents in tanks from one month post hatch up to 3.5 years of age resulted in 98% phenotypic females (Lange et al., 2011). Similarly, the discharge of estrogenic wastewater effluents caused a reduction in the percentage of male white suckers from upstream values between 36 and 46% to downstream values of 17 to 21% (Vajda et al., 2008). Nagler et al. (2001) found male genetic markers in phenotypically female Chinook salmon from the Columbia River, but in this case no direct link was made to endocrine disrupting chemicals.

In contrast, Larsson, Hollman & Förlin (2000) and Larsson & Förlin (2002) found sex ratios among the embryos of the viviparous blenny deviating from the normal 1:1 ratio in the vicinity of discharges from a pulp and paper mill in Sweden, with more male than female fish. A recent study of roach and perch, involving more than 3000 fish, which were caught at eight sampling sites, twice a year, in summer and late autumn/winter over a 2-year period found evidence of male-biased sex ratios. The sites, situated within the greater Upper Rhine catchment, were characterized by different degrees of anthropogenic impact. In addition, a significantly elevated proportion of male perch were obtained from a tributyltin-contaminated marina (Allner et al., 2010), although elevated proportions of male perch were also found at 3 sites with no documented contamination from tributyltin.

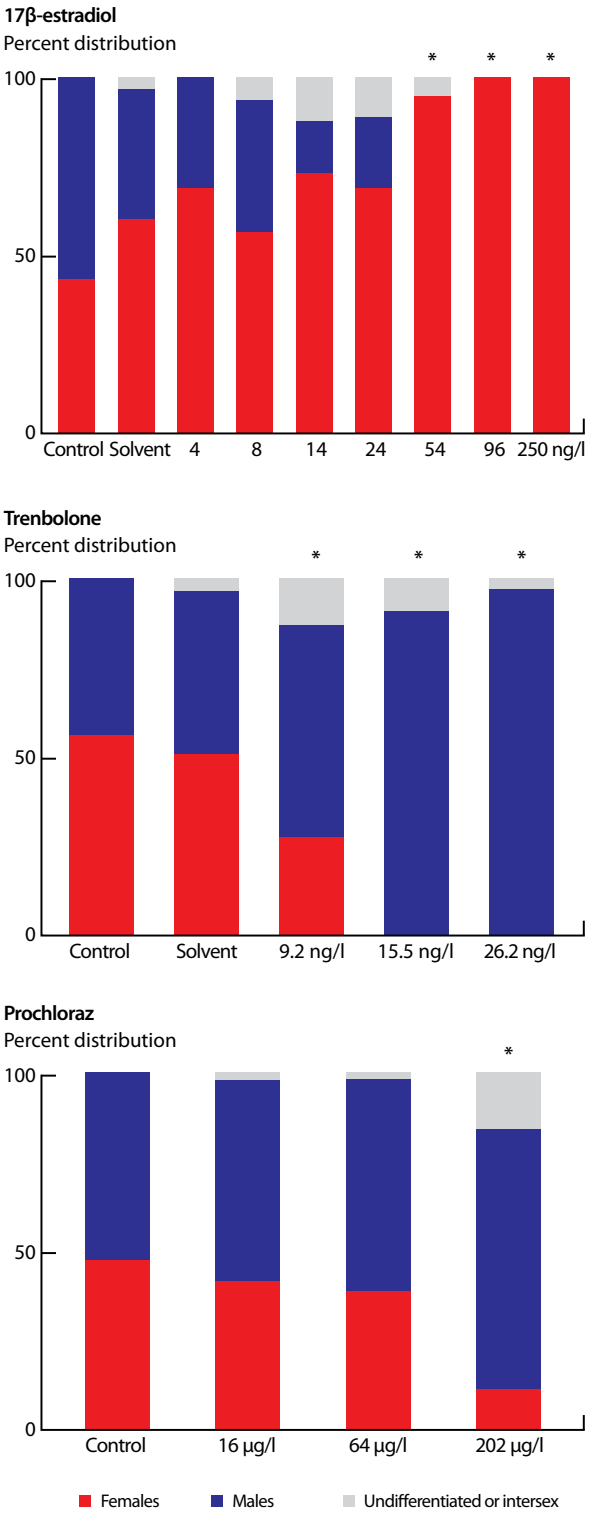
There is also some evidence of changes in the sex ratio of some bird species that are sexually size dimorphic, with males that are larger than females. Parents in poor body condition are able to switch their reproductive output towards the smaller sex. For example, a Norwegian colony of lesser black-backed gulls with high concentrations of organochlorines (PCBs, DDE, HCB, nonachlor, oxychlordane, heptachloroepoxide) in their blood had offspring with a sex ratio that was skewed towards females (Erikstad et al., 2009); no correlation was found between the blood concentrations of perfluorinated compounds or polybrominated diphenyl ethers and the sex ratio in the offspring, thus providing evidence for effects of specific chemicals and not others. The same phenomenon was expected in Arctic glaucous gulls (Erikstad et al., 2011) but, contrary to the expectation, females with high levels of organochlorines had offspring with a sex ratio strongly skewed towards males (most apparent among females in poor body condition). The mechanism underlying these changes in sex ratios is not known, but hatching failure in declining Swedish populations of tree sparrows was shown to result from embryo mortality, which was more likely to affect male embryos than females. The fledgling sex ratio was consequently highly female biased, however, the cause of this sex biased embryo mortality remains unknown (Svensson et al., 2007).

2.4.3.3 Invertebrates

Skewed sex ratios have been linked to exposure to EDCs in various mollusc species. Bacchetta & Mantecci (2009) found a higher proportion of female zebra mussels after a DDT-pollution incident in Lake Iseo, Italy, and Gagné et al. (2011) also found an elevated proportion of female freshwater mussels (*Elliptio complanata*) downstream of two municipal effluent outfalls in the Mille-Îles River (Quebec, Canada). At a tributyltin-contaminated site in the intertidal zone of the Saint Lawrence River (Quebec, Canada), Gagné et al. (2003) reported a decreased proportion of female softshell clams (*Mya arenaria*). Similarly, Leung et al. (2006) found an inverse correlation between the proportion of female neogastropods, *Thais clavigera*, and the body burden of TBT in Hong Kong waters.

2.4.3.4 Laboratory evidence for EDCs causing sex ratio changes in wildlife

Studies of sexual determination and differentiation in fish have revealed extraordinary sensitivity to external hormones or chemicals with endocrine properties (**Figure 2.8**). In the laboratory, populations of commonly-used test fish species such as zebrafish, Japanese medaka and fathead minnow can be made all female by exposure to estrogens during the period of sexual differentiation (Holbech et al., 2006; Nash et al., 2004; Zerulla



Sex ratios in zebrafish exposed to chemicals from hatch till 60 days after hatch when sexual differentiation is normally completed. Exposure to trenbolone (a potent synthetic androgen) and prochloraz (an aromatise inhibiting fungicide) masculinises the fish and the natural estrogen 17β-feminises them. Astrisks indicate significantly skewed sex ratios. Modified from Holbech et al. (2006), Kinnberg et al. (2007) and Morthorst et al. (2010).

Figure 2.8. Sex ratios of zebrafish exposed to chemicals (See legend in figure) (Figure reproduced with publisher's permission).

et al., 2002). In contrast, all male populations can be produced by exposure to androgens (Morthorst, Holbech & Bjerregaard, 2010) or aromatase inhibitors (Kinnberg et al., 2007; Zerulla et al., 2002). The sex ratio in these species is the dominant end point in OECD Test Guideline 234 (OECD, 2011), developed to identify endocrine disrupting chemicals. Sex reversal has also been induced experimentally in a number of other fish species, i.e. carp (Gimeno et al., 1996), Southern catfish (Liu, Zhang & Wang, 2010) and various salmonids (Piferrer, 2001).

Whilst there is extensive field-based evidence that exposure to estrogenic (and also possibly anti-androgenic) chemicals in sewage treatment works effluents is associated with female biased sex ratios (e.g. OECD, 2008; 2011; Lange et al., 2011; Vajda et al., 2008), a number of laboratory-based studies have demonstrated masculinizing effects of pulp and paper mill effluent also with effects on fish reproduction (e.g. Kovacs et al., 2011; Mower et al., 2011; Örn et al., 2006). Male bias was found in fathead minnows exposed to pulp and paper mill effluent during the period of

sexual determination and differentiation (Kovacs et al., 1995). The exact causative agent underlying this masculinizing effect of pulp and paper mill discharges is not known. Wastewater from pulp and paper mills contains complex mixtures of organic compounds including plant sterols with endocrine activity and, previously, when chlorine was used in bleaching processes, chlorinated dioxins and dibenzofurans were also discharged.

There is also experimental evidence that sex ratios can be affected in amphibians (Brande-Lavridsen, Christensen-Dalsgaard & Korsgaard, 2008; Pettersson & Berg, 2007) and reptiles (e.g. Bergeron, Crews & Mclachlan, 1994; Milnes et al., 2005) by exposure to hormones or chemicals with endocrine disrupting activity. Furthermore, laboratory-based experiments on birds have demonstrated that chickens are masculinized if the embryos are treated with an aromatase inhibitor (Yang et al., 2011).

Parthenogenesis, hermaphroditism, female-male cycling during life and gonochoristic sexual development illustrate the variability in sexual strategies among invertebrates, although the precise role of the vertebrate sex steroids within these organisms is not known. Most of the laboratory-based research into the effects of EDCs on sexual development has been done with arthropods, mainly crustaceans, where invertebrate-specific hormones, moulting hormone (ecdysone) and juvenile hormone (methyl farnesoate), also play important roles in development. Although the biochemical mechanisms are far from elucidated, laboratory studies suggest that exposure to EDCs may be able to change sex ratios among invertebrates.

The water flea, *Daphnia magna*, is a branchiopod crustacean, which is a common inhabitant in fresh water ponds in Europe and Asia. It is known to switch between parthenogenetic and sexual reproduction when environmental quality declines. The proportion of male *D. magna* increases upon exposure to atrazine (Dodson et al., 1999), the insecticides pyriproxyfen, fenoxycarb (Wang et al., 2005) and endosulfan (Palma et al., 2008), the miticide dicofol (Haeba et al., 2008), and the juvenile hormone methyl farnesoate (Rider et al., 2005; Wang et al., 2005). Whilst the insecticide methoprene (a juvenile hormone analog) has been reported to increase the number of males (Wang et al., 2005), it can also, conversely, reduce the number of all-male broods (Peterson, Uashian & Dodson, 2001). Similar contradictory results have been found for dieldrin in different species of *Daphnia* (Merritt et al., 1999; Wang et al., 2005).

In other invertebrate classes, bisphenol A exposure has been found to reduce the proportion of female houseflies (Izumi et al., 2008), and to increase the proportion of female isopods, *Porcellio scaber* (Lemos, Vab Gestel & Soares, 2009). The exposure of freshwater amphipods, *Gammarus pulex,* to EE2 resulted in a female dominated population (Watts, Pascoe & Carroll, 2002).

2.4.4 Evidence for a common cause of endocrine disruption of the sex ratio in humans and wildlife

The documented examples of EDC-related sex ratio imbalances in wild fish and invertebrates indicate direct interference of chemicals with sexual determination and/or differentiation, which is possible in these species. In contrast, it would appear that EDC-related sex ratio imbalances in bird populations are more likely to occur as a result of a negative selection of one sex. Humans may follow this model, with decreasing proportions of baby boys occurring as a result of negative selection on male embryos (or potentially sperm cells). Thus, the different mechanisms involved in sex ratio imbalances call for caution when making extrapolations from wildlife to humans about the causes of sex ratio imbalances.

2.4.5 Main messages

- EDC-related sex ratio imbalances resulting in fewer male offspring in humans do exist, e.g. in relation to dioxin and DBCP, although the underlying mechanisms are unknown.
- The effects of dioxins in humans are supported by results in experimental animals.
- EDC-related sex ratio imbalances have been seen in wild fish and molluscs and, in some of these species, are also supported by laboratory evidence.
- The mechanisms underlying EDC effects on sex ratios remain unknown for many species.

2.4.6 Scientific progress since 2002

The following progress has been made since the IPCS (2002):

- Skewed sex ratios in exposed fish and mollusc populations have been demonstrated.
- Skewed sex ratios in a dioxin-exposed human population have been corroborated by results obtained in a mouse model.
- In OECD Test Guidelines TG211 and TG234, sex ratios in *Daphnia magna* were included as endpoints to detect endocrine activity (OECD, 2008; 2011).

2.4.7 Strength of evidence

In wildlife, the changes in sex ratios are consistent with predictions from results of laboratory experiments for a number of the observed effects (e.g. more female fish downstream from estrogenic effluents than upstream) and the evidence that EDCs cause gender imbalances in some non-mammalian wildlife is sufficient. In the human population, the evidence that exposure to specific chemicals (e.g. dioxin and dibromochloropropane) causes gender imbalances in selected populations is sufficient. The evidence that the general decrease in sex ratios in a number of industrialized countries is related to exposures to EDCs is currently insufficient. However, based on the results of occupational exposures, it is plausible that exposure to EDCs can cause gender imbalances among humans.

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2.5 Endocrine disruptors and thyroid-related disorders and diseases

2.5.1 Overview of thyroid-related disorders and diseases in humans and wildlife and evidence for endocrine disruption

Thyroid diseases and disorders in humans (e.g. congenital hypothyroidism and adult autoimmune thyroid disease) have increased in incidence over the past several decades, such that the burden of thyroid disease is approximately two billion people worldwide.

- **Thyroid diseases and disorders represent a particularly high and increasing disease burden in children and adolescents** in several countries in which they have been studied (McGrogan et al., 2008).
- **Between 6-10% of adults have a thyroid disease or disorder.** Hypothyroidism is the most common thyroid disorder and is six times more common in women than men (Vanderpump, Turnbridge & French, 1995)
- **Population-wide testing of thyroid function in the absence of suggestive clinical features reveals a great proportion of "mild" thyroid abnormalities that have most likely gone unrecognized.** These studies suggest that there may be many more adults with undiagnosed thyroid conditions than is currently appreciated (e.g. Canaris et al., 2000).
- Slight decreases in thyroid function - sometimes referred to as "subclinical" or mild hypothyroidism - may have adverse health consequences (elevated cholesterol levels, heart disease and diabetes), especially over the long term and during pregnancy.

Both genetic and environmental factors play a role in thyroid health. However, observations in laboratory animals and wildlife suggest that exposure to endocrine disruptors, particularly during fetal life, could also play a role. Alongside the human health trends, studies describing thyroid dysfunctions in wildlife also exist. Sometimes, these wildlife observations are associated with exposures to contaminants. Examples include:

- **Relationships between body burden of persistent organic pollutants (PCBs, PBDEs and organochlorine pesticides) and thyroid-related effects in marine mammals;** in seals (Brouwer., 1989; Hall, Kalantzi & Thomas, 2003; Hall & Thomas, 2007; Routti et al., 2008), sea lions (Debier et al., 2005), beluga whales inhabiting the St. Lawrence estuary (DeGuise et al., 1995), the harbour porpoise (Schnitzler et al., 2008), and polar bear (Braathen et al., 2004, Skaare et al., 2001).
- **Significant thyroid disruption in monitoring studies of birds in the Great Lakes, Barents Sea, Tokyo Bay, linked with EDC (PBDE and PCB) burdens** (Scanes & McNabb, 2003; Verreault et al., 2004; Saita et al., 2004).
- **Thyroid disruption in salmonid fish living in heavily polluted regions of the Great Lakes in the United States during the 1970s and 1980s and, more recently, in mummichogs in New Jersey and San Francisco Bay** (reviewed in Jobling & Tyler, 2003; Zhou et al., 2000; Brar et al., 2010). Effects in mummichogs were positively correlated with PCB concentrations measured in the livers of the fish.

Hormonal mechanisms underlying thyroid disorders and diseases

The thyroid gland is located at the base of the throat and straddles the trachea. When it becomes physically enlarged in some diseases, it is visible to the eye or can be palpated (goitre). The major product of the thyroid gland is "thyroxine" (tetraiodothyronine, T_4). However, T_4 is not considered to be the most active form of the hormone; rather, it is converted to tri-iodothyronine (T_3), which then acts on the thyroid hormone receptor (TR) in cells.

Thyroid function itself is controlled by "Thyroid-Stimulating Hormone" (TSH, or "thyrotropin"). TSH is a large protein hormone secreted from the pituitary gland that binds to specific membrane receptors on thyroid cells and activates a biochemical pathway that stimulates thyroid hormone production and secretion (Taurog, 2004). The amount of TSH stimulation required to maintain blood levels of thyroid hormone within a "normal" range is controlled by a negative feedback relationship between serum T_4 and serum TSH (Larsen, Silva & Kaplan, 1981). The negative feedback action of T_4 occurs both at the level of the hypothalamus (Vella & Hollenberg 2009; Hollenberg 2008; Greer et al., 1993; Koller et al., 1987; Aizawa & Greer 1981) and pituitary (Wan, Farboud & Privalsky, 2005; Hodin et al., 1989; Chin & Carr, 1987; Carr, Need & Chin, 1987). Thus, under normal conditions, there is a negative correlation between serum levels of T_4 (specifically "free" T_4) and serum TSH.

For this reason, blood levels of T_4 and TSH form the principle clinical measures of thyroid function and disease. So-called "reference" ranges are developed for human populations because there are slight differences in the set-point around which thyroid hormone is regulated in different races, ethnicities and in pregnancy. These reference ranges are generated from a large sample of the population that is without other measures of thyroid disease (symptoms or the presence of anti-thyroid antibodies) (Haddow et al., 2004; Surks, 1991). Reference ranges have been developed for different populations (e.g. Zarkovic et al., 2011), for the different periods of pregnancy (Haddow et al., 2004), even for twin versus singleton pregnancy (Haddow, Palomaki & McClain, 2006), and for preterm versus term birth (Clark et al., 2001; Adams et al., 1995).

Thyroid hormones are important for normal development of the human brain (Bernal, 2007; 2011; Oerbeck et al., 2007), lungs (van Tuyl et al., 2004; Bizzarro & Gross, 2004), heart

(Stoykov et al., 2006; Grover, Mellstom & Malm, 2005; Danzi, Dubon & Klein, 2005), and other organs. Moreover thyroid hormones induce metamorphosis in some fish (Yamano et al., 1994) and in frogs (Buchholz, Paul & Shi, 2005), and they are essential for development in birds (McNabb, 2006) and mammals (Zoeller & Rovet, 2004). There is remarkable evolutionary conservation among vertebrates and some invertebrates in the chemistry of thyroid hormones, as well as in their role in development and adult physiology (Heyland, Reitzel & Hodin, 2004; Heyland & Moroz, 2005). Likewise, the molecular signalling pathways (involving thyroid hormone receptors) through which these hormones exert their actions are highly conserved across the vertebrate taxa (Buchholz, Paul & Shi, 2005; Bertrand et al., 2004; Whitfield et al., 1999).

Endocrine disruptors as risk factors in thyroid disease and dysfunction

Given the importance of thyroid hormone in human and wildlife physiology, and the life-long effects of thyroid dysfunction during development, it is reasonable to carefully consider the possibility that environmental chemicals may interfere with the ability of thyroid hormone to perform its functions. There is a very large list of environmental chemicals – mostly human-made – that can cause a reduction in circulating levels of thyroid hormone in experimental animals (Howdeshell, 2002; Brucker-Davis, 1998). Not all of these produce goitre, although they all reduce serum concentrations of thyroid hormone. Moreover, more environmental chemicals are being identified that can interfere directly with the receptor for thyroid hormone (Zoeller, 2010) or with other processes controlling thyroid hormone action (Gilbert et al., 2011; see **Figure 2.9**).

Thyroid hormone dependent mechanisms of nervous system development in animals and humans

Severe thyroid hormone deficiency produces severe brain damage (Chen & Hetzel, 2010) and moderate or even transient insufficiency can cause specific developmental defects in rodents (Auso et al., 2004; Crofton, 2004; Crofton et al., 2000; Goldey et al., 1995; Goodman and Gilbert, 2007), and in humans (Haddow et al., 1999; Kooistra et al., 2006; Oerbeck et al., 2003; 2007; Pop et al., 1995; 2003; Pop & Vulsma, 2005). Small differences (~25%) in point estimates of maternal T_4 or TSH during the early fetal period are associated with adverse outcomes in humans (e.g. reduced IQ scores), even though hormone levels are not outside the population reference range (Haddow, Palomaki & Williams, 2002; Morreale de Escobar, Obregon & Escobar del Rey 2000). However, in a hallmark study by Bongers-Schokking et al. (2000), the Mental Development Index of children with congenital hypothyroidism was affected by the age of onset of treatment with thyroid hormone, rather than the specific serum free T_4 concentration after treatment. Thus, the degree of

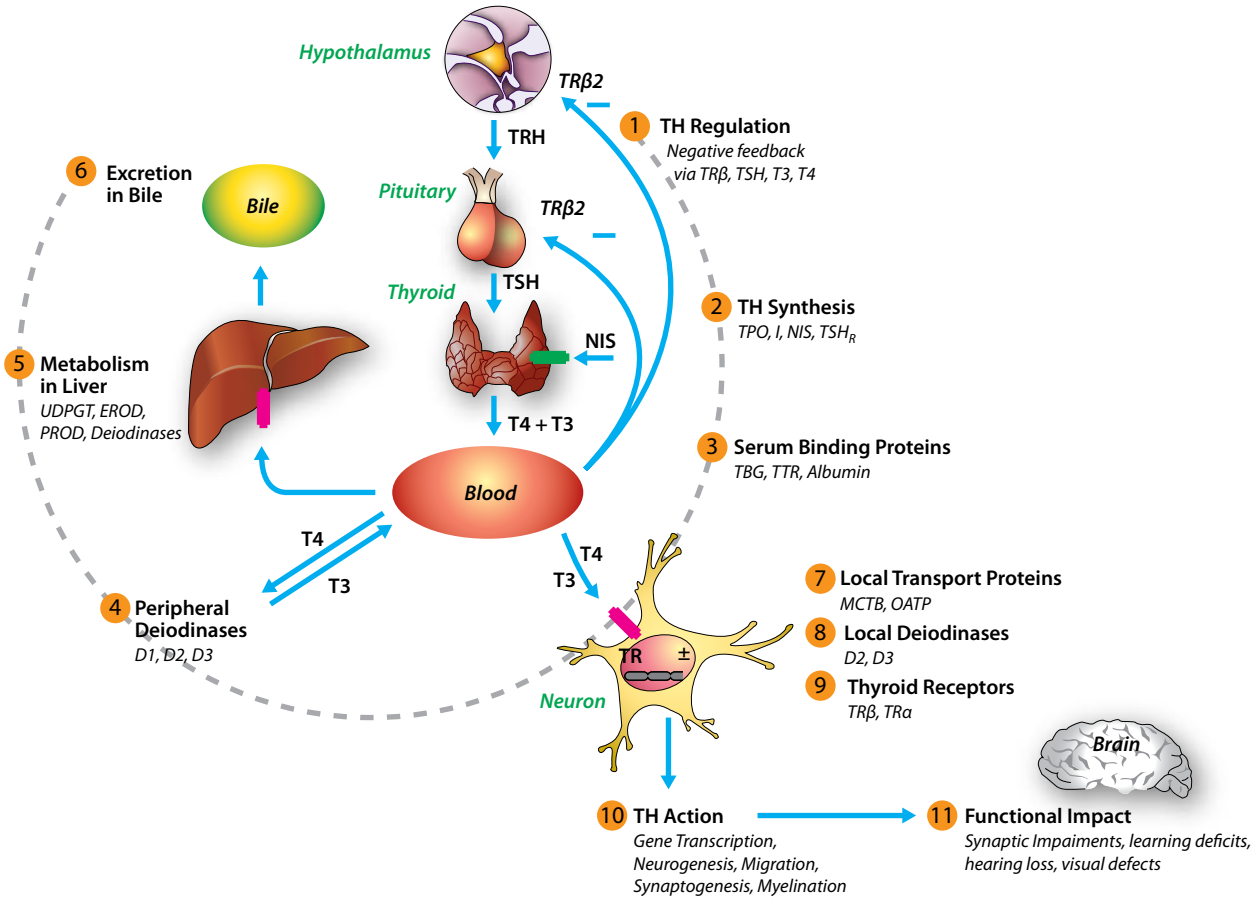

Figure 2.9. Possible sites of action of environmental contaminants on the HPT axis (Figure from Gilbert et al., 2011, redrawn; Used with publisher's permission).

thyroid hormone insufficiency is not the only variable affecting human development; the duration of the insufficiency and the developmental timing of the insufficiency are also important and may vary by species, presenting a challenge for risk assessment. This is discussed further in Chapter 1.2.4)

Experimental work in animals provides strong support for the hypothesis that moderate to mild thyroid hormone insufficiency can alter development in rodents. Integrating data over a series of studies, a decrease in serum total T_4 by 50% during the critical period for cochlear development in the ear was associated with a permanent hearing loss in adult offspring (Crofton, 2004). Moreover, Auso et al. (2004) found that less than a 30% decrease in serum total T_4 in female rodents, for only 3 days, was associated with structural abnormalities in the brains of their offspring. An average decrease in serum total T_4 of only 28% in 2-week-old pups given low doses of propylthiouracil was associated with marked reduction in cell density of the corpus callosum region of the brain (Sharlin et al., 2008). Interestingly, Gilbert & Sui (2008) found that a 28% reduction in circulating levels of T_4 in pregnant rats produced significant adverse effects on synaptic function of hippocampal neurons of their adult offspring despite no detected change in serum T_4 levels in the pups after birth. The US EPA has discovered a cluster of neurons that reproducibly migrates to an incorrect position in the brain of animals that have low thyroid hormone (Goodman & Gilbert 2007). Elements of this cluster very sensitive to prenatal thyroid hormone insufficiency have been characterized (a heterotopia) (Gilbert et al., 2012) . Finally, Sharlin et al., found a very strong inverse relationship between serum T_4 in rat pups and the numbers of myelin-forming oligodendrocytes in major white matter tracks in the brain (Sharlin et al., 2008), and this was not compensated for by elevated serum TSH (Sharlin et al., 2010). Thus, experimental findings confirm what has been observed in humans: small, even transient, decreases in serum total T_4 are associated with altered brain development.

In general, there is strong evidence to conclude that thyroid hormone plays the same general role in brain development of animals and humans (Zoeller & Rovet, 2004). This clearly indicates that rodents represent important test systems to provide information important for protecting public and wildlife health from chemical exposures. In animal studies, the investigator is able to measure the effect of environmental chemicals on blood levels of hormones, and can fully characterize the consequences of these changes on thyroid hormone action at the molecular, cellular and tissue level at various times during development. In addition, a variety of drugs and genetic lines of mice are available to experimentally confirm that environmental chemicals are specifically disrupting thyroid hormone action and not some other pathway of toxicity that could produce similar effects on apical endpoints. In contrast, in human studies, the investigator can only correlate measures of hormone levels in the blood with exposures and with various metrics of health and very few additional measures can be obtained to help interpret the relationship between these variables of interest. Therefore, it is critically important to consider animal studies in the interpretation of human studies.

Notwithstanding this, the current set of validated test methods in the USA and EU for evaluating the ability of chemicals to interfere with thyroid hormone action does not include testing whether the chemical can interfere with thyroid hormone action (Zoeller, Tan & Tyl, 2007a; Zoeller, Tyl & Tan, 2007b).

2.5.2 Evidence for endocrine disruption of the thyroid in humans and in mammalian models of humans

2.5.2.1 Human thyroid diseases and disorders

Thyroid disorders are amongst the most prevalent of medical conditions and include goitres or thyroid nodules (adults), congenital and adult hypothyroidism, autoimmune thyroiditis, hyperthyroidism or Graves' disease and thyroid cancer. In this section, we will deal mostly with congenital and adult hypothyroidism as well as Graves' disease, the remainder being covered in sections 2.11 (autoimmune diseases) and 2.7 (thyroid cancer). As already mentioned, thyroid hormone deficiencies during the development of the brain can also cause neurodevelopmental disturbances leading to mental difficulties, manifest as Attention Deficit Hyperactivity Disorder (ADHD), learning difficulties and possibly even autism. These are discussed further under section 2.6.

Hypothyroidism: This refers to an "underactive" thyroid gland such that it produces too little thyroid hormone. Symptoms associated with hypothyroidism are broad and can be somewhat non-specific including cold intolerance, weight gain, lethargy, and low mentation (Haddow, 2010). Moreover, the body consumes less oxygen and produces less body heat. Hypothyroidism can occur in both children and adults. In the adult population, studies in Northern Europe, Japan and the USA have found the prevalence of hypothyroidism to range between 0.6 and 12 per 1000 women and between 1.3 and 4.0 per 1000 men investigated, although the prevalence is higher in surveys of the elderly (Vanderpump, 2011).

Congenital hypothyroidism: Congenital hypothyroidism (CH) is one of the most common preventable causes of mental retardation caused by thyroid dysgenesis during fetal life. In the first trimester, the fetus is dependent on the trans-placental passage of thyroid hormones of maternal origin because the fetal thyroid gland does not produce thyroid hormone until the end of the first trimester, and then in sufficient quantities only at 20 weeks gestation (Smallridge et al., 2005). Thereafter, however, a hypothyroid fetus will synthesize around 70% less T4 than a normal fetus leading to CH (Olney, Grosse & Vogt, 2010). In 75-80% of all cases of CH, the underlying etiology is unknown, whilst the remaining 15-20% have genetic thyroid dyshormonogenesis. A daily iodine intake <25 µg, particularly in preterm infants, accounts for many cases of CH in Europe, Asia and Africa, but multiple other factors may also be causal elements.

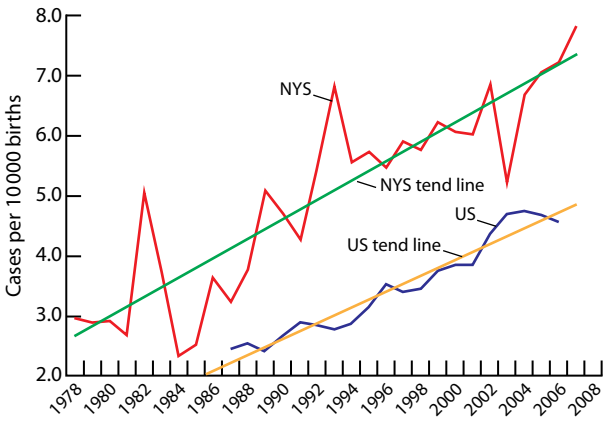

Figure 2.10. Incidence rate of CH in New York State (NYS), 1987–2007, and in the United States (excluding NYS), 1987–2006. (Figure from Hinton et al. (2010), redrawn; Used with publisher's permission).

Estimates of the birth prevalence of congenital hypothyroidism (CH) varies considerably throughout the world where universal screening programs are in place, as reviewed by Rendon-Macias et al. (2008). These estimates range from 1:1403 in Iran to 1:6450 in Latvia.

It was recently reported that the incidence of congenital hypothyroidism has nearly doubled over the past two decades in several countries in which it has been studied including the USA (Harris and Pass 2007; **Figure 2.10**), Western Australia (Kurinczuk et al., 2002), Italy (Corbetta. et al., 2009), the northern UK (Pearce et al., 2010b), and Greece (Mengreli et al., 2010). Some authors speculate that this is due to changes in the cut-off values for the neonatal screening system in the definition of this disorder (Mitchell, Hso & Sahai, 2011; LaFranchi, 2011). This will be an important issue to address.

Subclinical hypothyroidism (mild thyroid failure)

More widespread testing of thyroid function in the absence of suggestive clinical features suggests there are a great number of individuals, not diagnosed with thyroid problems, in which only TSH is abnormal (see **Figure 2.11**). A population study in Colorado, of over 25, 000 individuals of mean age 56 years, showed TSH excess in 9.5 % of the population and suppressed TSH in 2.2%; over half the group with suppressed TSH were taking thyroid medication. Similarly, in the Whickham survey in North East England, 8% of women and 3% of men had subclinical hypothyroidism and in the National Health and Nutrition Examination Survey (NHANES III), approximately 2% of adolescents aged 12–19 years had a serum TSH >4.5 mIU/L.

Prevalence data from one region do not necessarily apply to other populations, because of differences such as ethnic predisposition or variations in iodine intake. Several European studies have compared the effect of various levels of iodine intake on the prevalence of thyroid over- and under-function. Hypothyroidism is generally more common with abundant iodine intake, while goitre and subclinical hyperthyroidism are more common with low iodine intake.

2.5.2.2 Evidence for EDC exposures causing thyroid diseases and disorders.

It is possible that specific chemical exposures could lead to clinical thyroid disease and that this could be reflected in observed secular increases in the incidence or prevalence of thyroid disease. As reviewed above, thyroid disease is defined in large part by the presence of blood levels of T_4 and TSH that are outside the population reference range. For example, clinical hypothyroidism is defined as low T_4 and high TSH; both hormones need to be outside the reference range. However, the clinical symptoms associated with this hormone profile are highly variable in the population, and

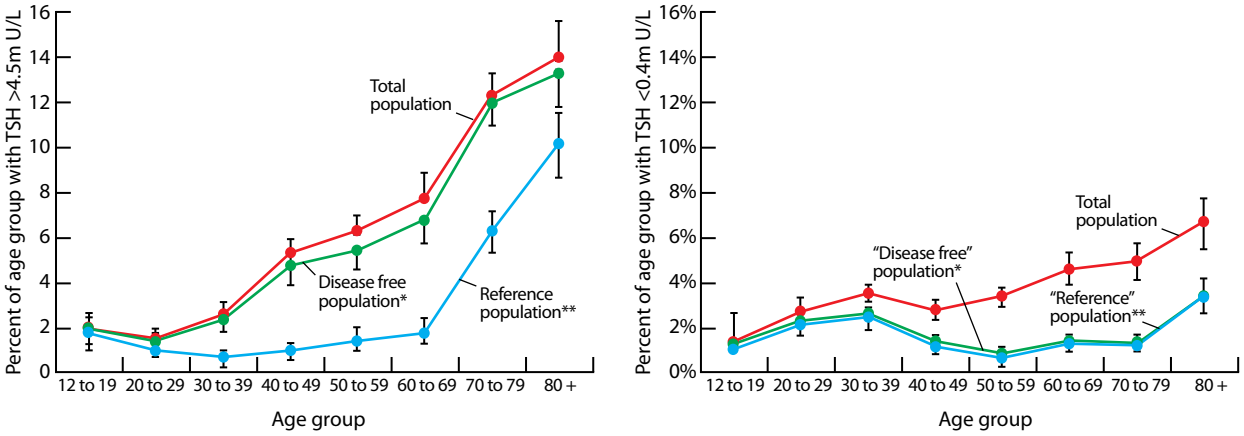

Figure 2.11. Percentage of the USA population (in 2002) with abnormal serum TSH concentrations as a function of age. The disease-free population excludes those who reported thyroid disease, goiter or thyroid-related medications; the reference population excluded, in addition, those who had positive thyroid autoantibodies, or were taking medications that can influence thyroid function. Note the much higher prevalence of TSH abnormalities in the total population, than in the reference population (Figure from Hollowell et al. (2002), redrawn; Used with publisher's permission).

as a result, a significant proportion of the general population can have undiagnosed thyroid disease. In fact, in addition to the 14 million adults in the USA with diagnosed thyroid disease, a further 13 million are estimated to be undiagnosed (Blackwell, 2004). With such a large proportion of likely undiagnosed disease, it is clear that reported changes in incidence or prevalence would not be meaningful. Moreover, because thyroid hormone levels are variable within individuals (Andersen et al., 2003; Andersen et al., 2002), it will be difficult to identify relationships between clinical disease and chemical exposures; in contrast, it may be more likely that chemical exposures will be related to thyroid hormone levels within the reference range. Risk assessors should not disregard such relationships for several reasons:

- First, a large number of chemicals can affect circulating levels of thyroid hormone in animals (Howdeshell, 2002; Brucker-Davis, 1998). Although there are differences between rodents and humans in some characteristics of the thyroid system (see below), rodent systems still provide important fundamental information for the pharmaceutical development of therapeutics for humans. Therefore, it seems inefficient to employ rodent systems to develop drugs but to fail to use rodent systems to protect public health.
- Second, serum TSH levels within the reference range have been identified as a risk factor for blood pressure and serum cholesterol (reviewed in Miller et al., 2009) as well as for bone in postmenopausal women (Morris, 2007). This suggests that serum thyroid hormone levels – TSH and possibly total or free T_4 – will be useful measures to link chemical exposures to various diseases.
- Finally, small differences in serum thyroid hormone levels during pregnancy or at birth are associated with deficits in cognitive function (LaFranchi, 2010). Therefore, if the fetus or neonate is as sensitive to chemical exposures as are adults, then even weak relationships between chemical exposure and hormone levels could produce permanent adverse effects.

A comprehensive review of this literature has recently appeared (Boas, Feldt-Rasmusssen & Main, 2011; Boas, Main & Feldt-Rasmusssen, 2009). There is now reasonably firm evidence that PCBs have thyroid-disrupting effects and that several other common contaminants also have such properties. These include brominated flame retardants, phthalates, bisphenol A and perfluorinated chemicals. In all cases, chemical exposure has been associated with serum thyroid hormone levels. Chemicals may affect circulating levels of thyroid hormone by interacting with the thyroid system in different ways (**Figure 2.9**) and there is currently little information about exactly how these may interact. A key issue is the extent to which changes in circulating levels of thyroid hormone reflect changes in thyroid hormone action in tissues (e.g. Zoeller, 2003). Human exposure to these chemicals (listed in Chapter 3, Table 3.1) is comprehensively reviewed in Chapter 3.2.2.

2.5.2.3 Polychlorinated biphenyls (PCBs)

PCBs are a family of biphenyls that have been randomly chlorinated, producing a mixture of chemicals that have as many as 209 different chlorination patterns. Their biological activity is altered by these patterns; in general, chlorination patterns that stabilize the ring structures into a planar conformation have dioxin-like activity (Kafafi et al., 1993; Kafafi et al., 1992) and those whose chlorination pattern stabilizes the ring structures into a non-coplanar conformation have a variety of activities (Lyng, 2007; Zoeller, 2001; Seegal & Shain, Snyder-Keller & Seagal, 1992; Shain, Bush & Seegal, 1991). Although PCB production was banned in the 1970s, PCBs remain common contaminants in the environment and in humans and wildlife both because of their chemical stability and because of the widespread use from heavy industrial applications to home products such as floor finishes and window caulking.

A number of studies have reported associations between PCB exposure and measures of thyroid function in humans that support the hypothesis that PCBs can reduce circulating levels of thyroid hormone (Abdelouahab et al., 2008; Hagmar et al., 2001a; 2001b; Persky et al., 2001; Schell et al., 2008; Turyk, Anderson & Persky, 2007). Some studies indicate that PCB body burdens suppress serum T_4, whilst others indicate serum T_3. In some cases, the findings are in men, in other cases in women. Overall, it is not a uniform picture. In studies of pregnant women, PCB body burden is positively associated with serum TSH (Chevrier et al., 2007; Takser et al., 2005). Studies of newborns also indicate that PCB body burden suppresses thyroid function (Chevrier et al., 2007; Herbstman et al., 2008). However, a number of studies report no associations between PCB body burden and measures of thyroid function (e.g. Dallaire et al., 2009; Dallaire et al., 2008; Longnecker et al., 2000).

There are a very large number of variables that must be considered to identify a relationship of interest between PCB exposures and measures of thyroid function. These include the fact that PCBs have a very long half-life in the human body and that there are many different PCB congeners that could influence thyroid function differently. There are also slightly different congener profiles in different populations. Measures of thyroid function are also variable across the population (serum total and free T_4 and T_3 and TSH) and this is exacerbated when time-of-day (with which thyroid hormone levels vary) is not standardized. Likewise, there are small gender and population differences. In one study of newborns, the birth mode (caesarean versus vaginal delivery) was important in identifying a relationship between serum PCBs and measures of thyroid function (Herbstman et al., 2008).

Evidence for PCB exposures causing thyroid diseases and disorders in rodent models

Considering these issues, it should be expected that not all studies will find exactly the same relationships. The issue is whether observed correlations between PCB body burden and various measures of thyroid function are consistent with an effect on population health that is mediated by effects on

thyroid hormone action. This is where experimental studies in animal models can be revealing. PCB exposures nearly uniformly cause a reduction in serum total and free T_4 (Gauger, Sharlin & Zoeller, 2007a). However, serum TSH is not often reported to be elevated in response to this decrease (Hood and Klaassen). In addition, different PCB congeners appear to be differentially potent at causing serum T_4 reductions (e.g. Giera et al., 2011), although it is not clear why this is observed. In a controlled study comparing the effects of reduced serum T_4 produced by either propylthiouracil (PTU), which blocks thyroid hormone synthesis, or various PCBs, which presumably induce liver microsomes and decreases the serum half-life of T_4, Giera et al. (2011) found very different effects of PCB exposure compared to PTU exposure. Despite the fact that both exposures brought serum total T_4 to the same concentration in blood, the two exposures had very different effects on the expression of known thyroid hormone response genes in the liver. Thus, the effect of PCB exposure on serum thyroid hormone levels cannot be interpreted the same way as the effect of PTU on serum thyroid hormone levels. This conclusion is supported by other studies (Bansal & Zoeller, 2008; Roegge et al., 2006; Bansal et al., 2005).

These findings also indicate that PCBs, or at least some congeners or metabolites, can interact directly with the thyroid hormone receptor. This hypothesis has been supported by a variety of studies. Several hydroxylated PCBs have been shown to displace T_3 from the TR (You et al., 2006; Kitamura et al., 2005), or to increase (Freitas et al., 2011; Gauger et al., 2007) or decrease (Amano et al., 2010; Miyazaki et al., 2008) thyroid hormone receptor activation in expression systems. Likewise in vivo, PCBs produce effects that are consistent with the hypothesis that they can interfere with thyroid hormone action; in a recent study, PCB body burden in killer whales was highly correlated with the expression of the thyroid hormone receptor (Buckman et al., 2011), a known target of thyroid hormone itself.

Taken together, these findings reveal relatively inconsistent relationships between PCB exposure and measures of thyroid function in humans, but very strong evidence in animals and in molecular studies indicating that PCBs can interfere with thyroid hormone action. The complexity of the human data has been interpreted by some to indicate that there is no convincing evidence that PCBs interfere with thyroid function in humans (Kimbrough & Krouskas, 2003). Moreover, these authors suggest that even if the current data indicate that PCBs can interfere with thyroid function in humans, it is not clinically relevant. Importantly, this review did not include aspects of thyroid measurements that would provide insight into the difficulty in observing PCB effects of interest or the kind of statistical analysis that would be required.

All studies of endocrine disruptors in humans will likely have elements of the dataset observed with PCBs. Specifically, environmental chemicals may produce effects on endocrine systems that are either dissimilar to that of overt disease states, or that are inconsistent from one study to the next due to the difficulty in standardizing exposure measures and measures of hormone levels.

2.5.2.4 Other environmental chemicals

Boas, Feldt-Rasmussen and Main (2011) have also reviewed the literature linking a variety of chemical exposures to thyroid function in humans. These include PBDEs, pesticides, perfluorinated chemicals, phthalates, bisphenol A, UV-filters and perchlorate. With the possible exception of perchlorate, none of these chemicals have been as extensively for their relationship to thyroid function as that of PCBs. Human exposure to these chemicals is, however, extensive (Chapter 3.2.2). Suvorov and Takser (2008) suggest that the PCB story can further inform the number of publications (and time) required to generate enough data to make informed decisions about human and wildlife health.

2.5.2.5 The perchlorate controversy

Perchlorate is an oxidant used in a variety of industrial applications, from the production of solid rocket fuels, to explosives used in automobile airbags, fireworks and blasting caps (reviewed in Oxley et al., 2009). Perchlorate is also naturally occurring (Dasgupta et al., 2006), though the relative degree to which environmental contamination is caused by human-made or naturally occurring perchlorate is not clear. Perchlorate is chemically stable when wet and persists for long periods in geological systems and in groundwater. Largely because of disposal practices during the 1960s – 1990s, perchlorate became a common contaminant of groundwater in the United States (Urbansky, 2002).

The best known biological effect of perchlorate is the inhibition of iodide uptake by the sodium/iodide symporter (NIS) (Wolff, 1998), although it has recently been reported that perchlorate also interacts with Pendrin, another iodide transporter (Attanasio et al., 2011). NIS is responsible for transporting iodide into the thyroid gland, which is required for the production of thyroid hormone (Carrasco, 2000). In addition, this protein is expressed in the gut (Nicola et al., 2009; Vayre et al., 1999), lactating breast (Nicola et al., 2009; Dohan et al., 2003; 2007), placenta (Mitchell et al., 2001), and choroid plexus (Carrasco, 2000), all presumably as a delivery mechanism for iodide to the thyroid gland. In this regard, it is important that the expression of NIS in the human fetal thyroid gland is the limiting step in the production of thyroid hormone (Szinnai et al., 2007).

Given the essential role of thyroid hormone in development, it is important to determine whether perchlorate exposure is associated with measures of reduced thyroid function in the human population. Early studies sought to test this by comparing T_4 or TSH levels in blood spots taken as part of the neonatal screening program with a proxy measure of perchlorate exposure – i.e. the city in which the infant was born (Las Vegas compared to Reno, Nevada, USA) (Li et al., 2000a; 2000b; Crump et al., 2000; Lamm and Doemland, 1999). The hypothesis was that because municipal drinking water was contaminated with perchlorate in Las Vegas but not in Reno, pregnant women and neonates would be exposed to perchlorate in Las Vegas but not in Reno. These studies uniformly found no association between the city of birth and neonatal thyroid

hormone. This was further supported by studies in Chile, in which perchlorate of natural origin is high, and again found no association between neonatal measures of thyroid function and the city of birth (Crump et al., 2000; Tellez Tellez, 2005). It was later shown in national biomonitoring data that almost everyone in the USA is exposed to perchlorate on a continual basis (Blount et al., 2006a; 2006b) and that much of this is derived from food (Huber et al., 2010; Sanchez et al., 2009). Therefore, studies using point estimates of exposure (i.e. city of birth) were confounded by large misclassifications of exposure and provide little useful information concerning the relationship of interest, i.e. perchlorate exposure and thyroid function.

A separate series of studies were performed to determine the efficacy of perchlorate exposure on iodide uptake inhibition in human volunteers (Greer et al., 2002; Lawrence, Lamm & Braverman, 2000; 2001), with the idea that this would help determine whether human exposures could influence thyroid function in the general population. These studies indicated that adults would have to consume 2L of drinking water daily that was contaminated with at least 200 ppb (µg/L) perchlorate to reach a level in which iodide uptake would begin to be inhibited (Greer et al., 2002). Of course, the relationship between iodide uptake inhibition, thyroid hormone synthesis and serum concentrations of thyroid hormone is not known, but was believed to require significant iodide uptake inhibition for extended periods before thyroid function would be impaired. Based on these studies, a USA National Academy of Science (NAS) committee recommended a reference dose (RfD) of 0.0007 mg/kg per day (National Research Council, 2005), which the US EPA used to set a provisional drinking water standard of 15 ppb.

Several authors disagreed with EPA's drinking water standard of 15 ppb and perchlorate remediation goal of 24.5 ppb on the basis that it did not consider infants (Ginsberg et al., 2007). The reason for this was that infants must synthesise their supply of thyroid hormone each day (van den Hove et al., 1999); thus, if environmental factors reduce thyroid hormone synthesis and hormone levels decline, adverse effects on cognitive function would develop. Infants are very sensitive to thyroid hormone insufficiency (Zoeller & Rovet, 2004) and small differences in circulating levels of thyroid hormone in infants have been associated with differences in measures of cognitive function into adulthood (LaFranchi & Austin, 2007; Oerbeck et al., 2003; Heyerdahl & Oerbeck, 2003). Ginsberg et al. (2007) calculated that as many as 90% of nursing infants may exceed the RfD, although later empirical measurements indicate that this number is probably closer to 10% (Valentin-Blasini et al., 2011).

Blount et al. (2006b) showed a significant and sizable association between urinary perchlorate and serum thyroid hormones in a statistically representative sample of the USA population as part of the NHANES survey. This association was observed for women, but not for men. Importantly, the associations observed are plausibly consistent with a cause-effect relationship. That is, urinary perchlorate was positively associated with serum TSH and this association was stronger when urinary iodide was low. In addition, urinary perchlorate was negatively associated with serum T_4 levels when urinary iodide was low. Thirty percent of women in this study had low urinary iodide (below 100 µg/L). Using this same dataset, Steinmaus, Miller & Howd, (2007) showed that women who smoked had elevated levels of thiocyanate, which also inhibits iodide uptake by the NIS, and that in women with low urinary iodide, the association between perchlorate exposure and measures of thyroid function was much stronger.

Conclusions from the NHANES 2001-2002 data are not easily reconciled with the earlier studies of human volunteers or with other population studies (Pearce et al., 2010a; 2011). If these studies reflect a true relationship between very low levels of perchlorate exposure and thyroid function, it would mean that data derived from short-term, high-dose experiments in humans do not accurately predict effects of chronic low-dose exposures.

The conflicting findings among epidemiological studies of the relationship between perchlorate exposure and thyroid function should highlight features of the thyroid system that do not appear to be commonly taken into consideration. One of the most important of these is that circulating levels of thyroid hormone are somewhat variable in each individual (Andersen et al., 2002). In fact, Andersen et al. (2002) estimate that it would require 25 separate tests to estimate the "set point" for serum T_4 in a single individual with a precision of 5%. Thus, the known variability in measurements of T_4 and TSH should be employed to estimate the number of subjects needed to test whether there is a relationship between serum T_4 and perchlorate. Likewise, consideration needs to be given to the known variability of estimates of perchlorate exposure. None of the current studies formally calculate the number of participants that would be required to identify a relationship between serum T_4 (or TSH) and urinary perchlorate. The Blount study included over 1,111 women in their study – the largest to date.

The story of perchlorate contamination should be used to inform studies of other contaminants and their relationship with thyroid function. For those exposures that will act by changing circulating levels of thyroid hormone, perchlorate can serve as a direct example and it will be important to ensure that the study has enough subjects to provide adequate statistical power. This is important because there are known associations between circulating levels of thyroid hormone in pregnant women and, especially, neonates that provide very strong evidence linking hormone levels to adverse outcome. However, for exposures to chemicals that can interfere with thyroid hormone signalling without affecting serum hormone levels, there is clearly a lack of approach at this moment to test these associations in the human population.

2.5.3 Thyroid hormone and other organ systems

It is important to recognize that thyroid hormone concentrations are correlated with adverse effects in organ systems other than the nervous system in the adult, including the cardiovascular system and control of serum lipids (Asvold et al., 2007a; Biondi et al., 2005; Osman et al., 2002), pulmonary system (Krude et al., 2002; Lei et al., 2003; Mendelson & Boggaram, 1991) and

kidney. Total cholesterol, low density lipoproteins (LDL), non-high density lipoproteins (non-HDL), and triglycerides increase linearly with increasing TSH, and HDL decreases consistently with increasing TSH across normal reference ranges without evidence of any threshold effect (Asvold et al., 2007b). Similar trends in lipid profiles can be identified across clinical categories from hypothyroid to euthyroid to hyperthyroid individuals (Canaris et al., 2000). Within the reference ranges for TSH, there is a linear positive association between TSH and both systolic and diastolic blood pressure (Asvold et al., 2007b). Intimal medial thickness (IMT), a measure of atherosclerosis and predictive of coronary vascular disease and stroke, is inversely related to free T_4 after controlling for lipids, clinical factors, and thyroid autoantibodies (Dullart et al., 2007). Some of these measures are ameliorated by treatment with thyroxine. Not surprisingly, deficits in thyroid homeostasis are associated with cardiovascular risk in multiple epidemiologic studies. A meta-analysis of 14 epidemiologic studies (Rodondi et al., 2006) found an overall increase in risk of coronary heart disease (CHD) of over 65% in those with subclinical hypothyroidism (elevation in TSH with normal T_4). A higher risk was noted in those studies that adjusted for most cardiovascular risk factors. Treatment with L-thyroxine of patients with subclinical hypothyroidism resulted in improvements in cardiovascular risk factors including total cholesterol and endothelial function (Razvi et al., 2007). In addition, environmental exposure to at least one thyroid disrupting chemical (PCBs) has been shown to have an inverse association with T_3 in men (Meeker, Altshu & Hauser, 2007) and was associated with both unfavorable lipid profiles and self reported cardiovascular disease in men and women (Goncharov et al., 2008). Therefore, epidemiologic as well as mechanistic and therapeutic evidence substantiates the concern that thyroid disrupting chemicals may adversely affect cardiovascular risk in humans by reducing serum T_4.

2.5.4 Evidence for endocrine disruption of the thyroid in vertebrate wildlife

Thyroid hormone is produced in all vertebrate classes and the chemistry of the hormone is identical in all of these species. In addition, thyroid hormones play a role in development in at least some members of all vertebrate classes. For example, in the flounder, metamorphosis is thyroid hormone dependent. This is also the case for amphibians. Much less is known about the capacity for thyroid dysfunction by EDCs in reptiles and in birds (with the exception of chick development, which provides an important developmental model). Thyroid hormone receptors (both TRα and TRβ) are highly conserved among the vertebrates, suggesting that thyroid disruptors in any vertebrate may exert similar effects across all vertebrate species. However, metabolism of chemicals and subsequent exposures may differ considerably among the vertebrates and there may be other important differences that would suggest that caution be used when extrapolating information from one vertebrate class to another.

Thyroid hormone disruption reported in vertebrate wildlife species includes cetaceans and other sea mammals, as well as a range of fish and birds. Some examples are given in the following sections. Effects on invertebrate wildlife have not been included: whilst thyroid hormone receptor orthologues have been reported across a range of invertebrate species, including the platyhelminths, *Schistosoma japonium* and *Schmidtea mediterranea*, the mollusc, *Lottia gigantea,* and the arthropod, *Daphnia pulex* (Wu, Niels & LoVerde, 2007), the capacity for thyrotoxic chemicals to exert effects on invertebrates is, as yet, unknown. Exposures of wildlife to thyroid hormone disrupting chemicals are comprehensively reviewed in Chapter 3.2.1.

2.5.4.1 Wild mammals

Many studies have reported relationships between individual body burdens of persistent organic pollutants and thyroid-related effects in seals (Brouwer, 1989; Hall, Kalantzi & Thomas, 2003; 2007; Routti et al., 2008), sea lions (Debier et al., 2005), beluga whales inhabiting the St. Lawrence estuary (DeGuise et al., 1995), the harbour porpoise (Schnitzler et al., 2008), and the polar bear (Braathan et al., 2004, Skaare et al., 2001), suggesting contaminant-mediated disruption of thyroid homeostasis. In some studies, interfollicular fibrosis could be seen in the thyroid gland itself, associated with severe pathological dysfunction in other animals. PDBEs and PCBs particularly affect thyroid hormone transport and metabolism (Hallgren et al., 2001; Zhou et al., 2001; Zhou et al., 2002). Thyroid hormones are described as having a permissive role in the effects of other hormones and various enzymes, are important for metabolic regulation and are necessary for adequate growth. They control some aspects of fasting and may play a role in moulting cycles (Bentley et al., 1998). They are therefore key components of the endocrine system of wild mammals and any effects on their production, secretion, metabolism and target sites will have consequences for a range of physiological processes.

2.5.4.2 Non-mammalian vertebrates

Fish in contaminated locations are known to have impaired thyroid systems. The most famous historical examples of thyroid disruption were in the salmonids living in heavily polluted regions of the Great Lakes area in the United States during the 1970s and 1980s (e.g. Leatherland & Sontesgard, 1980a; 1980b; 1982a; 1982b; reviewed in Jobling & Tyler, 2003). Moreover, in the last decade, thyroid abnormalities were also reported in mummichogs from a polluted site in New Jersey, USA (Zhou et al., 2000) and in San Francisco Bay, California, USA (Brar et al., 2010). In the latter study, plasma concentrations of T_4 were significantly reduced in two species of fish from highly contaminated areas, compared with fish from cleaner locations in the same estuary and both the T_3:T_4 ratio and T_3 concentrations were positively correlated with PCB concentrations measured in the livers of the exposed fish whilst T_4 concentrations were inversely correlated. Taken together, the results support the conclusions from laboratory experiments and the general hypothesis already indicated in some marine and terrestrial mammals that environmental PCBs may alter T_4 deiodination or turnover. Relationships between exposure to other chemicals

and thyroid hormone disruption in fish are less common, albeit increasing in the last decade, especially in relation to exposure to flame retardants (PBDEs).

In birds, biomarkers of exposure to thyroid-disrupting chemicals have also been evaluated by McNabb (2005), Panzica, Viglietti, Panzica & Ottinger (2005), and Grote et al. (2006). However, the exact extent to which EDCs exert effects on bird populations is still not established and field studies do not always support extrapolation from laboratory studies (e.g. Fernie et al., 2003; Fernie, Bortolotti & Smiths, 2003a; Fernie, Smiths & Bortolotti, 2003b), possibly because of between-species differences in susceptibility. Notwithstanding this, the relationships between the PCB concentrations and thyroid dysfunction in various bird species conducted over a long period strongly suggest that some PCBs can modulate this system in wild birds. This suggestion is now also supported by results from experimental studies on various model species. Long-term monitoring of herring gulls in the Great Lakes revealed significant thyroid dysfunction linked with PCB burden (Scanes & McNabb, 2003), and structural thyroid abnormalities detected in great cormorants from Tokyo Bay were also associated with PCDF and PCB contamination (Saita et al., 2004).

In addition, other studies on birds have found negative correlations between blood T_4 and T_4:T_3 ratio and levels of organochlorines, particularly hexachlorobenzene and oxychlordane, in glaucous gulls from the Barents Sea (Verreault et al., 2004). Similarly, reduced T_4 levels were reported in white stork nestlings exposed to pollution from a copper smelter (Kulczykowska et al., 2007). In contrast, an increase in T_3 and T_4 levels were detected within the thyroid glands of tree swallow nestlings from reclaimed wetlands partly filled with mine tailings from oil sands processing in Alberta, Canada (Gentes et al., 2007). It was postulated that the modulation of thyroid function in these birds may adversely affect metabolism, behaviour, feather development and moulting, ultimately compromising the survival of fledglings. High body burdens of PCBs in the European shag were associated with increased fluctuating wing asymmetry and also with disruption of the thyroid hormone, vitamin A (retinol) and vitamin E (tocopherol) homeostasis (Jenssen et al., 2010). Intergenerational effects of PCB exposure have also been demonstrated in kestrels, primarily via maternal transfer but also attributable to behavioural effects in the male parent. Where one or both parents had been exposed in ovo to PCBs, the progeny exhibited effects on development and growth, and sexually dimorphic effects on plasma T_3 levels (Fernie et al., 2003b).

2.5.5 Evidence for a common EDC mechanism of thyroid disruption for human and wildlife

From the above, it is apparent that many of the symptoms associated with thyroid hormone disorders in humans, namely alterations in the levels of circulating thyroid hormones and changes in the structure of the thyroid gland, have also been reported in wildlife. However, although probable, as yet there is no evidence that directly links the disruption of thyroid function via chemical exposure to adverse ecological effects in any wildlife species. In contrast, evidence of adverse effects is beginning to emerge from laboratory-based studies and will be discussed in the following section.

2.5.5.1 Evidence for EDC causation of thyroid disruption in laboratory studies with rodents and other vertebrates

Much of the laboratory-based research into the implications of EDC exposure for thyroid function in humans stems from studies using rodent models. For example, the rat has been extensively used to explore the health effects of exposure to PBDEs, with most studies consistently reporting a negative correlation with T_4 concentrations (Zhou et al., 2002; Kodavanti & Derr-Yellin, 2002; Darnerud et al., 2007). Indeed, Kuriyama et al. (2007) demonstrated that BDE-99 has the capacity to reduce T_4 levels in rats, even at low and environmentally relevant doses, with adipose tissue concentrations of BDE-99 in rats close to those reported in non-occupationally exposed humans and also at equivalent doses to those associated with other adverse outcomes in male and female rats, including permanent changes in neurobehaviour, locomotor activity and fertility (Kuriyama et al., 2005). Thus, it would appear that, in rodents, effects on thyroid function occur at EDC concentrations close to current human body burdens.

There is also laboratory-based evidence to support the assertion that EDCs are involved in the causation of thyroid disorders in wildlife species. For example, the suggestion that organochlorine pesticides, PCBs and flame retardants are causing thyroid disruption in arctic wildlife is supported by data from experimental studies on various model species such as domesticated arctic foxes, Greenland sled dogs and goats (e.g. Lyche et al., 2004; Oskam et al., 2004; Ropstad et al., 2006 Sonne et al., 2009). As a model of high trophic level carnivores, Kirkegaard et al. (2011) exposed female Greenland sled dogs and their pups to whale blubber contaminated with organohalogen compounds from 2-18 months of age and then examined thyroid hormone status. Although the sample numbers were low, the results supported observational data in other wildlife and humans, by showing that long term exposure to EDCs may result in detectable effects on thyroid hormone dynamics by lowering both free and total T_3.

In non-mammalian vertebrates, there are many laboratory studies reporting the effects of EDCs on thyroid hormone homeostasis, particularly in amphibians, due to the role of thyroid hormone in inducing metamorphosis. In this respect, BPA has been shown to block thyroid hormone-induced metamorphosis, indicating anti-thyroid activity (Iwamuro et al., 2003), which is consistent with its antagonism of T_3 binding in *Xenopus* tadpoles (Goto et al., 2006). The herbicide acetochlor was also found to accelerate T_3-induced metamorphosis of *Xenopus* (Crump et al., 2002), a process that was preceded by disruption of T_3-

dependent expression of thyroid hormone receptor genes in the tadpole tail. Nonylphenol had an overall inhibitory effect on the rate of bullfrog tadpole metamorphosis (Christensen et al., 2005). Gutleb et al. (2007) developed a synchronized amphibian metamorphosis assay, which is based on a the analysis of a range of endpoints, including the percentage of metamorphosed froglets by the end of the 60-day experimental period and the percentage of tadpoles at different stages of development, using *Xenopus laevis* as a model. Using this assay as a tool, a range of thyroid hormone disturbances were observed in response to a mixture of PCBs.

Although differences in sensitivity have been reported, depending on the model in question, in general, it would appear that the same chemicals, or groups of chemicals, elicit similar response patterns regardless of the species in question and the test system used. Laboratory-based studies using mammalian (mainly rodents) and non-mammalian species (most notably amphibians) have been invaluable in demonstrating the capacity for EDCs to affect thyroid development and in helping to identify critical periods of exposure during development. The data generated by these studies support the theory concerning the involvement of EDCs in the causation of thyroid disorders in wildlife and, in many cases, mirror the evidence concerning the etiology of these disorders in humans.

2.5.5.2 Interspecies extrapolation

Interspecies extrapolation of adverse effects of EDCs requires careful consideration. An example in which cross-species extrapolation is warranted is that of perchlorate. Perchlorate competitively inhibits iodine uptake into the thyroid gland, with subsequent decreases in TH synthesis and declines in circulating TH concentrations (Wolff, 1998). The kinetics for perchlorate inhibition of iodine uptake in humans and rats are extremely similar (US EPA, 2002), indicating the homologous nature of the initial toxic event. Although this is a clear example of a situation in which the toxic event (i.e. iodine uptake into the thyroid gland) exhibits similar kinetic profiles for rodents and humans, the impact of reduced serum thyroid hormone in rodents and humans may differ in some characteristics. For example, rodents or humans may possess robust compensatory mechanisms that would ameliorate the impacts of perchlorate exposure or low T_4 (National Research Council, 2005). However, it is not at all clear that this is the case. Studies in humans indicate that even mild iodine insufficiency is associated with lower IQ in children (Berbel et al., 2009; Zimmermann, 2007; Aghini Lombardi et al., 1995), which does not support the notion that compensatory mechanisms are robust or available to the developing brain. Moreover in animals, Gilbert & Sui (2008) found that perchlorate exposure of pregnant rats can significantly affect synaptic transmission in the adult offspring, which also indicates that robust compensatory mechanisms to low thyroid hormone are not available. In addition, Sharlin et al. (2010) failed to identify compensatory responses to low levels of thyroid hormone in the developing rodent brain.

In contrast to the above, some studies do not support direct extrapolation between species (Crofton, 2004). To illustrate this kind of situation, both in vivo and in vitro studies suggest that PCBs activate the pregnane X receptor (PXR) in rodent liver, which leads to upregulation of hepatic catabolic enzymes and subsequent declines in circulating concentrations of T_4 (Schuetz, Brimer & Schuetz, 1998). The steroid X receptor (SXR) is the human equivalent of rodent PXR (Blumberg et al., 1998) and there are species differences between these two proteins. Rodent PXR is activated by pregnenolone-16α-carbonitrile (PCN), but not by rifampicin, whereas human SXR is activated by rifampicin but not by PCN (Kliewer, Goodwin & Willson, 2002). In addition, in vitro data suggest that high concentrations of CB-153 act as an antagonist at the human SXR rather than an agonist on the PXR in rodents (Tabb et al., 2004). Thus, PCBs may cause serum T_4 to decline in animals but not in humans. While these data appear to support the conclusion that rodent data for PCBs are not relevant to humans, it does not appear to be that simple. First, if the hypothesis is correct that PCB, increase T_4 clearance in a manner similar to that of phenobarbital, then serum TSH should increase as it does in response to phenobarbital (Hood & Klaassen, 2000). Because TSH does not increase in response to PCB exposure in rodents, the mechanism(s) by which PCBs cause a reduction in serum T_4 may not be well understood. In addition, we know that some PCB congeners or metabolites can interact directly with the TR (see above), which is not related to a PXR/SXR pathway. Thus the mechanisms by which PCBs cause a reduction in serum T_4 even in animals are not fully understood, nor have the most important pathways of toxicity in animals or humans been identified. Thus, the information required to exclude animal studies for consideration in risk assessment for PCBs is not available. Moreover, there are few other chemicals for which so much information is available. Therefore, it is unlikely to be the case that animal-to-human extrapolation should be excluded.

Finally, some authors propose that there are differences in circulatory transport proteins for thyroid hormones (e.g. transthyretin and thyroid-binding globulin) in rodents compared to humans and that this renders rodents much more sensitive to thyroid hormone reducing agents than are humans (Capen, 1997; Hill et al., 1998). However, it is not clear that these differences are meaningful for two reasons. First, pregnant and neonatal rodents have high levels of all transport proteins including thyroxine binding globulin (TBG) (Savu et al., 1991; Vranckx, Savu & Nunez, 1989; Savu et al., 1989; 1987). Rat TBG has been cloned (Tani et al., 1994) and its regulation studied (Vranckx et al., 1994). Thus, the contention that rodents do not have the same serum binding proteins as humans may not be correct. A further difference between rodents and humans is that the serum half-life of T_4 in rodents is much shorter than that of humans (1 day in rodents versus 7-10 days in humans), although it is not at all clear that this issue renders rodent studies of thyroid function irrelevant to humans either; there are considerable data that suggest just the opposite.

2.5.6 Main messages

- Thyroid hormone is important in development and in adulthood in both wildlife and humans.
- Aside from thyroid cancer and congenital hypothyroidism, it is difficult to identify trends in the incidence of human thyroid disease.
- There are many chemicals that can interfere with thyroid function.
- Similarly, there are chemicals that can interfere directly with thyroid hormone action.
- Many chemicals interfere with thyroid function in a manner that will not be captured by evaluating only serum hormone levels.
- Despite the recognition that thyroid hormone is essential for brain development in humans, few if any chemicals are tested for their ability to interfere with thyroid hormone action.
- Relationships between exposure to chemicals and thyroid hormone disruption in wildlife species have increased in the last decade, especially in relation to exposure to flame retardants (PBDEs) and PCBs.
- The strength of evidence supporting a role for endocrine disrupting chemicals in disrupting thyroid function in wildlife adds credence to hypothesis that this could also occur in humans.
- Thyroid disruption is acknowledged to be poorly addressed by the chemical tests currently listed in the OECD Conceptual Framework.

2.5.7 Scientific progress since 2002

Since the *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002), the following advances have been made:

- Increasing numbers of human studies establish a link between chemical exposures and thyroid function, including in pregnant women.
- However, few studies have focused on the relationship between chemical exposures in pregnant women, thyroid measures in those women (or in the cord blood of their offspring), and cognitive function in neonates.
- Genetic lines of mice have become widely available that should be coupled with toxicology studies to help clarify the mechanisms by which chemical exposures can interfere with thyroid hormone action.
- Relationships between exposure to chemicals and thyroid hormone disruption in wildlife species have increased in the last decade, especially in relation to exposure to the flame retardants (PBDEs) and PCBs, but other chemicals are insufficently studied.

2.5.8 Strength of evidence

There is sufficient evidence that some thyroid diseases are increasing in the human population and that this baybe related to environmental exposures. These diseases include congenital hypothyroidism and thyroid cancer. This evidence is considered to be sufficient because several authors report an increased incidence using screening data that reflect population-wide surveys. However, there are insufficent data linking these increases in thyroid disease to specific environmental factors.

There is limited evidence from wildlife studies and sufficient evidence from laboratory experiments that endocrine disrupting chemicals can interfere with thyroid hormone signalling, leading to diseases and disorders in wildlife species. The data generated by these studies support the theory concerning the involvement of EDCs in the causation of thyroid disorders in wildlife and mirror some of the evidence seen in humans. For many wildlife species, however, no studies have been done.

There is insufficient direct evidence in the human literature supporting the hypothesis that effects on thyroid hormone signalling mediate the association between chemical exposures and human disease/disorders. Perhaps the best example of this is focused on PCBs. There is sufficient evidence linking PCB body burden to reduced measures of cognitive function in children (Schantz, Widholm & Rice, 2003) and this evidence is deemed to be sufficient because a number of authors have reported similar findings and because it is consistent with studies in animals. In animal studies, PCBs clearly reduce circulating levels of thyroid hormone (Brouwer et al., 1998) and can affect brain development (Roegge et al., 2006). There are some studies indicating that PCB body burden is linked to reduced measures of cognitive function, but the evidence demonstrating a causal relationship is limited. Few studies have evaluated the relationship between PCB exposure, cognitive development, and thyroid hormone; therefore, there is overall insufficient evidence to demonstrate that PCBs interfere with thyroid hormone signalling and cause an adverse effect. Animal studies indicate that PCBs can exert effects on thyroid hormone signalling in development that are not consistent with effects on serum hormone levels (Bansal & Zoeller, 2008; Giera et al., 2011). Therefore, while considerable evidence exists in animal studies that chemicals can interfere with thyroid hormone signalling during development and produce adverse outcome, we have not developed the approach to fully test this hypothesis in human populations.

Thus, there are insufficient data linking chemical exposures to altered thyroid hormone signalling and the occurrence of disease or dysfunction in humans. Clearly, considering the importance of thyroid hormone during development, the large knowledge gaps, animal data, and the economic cost of population wide impacts on thyroid function during development (Dosiou et al., 2008), these are issues that need to be addressed quickly.

2.5.9 References

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2.6 Endocrine disruptors and neurodevelopment in children and wildlife

2.6.1 Overview of neurodevelopmental problems in humans and wildlife and evidence for endocrine disruption

There is currently considerable concern about a potential relationship between increasing prevalence of neurodevelopmental disorders and the increase in exposure to pollutants over the past several decades (Landrigan & Goldman, 2011a; 2011b; Weiss & Landrigan, 2000). Since the 1970s, there have been dramatic increases in previously rare neurodevelopmental disorders. For example, in the 1970s autism prevalence was estimated to be between 4 and 5 in 10,000 children (Wing et al., 1976) but today this value is estimated to be 1 in 110 children (Rice, 2007). Similar trends have been observed for other neurobehavioural problems such as ADHD (attention deficit hyperactivity disorder) and autistic disorder (**Figures 2.12** and **2.13**), learning disabilities and childhood and adult depressive disorders. Predominant among these disorders are attention deficit disorders (ADD) – with or without hyperactivity – with a worldwide pooled prevalence estimate of about 5.3% (Polanczyk et al., 2007). Whilst the increase in autism spectrum disorders is indisputable, questions remain as to whether the increase in the incidence and prevalence of ADHD represent a true increase rather than an artefact due to more agressive disagnosis and reporting.

There are also questions regarding whether there are biological determinants of ADHD that may be impacted by the environment. There are brain imaging studies that support the concept that there are biological differences between children with ADD compared to those children without ADD (Aguiar, Eubig & Schantz, 2010). In addition, genetic studies show a link between ADHD and genotype, though this is modified by environment (Khan & Faraone, 2006). Therefore, it remains a significant challenge to identify the possible causes of the increased incidence – either geographical or temporal – and to determine the extent to which environmental factors play a role (Aguiar, Eubig & Schantz, 2010).

The observation of Paracelsus that women with goitre gave birth to children with severe mental retardation was an early indication that environmental factors could affect brain development and neurobehaviour (Cranefield & Federn, 1963). Likewise, lead poisoning has been known to cause neurotoxicity for millennia, though this was believed to be a disease of adults working in occupations in which lead exposure was very high (Needleman, 2009). Since then, our knowledge of the relationship between neurodevelopmental disorders and chemical exposure has advanced. It is now clear that children – especially during fetal development – are sensitive to the neurotoxic effects of lead and mercury, and at low levels (e.g. Needleman, 2009). It is less widely appreciated that hormones play many critical roles in neurodevelopment and, therefore, associations between chemical exposures and neurobehavioral disorders in humans and wildlife could be plausibly related to disruptions of endocrine pathways. Perturbations in thyroid hormone homeostasis during early life can alter the neuroendocrine circuits that co-ordinate sex-

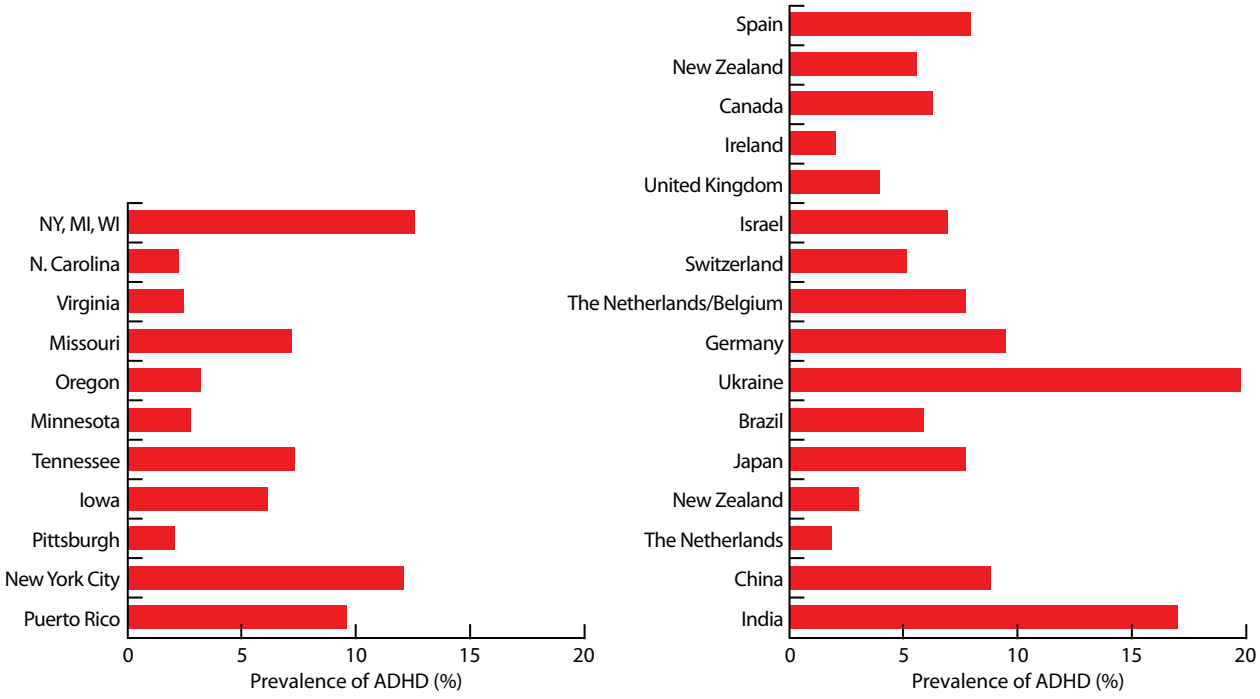

Figure 2.12. Worldwide prevalence of ADHD in children (http://www.medscape.org/viewarticle/547415). Figure reproduced with publisher's permission.

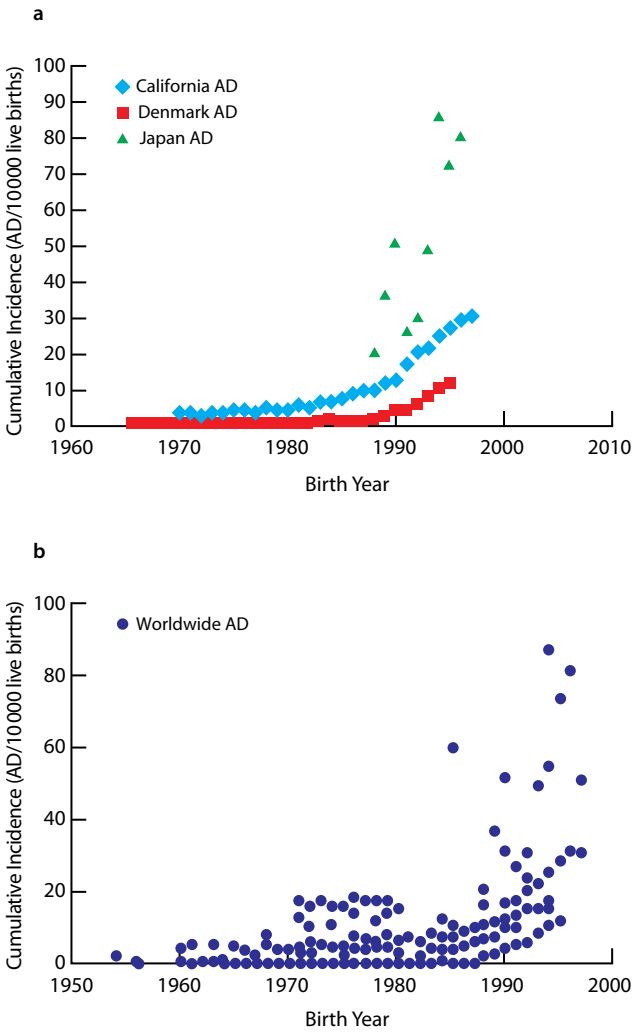

Figure 2.13. Autistic disorder (AD) cumulative incidence time series by cohort birth year from the literature for (a) Denmark, California, and Kohoku Ward, Japan and (b) worldwide AD cumulative incidence. Figure taken from: Timing of Increased Autistic Disorder Cumulative Incidence. (Figure from McDonald & Paul (2010), redrawn; Used with publisher's permission)

specific physiology and behaviour (Jugan, Levi & Bloneau, 2010) and lead to a series of psychiatric and behavioural conditions that are becoming increasingly evident in our society (Gore, 2008, Mclachlan, 2001). The reproductive hormones – estrogens, androgens, progestins – likewise have important effects on neurodevelopment and childhood behaviour, as well as effects on the brain of adults and adult disease. Receptors of these hormones are expressed in the developing brain – in some cases under regulation by other hormones - indicating the great number of interactions hormone systems have on the developing brain. Not only are these hormones involved in the development of sex-typical behaviours – critical attributes in wildlife populations – but they are also involved in the development of other brain structures.

Evidence suggests that endocrine disrupting chemicals can interfere with neurodevelopment affecting cognition and sexual behaviour in both wildlife and humans:

- **There are sufficient data in human populations to conclude that exposures to PCBs during fetal development are linked to general cognitive deficits (e.g., lower global intelligence quotient).** Even in studies of relatively low exposures, PCBs are correlated with measures of cognitive function.
- **Alterations in sexually dimorphic behaviours are seen in human populations highly exposed to PCBs.**
- **Limited data exist to show that *in utero* exposure to other EDCs also affects cognition and sexually dimorphic behaviours in animal studies.**
- **Recent studies of aquatic birds and fish suggest that methylmercury exposure at environmentally relevant levels can interfere with reproductive success** due either to overt neurotoxicity or more subtle neuroendocrine disruptive effects. Methylmercury-exposed birds in the field and in the laboratory have shown altered testosterone and estradiol concentrations (Frederick & Jayasena, 2011), as well as altered courtship behaviour, altered song (Hallinger et al., 2010), high levels of male-male pairing and reduced reproductive success (Frederick & Jayasena, 2011). Reproductive behaviours are also affected in fish exposed to environmentally relevant concentrations of methylmercury (Hammerschmidt et al., 2002; Sandheinrich & Miller, 2006), likely due to its effects on the endocrine system (Crump & Trudeau, 2009).
- **Many areas of the world are still inhabited by wild mammals with levels of methyl-mercury in their tissues that would be unsafe for rodents and humans** (Basu & Head, 2010; Mergler et al., 2007).

2.6.1.1 Thyroid hormone insufficiency and brain development

The neurobehavioural impacts of thyroid hormone insufficiency in humans are so clear that there is universal screening of thyroid function in all regions of the world (LaFranchi, 2010). To understand the ways in which exposure to endocrine disruptors could affect brain development, it is necessary to understand the complexities of the development of both the neurologic and thyroid systems and how thyroid hormones regulate brain development.

Figure 2.14 illustrates the three stages of neurological development in relation to thyroid hormone during fetal development, thus highlighting why the fetus is sensitive to thyroid hormone disruption (Williams, 2008). There are three stages of thyroid hormone-dependent neurological development depicted in the figure: the first is before the onset of fetal thyroid hormone synthesis (16-20 weeks post conception in humans), the second is during the rest of pregnancy, when the developing brain derives thyroid hormones from both the mother and the fetus, and the third is in the neonatal and post-natal period when thyroid hormone supplies are derived from the child. Thyroid hormone plays

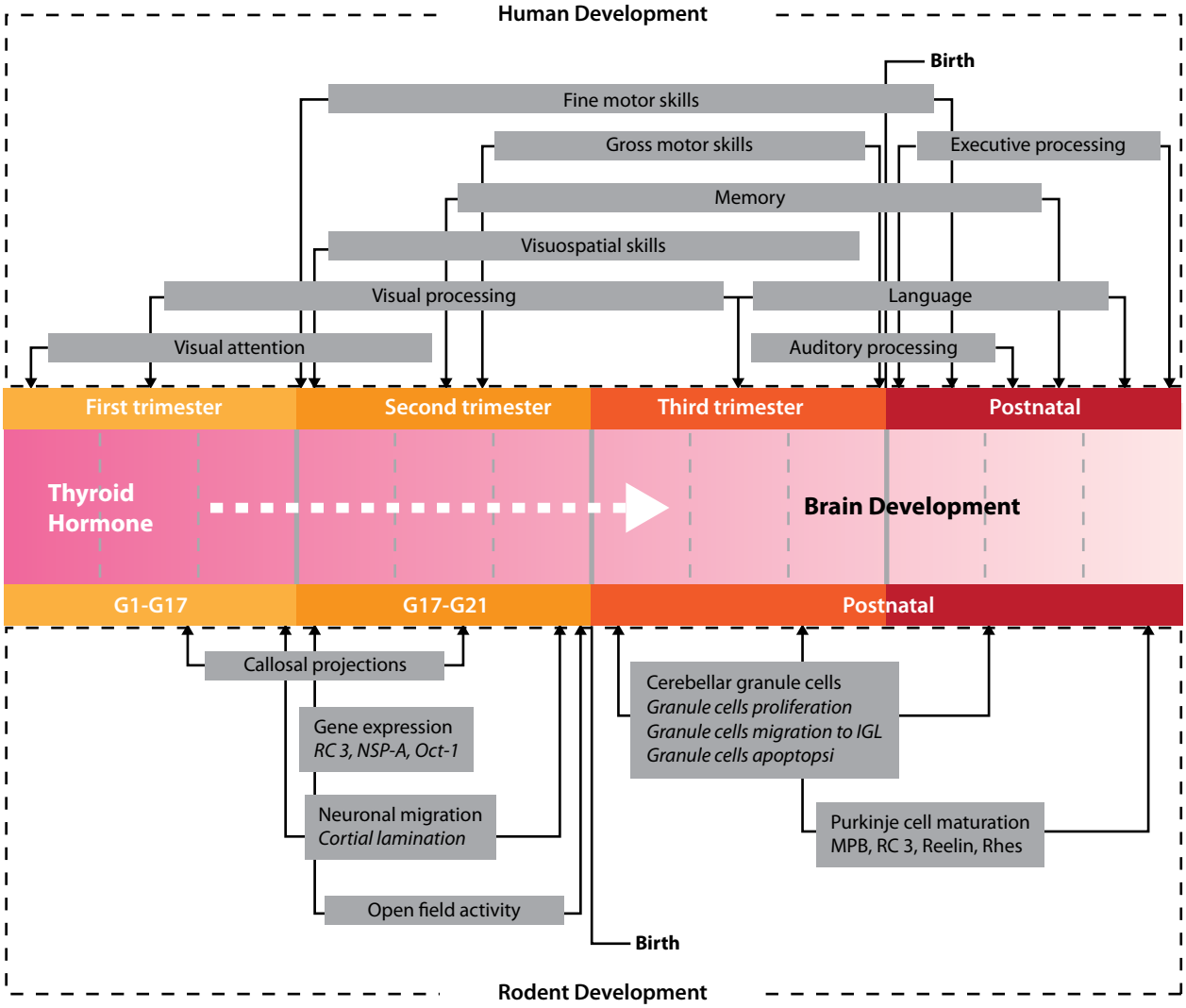

Figure 2.14. Relationship between thyroid hormone action and development of the brain. In the first trimester of pregnancy, early neuronal proliferation and migration is dependent on maternal thyroxine (T4). By the end of the first trimester, development of the hypothalamic-pituitary axis has occurred and a surge in thyroid-stimulating hormone (TSH) secretion results in the onset of fetal thyroid hormone production and increasing occupation of thyroid hormone receptors (TRs) by T3. Continuing development of the brain in the second and third trimesters relies increasingly on T4 produced by both the fetus and mother. Continued post-natal development is entirely dependent on neonatal thyroid hormone production (Figure based on Zoeller & Rovet, 2004).

different roles in different parts of the brain at various times during development (Zoeller & Rovet, 2004). It is delivered to the brain in a complex shuttling system. Largely, T_4 is transported across the blood brain barrier by specific transporters, converted to T_3 in the supporting (glial) cells, and then further transported to neurons (Bernal, 2005). Thus, it is a combination of transporters and enzymes that regulate the delivery of the hormonally active T_3 to its targets. These targets are both glial cells and neurons. For example, the cells that produce the insulating sheath (myelin) around the axons in the brain are dependent upon thyroid hormone (Billon et al., 2002) and animals with low thyroid hormone show progressively fewer of these cells in the major bridge between the two hemispheres of the brain (Sharlin et al., 2008). Genetic defects of the T_3 transporter in humans cause several mental deficits to occur (Maranduba et al., 2006; Schwartz et al., 2005; Visser et al., 2009).

2.6.1.2 The role of hormones in brain development

Sex steroids

In addition to the changes in thyroid hormone exposure during early development, both male and female rodent embryos are exposed to a changing milieu of sex steroid hormones during the late embryonic and early postnatal period that cause permanent sexually dimorphic differences in the size, cell number and neurochemistry of hypothalamic regions of the brain (reviewed in McCarthy, 2009). Because male and female embryos are exposed to different genetic and hormonal environments, the brains of male and female newborns are substantially different from the day of birth. The existence of a genetic contribution to the control of sexual dimorphisms in neurobiology and sexual orientation is firmly established, although the specific genes implicated in the process are not known. The role of hormones in human sexual

orientation is less well understood. Balthazart (2011) reviews the literature indicating that sex steroid hormones may act in concert with genetic factors as well as features of the social postnatal environment to influence sexual orientation.

Balthazart (2011) reviews the literature indicating that sex steroid hormones may act in concert with genetic factors - and features of the postnatal social environment - to influence sexual orientation in humans. This review emphasizes the heuristic value of studies of girls with a medical condition known as congenital adrenal hyperplasia (CAH). These girls are exposed in utero to high levels of androgens from the adrenal glands and exhibit, as a population, masculinization of various behaviors including aggressive play and increased probability of homosexual relationships (30-40 % in some studies compared to 10% in case controls or unaffected sisters) (Balthazart, 2011).

Other morphological and physiological characteristics also appear to be influenced by prenatal testosterone in CAH women, such as the ratio of the lengths of the second to fourth fingers which differs between males and females (Hampson, Ellis & Tenk, 2008) and is clearly masculinized in these women (Breedlove, 2010).

In rodent models of human physiology, studies show that sex differences in brain structure are caused by differences in hormone action early in fetal development. These structural differences are irreversible and are parallel to the effects of hormones early in development on adult sex behavior. Thus, hormones act early in development to organize the nervous system in such a way that hormones in the adult can activate sex-typical behaviours. In rodents, exposure to the male hormone testosterone induces the preference for a feminine sexual partner. Thus, genetic males or genetic females exposed to testosterone will orient toward a female. In contrast, the absence of testosterone leads to preference for a masculine partner; genetic males or females deprived of testosterone during development will orient toward a normal male. It is also important to recognize that testosterone is converted to estrogen in the male brain, and it is estrogen that is responsible for sexual orientation (Henley, Nunez & Clemens, 2009; 2011).

Hypothalamic and pituitary hormones

Multiple hypothalamic neuropeptides and neurotransmitters as well as pituitary hormones exert control over sexual behaviour and reproduction in vertebrate animals including humans (reviewed in Dickerson & Gore, 2007). The reproductive neuroendocrine axis in vertebrates is regulated by the gonadotropin releasing hormone (GnRH) neurosecretory system, located at the base of the hypothalamus. In the pituitary, GnRH binds to its receptors and stimulates the synthesis and release of the gonadotrophic hormones LH and FSH into the general circulation. The gonadotrophins act at the gonads (ovaries and testes) to stimulate sex steroid production, gonadal maturation and sperm and egg production. These three levels of the hypothalamic, pituitary gonadal axis function both independently and interdependently and thus a dysfunction at one level has consequences for the other levels. Moreover, neurotransmitters also modulate the release of GnRH.

Each neurotransmitter may have more than one type of receptor on more than one type of cell and therefore, alterations in the level of a single neurotransmitter may affect multiple cells in different ways. Chemical contaminants can affect both neurotransmission and neurosecretion via various mechanisms. Minor changes in neuronal function may cause major changes in sexual behaviour.

2.6.2 Evidence for endocrine disruption of neurodevelopment in children and in rodent models

2.6.2.1 Attention deficit disorders

There are a number of studies that support the hypothesis that specific environmental factors represent risk factors for ADD. Lead and PCB exposures represent important cases of environmental contaminants associated with ADD in children (Eubig, Aguiar & Schantz, 2010). Exposure to lead is particularly high in developing countries like in many parts of Africa where more than one third of the children still suffer high levels of lead exposure (Falk, 2003). In developed countries, on the other hand, only a small minority of children (mainly the urban poor) are still affected by high levels of lead (reviewed in WHO, 2003). Likewise, attention deficit is over represented in children whose mothers exhibited low thyroid hormone in the first trimester of pregnancy (Haddow et al., 1999) or in children with prenatal ethanol exposure (Mattson, Crocker & Nguyen, 2011). Finally ADD has been linked to elevated exposure to a variety of organophosphate pesticides (Bouchard et al., 2010; Kuehn 2010; Marks et al., 2010; Riccio, Avila & Ash, 2010; Schettler 2001; Xu et al., 2011) still found in relatively large segments of human populations (see Chapter 3.1.1.6 & 3.2.2.2). Thus, overall, although there is uncertainty about causes of the increased incidence and prevalence of ADD in children worldwide, there is plausible evidence to conclude that some environmental chemicals are associated with this disorder.

2.6.2.2 General cognitive deficits and PCB exposure

A very large number of studies have been published over the past 10 years designed to characterize exposures of children to industrial chemicals and to test whether they are related to measures of cognitive deficits in children. In particular, the relationship between exposures to PCBs and measures of cognitive function has been well-studied. Despite the fact that PCB production was banned in the late 1970s, they are still found in all environments and all human and animal tissues (see Chapter 3 for a comprehensive review). The relationship between PCB exposure and cognitive function is an important topic (reviewed by Carpenter, 2006) for the following reasons: 1) a large number of high quality studies have been published on human populations around the world, 2) exposure assessment has become quite sophisticated, and 3) cell-free,

cell-based, and animal studies provide important insights into the mechanisms by which these and other endocrine disrupting chemicals can produce neurotoxic effects.

There are sufficient data in human populations to conclude that exposures to PCBs during fetal development are linked to general cognitive deficits (e.g., lower global intelligence quotient). In highly exposed populations, the most consistent effects across all studies were impaired executive functioning, followed by processing speed, verbal ability and visual recognition memory. These populations include: the Yu-Cheng children in Taiwan (born to mothers exposed to thermally degraded PCBs between 1978 and 1979; Chen et al., 1992), the Dutch cohort (Patandin et al., 1999), the Lake Michigan cohort of children born to mothers who ate PCB contaminated fish (Jacobson & Jacobson 1996; 2003), the Dusseldorf cohort (Walkowiak et al., 2001; Winneke et al., 1998) and the Slovakian cohort (Park et al., 2009). Moreover, even in studies of relatively low exposures, PCBs are correlated with measures of cognitive function, including impulse control (Stewart et al., 1999; 2000; 2003a; 2003b; 2005; 2000; 2008; 2006).

In addition to the cognitive deficits observed, the Yu-Cheng children also exhibited alterations in sexually dimorphic behaviour with exposed boys having a deficit in spatial abilities (Guo et al., 2004). Exposure to higher ambient levels of PCBs has also been associated with less masculinized play in boys and more masculinized play in girls in a group of Dutch school children (Vreugdenhil et al., 2004).

PCB levels detected in blood today are markedly lower than they were in the 1970s – 1990s and a study carried out in Germany recently suggested that exposure to PCBs at current exposure levels does not impair neurodevelopment. This conclusion was based on studying two populations in close proximity to each other in Germany (Wilhelm et al., 2008). Taken together, the available epidemiological evidence is sufficient to conclude that PCB exposures during fetal development are linked to measures of cognitive deficits (Schantz, Widholm & Rice, 2003).

2.6.2.3 Animal studies with PCBs

The mechanism(s) by which PCBs produce developmental neurotoxic effects have been studied extensively. A dominant theory is that PCBs can interfere with thyroid hormone signalling during development. Many of the cognitive deficits linked to PCB exposures are similar to those associated with pre- and post-natal thyroid hormone insufficiency (Zoeller and Rovet, 2004). Rodent studies almost uniformly show that PCB exposures decrease serum thyroid hormone levels (Bastomsky 1974; Goldey et al., 1995; Zoeller, Dowling & Vas, 2000) and produce effects on brain development that are similar to those seen in PCB-exposed human populations (Goldey & Crofton, 1998; Goldey et al., 1995; Herr, Goldey & Crofton, 1996). Cell-based studies show that some PCB congeners can interfere directly with the thyroid hormone receptor (Gauger et al., 2007; Iwasaki et al., 2002; Koibuchi & Iwasaki, 2006; Miyazaki et al., 2004; Miyazaki et al., 2008).

Even in rodent animal models of humans, however, it is difficult to say with certainty that behavioural or developmental effects of PCB exposure are caused directly by effects on thyroid hormone signalling. Specific PCB congeners can affect the intracellular regulation of calcium in rodent brain that is very important in nerve cell development and function (Pessah, Cherednichenko & Lein, 2010). They can also influence neurogenesis, neuron proliferation and differentiation (Fox et al., 2010), and the dopaminergic system, in vitro and in vivo (Barkley 1998; Kirley et al., 2002; DiMaio, Grizenko & Joober, 2003), thought to be crucial for the pathogenesis of ADHD. Very low doses of PCBs can impact sex steroid-related endpoints in the rodent brain (Dickerson et al., 2011a; Dickerson, Cunningham & Gore, 2011). Therefore, it is not possible to directly demonstrate in animals that PCBs produce neurotoxic effects by acting on thyroid hormone signalling alone or whether in combination with other mechanisms.

Considering this, it is even more difficult to prove that thyroid disruption mediates the effect of PCB exposure on developmental neurotoxicity in humans. A number of studies have evaluated the relationship between serum thyroid hormone and PCB body burden in humans (Miller et al., 2009; Longnecker, 2000). These are challenging studies to review both because the technology associated with PCB measurement has changed over the years, and because there are different measures of thyroid function that have been employed in these studies – as well as differences in the timing of sample collection relative to periods of exposure. In addition, different PCB congeners have different potencies for reducing thyroid hormone levels in rodents (e.g. Giera et al., 2011), and this needs to be considered also in epidemiological studies (Chevrier et al., 2007).

There are several important lessons from the PCB story:

- The only clinical measure of thyroid disruption currently available in humans is serum hormone levels, and therefore it is not currently possible to demonstrate that an association between chemical exposure and hormone level mediates specific adverse effects.
- Thyroid hormone insufficiency produces different effects on cognitive development when it occurs at different times during development (Zoeller & Rovet, 2004). Therefore, the timing of measurements of thyroid function and chemical exposure and the cognitive domains that may be affected by these exposures are critical.
- If chemicals can interfere with thyroid hormone action in a manner that is not revealed by changes in thyroid hormone levels, (as has been shown in animal studies, e.g. Giera et al., 2011), then we currently have no way of testing for this in human studies. Therefore, biomarkers of thyroid hormone action should be developed both for use in the clinic and epidemiological studies.
- No guideline study validated for use in screening or testing evaluates measures of thyroid hormone action; therefore, these chemicals would be missed by regulatory tests designed to screen chemical safety.

2.6.2.4 PBDEs and cognitive disorders

Knowledge of developmental toxicity of PBDEs is limited, although human in vitro and epidemiological studies indicate that they work through the same mechanisms as PCBs to induce effects on neurodevelopment via thyroid hormone disruption (Schreiber et al., 2010; Chevrier et al., 2010). Johnson-Restrepo & Kannan (2009) determined that infant daily exposure dose of PBDEs in the USA due to inhalation, incidental oral ingestion and dermal absorption of house dust were significantly higher than in adults. Moreover, serum samples of infants aged 0-4 years contained significantly higher PBDE concentrations as compared to children of 5-15 years of age in an Australian population (Toms et al., 2008). The major exposure route to PBDEs, however, is through maternal exposure in breast milk (reviewed in Chapter 3.2).

There are few epidemiological studies on the neurodevelopmental effects of PBDEs. A single study of 329 mothers in lower Manhattan, New York, examined 210 cord blood samples for PBDEs and neurodevelopmental effects in the children at 12-48 and 72 months of age. The findings indicated associations between high concentrations of BDE-47, -99 and -100 and lower psychomotor and mental development and IQ (Herbstman et al., 2010). An earlier study (Roze et al., 2009) also reported a similar association.

2.6.2.5 Animal studies with PBDEs

In vivo experimental studies show that maternal exposure of rodent models to individual PBDE congeners or commercial pentabrominated mixtures causes dramatic changes in thyroid hormone levels (Darnerud et al., 2007; Kodavanti et al., 2010) as well as subtle changes in neurobehaviour and both male and female reproductive endpoints (Kodavanti et al., 2010). Various studies also report long lasting hyperactivity and reduced performance in learning and memory tests (e.g. Branchi et al., 2003; 2005; Viberg 2009a; 2009b; Viberg, Fredriksson & Eriksson, 2003; 2004; Viberg, Mundy & Eriksson, 2008; Kuriyama et al., 2007; Hallgren & Darnerud, 2002; Zhou et al., 2002) in a similar fashion to that described for PCBs. Moreover, feminization of sex-steroid dependent behaviour, such as of the sexually dimorphic sweet preference of male rats, was observed following prenatal exposure to BDE-99 (Lilienethal et al., 2006) and to a PCB mixture resembling that found in human breast milk. In both of these cases, the effects on behaviour appeared to coincide with decreasing aromatase activity in the hypothalamic/pre-optic area of the brain, inhibiting the local production of the hormone estradiol (one of the main processes by which the brain becomes male-like). Parallel to these behavioural changes, alterations in proteins involved in neuronal survival, growth and synaptogenesis are seen (Dingemans, Van den Berg & Westerink, 2011). It is important to note that behavioural toxicity in rodents can also occur without alternations in maternal serum T4 (Gee & Moser, 2008; Gee, Hedge & Moser, 2008).

2.6.2.6 Mercury and neurodevelopment

Metals such as lead and mercury can also impair neurodevelopment through direct neurotoxic effects, through effects on thyroid function or through epigenetic mechanisms (Ellingsen et al., 2000; Takser et al., 2005). Methylmercury induced disruption of GABAergic signalling in the brain under probable and relevant exposure scenarios can have profound consequences as GABA (A) is the main inhibitory neurotransmitter in the mammalian brain, accounting for 50% of synapses in certain brain regions. Exposure to methylmercury results in build-up of GABA (A) neurotransmitter levels in the synapse and a corresponding decrease in GABA (A) receptor levels (Basu et al., 2010). It also reduces the availability of selenium which is essential for deiodinase activity that in turn activates and inactivates thyroid hormone in the brain and other tissues.

There is a particular current concern about methylmercury because of its high levels in the diet (Trasande et al., 2006; see Chapter 3.1.3). Historical incidences of methylmercury poisoning led to neurodevelopmental impairments in prenatally exposed children.

Consumption of fish is the primary route via which humans are exposed to methylmercury and it is estimated that 8-16% of USA newborns have cord blood levels higher than acceptable limits (Trasande, Landrigan & Schechter, 2005), although this percentage is higher in populations in all parts of the world that rely more heavily on fish for sustenance (Hightower, O'Hare & Hernandez, 2006), particularly in developing countries where fish-eating communities may be exposed to pollution from mercury processing plants (Oosthuizen & Ehrlich, 2001).

In West Greenland, for example, the levels of mercury in the human diet exceed acceptable tolerable daily levels by 50%, much of which comes from consumption of seal tissues (Johansen et al., 2004). The median concentration in the human brain of 17 Greenlanders was 0.17 µg/g wet weight, although levels of 4mg/g were found in some humans (Pedersen et al., 1999). Furthermore, a study of 43 Inuit children reported that mercury exposure might be related to neurological deficits (Weihe et al., 2002).

In Africa, Nweke & Sanders (2009) report that an important source of direct mercury exposure is the artisanal gold mining and processing when exposure to vaporized elemental mercury occurs during burning to separate the gold-mercury amalgam (Savornin, Niang & Diouf, 2007). Workers typically not equipped with personal protective equipment are at risk as well as children under their care (Van Straaten, 2000). In some countries, the mining and sale of gold are a female-only activity and this may include a workforce between 500 and >100,000 women and children (Hentschel, Hruschka & Priester, 2003).

2.6.2.7 Bisphenol A and phthalates may affect sex-specific behaviours and sex dimorphism in neural development

Yolton et al. (2011) recently showed that the concentrations of BPA and phthalates in maternal urine during early pregnancy

were associated with higher hyperactivity and aggression in 2 yr old girls, but not in boys, consistent with rodent data, suggesting an effect of BPA on sexual dimorphism of these types of behaviour, (Kubo et al., 2003, Rubin et al., 2006). In a further study, juvenile female rats exposed to BPA during gestation and lactation exhibited defeminization of social interactions, including reduced play with males, decreased social grooming, increased play with females and increased sociosexual exploration (Farabollinii, 2002, Porrini et al., 2005). Males exhibited increased aggression at sexual maturation (Kawai et al., 2003) and increased anxiety-related behaviour.

The effects of BPA on the brain and behaviour are assumed to be attributed to its estrogen receptor (ER)-mediated action, but it is not clear how its low potency could account for the strong effects that are observed in many tissues after exposure to relatively low doses. There is also evidence that changes in gene expression in utero persist into adulthood (e.g. Smith & Taylor, 2007; also reviewed by WHO, 2011) and hence possibly involve epigenetic mechanisms (see Chapter 1.3.6). This is supported by evidence that estrogen and some endocrine disruptors have been reported to dynamically change the methylation status of their target genes and that this is of critical importance for the function of the central nervous system; epigenetic mechanisms play a crucial role in neuronal plasticity (Borrelli et al., 2008) and thus are potential targets for neurodevelopmental effects of chemicals that induce cognitive dysfunction in human populations, mainly when the exposure takes place during prenatal and early postnatal development (Vahter, 2008; Bellinger, 2008). Given the widespread use and human exposure to chemicals such as phthalates and BPA (reviewed in Chapter 3.2), this is an important area for further study.

2.6.2.8 Are mixtures of different neuroendocrine disruptors a concern for human health?

There is almost no information concerning the effects of mixtures of neuroendocrine disruptors, even though there is little doubt that PBDEs, PCBs, mercury and several pesticides will co-occur in human tissues. Examples of situations where interactive effects of mixtures been suggested to occur include the combination of methylmercury and PCBs in two large cohorts of children in the Faroe islands (Grandjean et al., 2001; 2004; Roegge et al., 2004). These suggestions are supported by a very limited number of rodent studies in which synergistic changes in neurochemical measures (e.g. Bemis & Seegal, 1999) and increases in neurotoxic effects (Eriksson et al., 2003) have been reported as a result of combined exposures to PCBs and methylmercury.

2.6.3 Evidence for endocrine disruption of neurodevelopment in wildlife

In comparison with the evidence of neurodevelopmental diseases and disorders in humans, data describing patterns of neuroendocrine dysfunction in wildlife are less prevalent, despite the fact that studies on wildlife (particularly mammalian wildlife) can provide important information on environmental exposures, early and sub-clinical effects and clinical neurotoxicity of chemicals in the environment. There is, however, a wealth of literature on the neurotoxicology of mercury, the pesticide DDT, PCBs and PBDEs. Some case studies are highlighted here that add weight to the evidence presented in the human health section of this review on the environmental contaminants of neurotoxic concern to humans.

2.6.3.1 Mammals

Methylmercury, PCBs and PBDEs biomagnify (concentrate) up through aquatic food webs, resulting in high concentrations in fish and other top predators (Chapter 3.1.3 & 3.2). As such, consumption of contaminated fish represents the primary route via which wildlife and humans are exposed to these chemicals.

Methylmercury

Much of our knowledge concerning neurotoxicology of methylmercury was obtained following the human poisoning event in Minimata Bay, Japan, alerted 5 years earlier by the frenzied behaviour of cats, rats, crows and fish (Aronson, 2005). Around this time, population declines in other wildlife species were particularly noticeable in regions that used organomercurial fungicides, or that were located downstream of pulp and paper mills using mercury.

The structural brain lesions and effects of methylmercury are similar across mammals (reviewed in Basu & Head, 2010).The organic form of mercury, methylmercury, crosses the blood-brain barrier and can cause a range of effects on brain tissues in vertebrate wildlife. Lower exposures reduce the levels of key enzymes (cholinesterase and monoamine oxidase) in wild otters (Basu et al., 2007a) and *N*-methyl-D-aspartate (NMDA) glutamate receptors in wild mink (Basu et al., 2007a; 2007b), bald eagles (Rutkiewicz et al., 2011) and polar bears (Basu et al., 2009). These effects have been corroborated in laboratory studies of mammals and fish exposed to methylmercury (Basu et al., 2006; 2007c; 2010; Coccini et al., 2006; Berntssen, Aatland & Handy, 2003) and are of both ecological and physiological concern because these enzymes and receptors are parts of critical neurochemical pathways that control reproduction, cognition, growth and development (Manzo et al., 2001). At the present time, overt episodes of mercury poisoning are rare but there is evidence that lower levels of exposure can affect growth, reproduction and development in wildlife. These effects are much more common in longer-lived species that are higher up in the food web because of the biomagnification of methylmercury through aquatic systems (see Chapter 3.1.3). It is entirely possible that even subtle neurological damage in fish-eating wildlife may be having more severe consequences than we can currently ascertain.

Mammalian wildlife species also accumulate mercury in their brains where it can have subtle effects on the brain neurochemistry (Manzo et al., 2001; Scheuhammer et al.,

2007). Many areas of the world are still inhabited by wild mammals with levels of methylmercury in their tissues that would be unsafe for rodents and humans (Basu & Head, 2010; Mergler et al., 2007). However, it is important to note that there are differences in susceptibility between different species of mammals. For example, levels of mercury in the livers of polar bears in the Canadian Arctic exceeded those in the livers of humans that succumbed to Minimata disease, but there is little observational or experimental evidence of neurological damage in the brains of these bears (Sonne et al., 2007), probably because the levels in the brain stem were markedly lower than in the other tissues of the body (Basu et al., 2009). Notwithstanding this, mercury associated changes in brain NDMA receptor levels were found in these bears, one of the earliest known responses to mercury exposure. In addition, a subsequent study reported an inverse association between mercury exposure and DNA methylation in the lower brain stem of male (but not female) polar bears, suggesting possible long term consequences of mercury exposure for chromosomal stability, disease progression and reproductive function (Pilsner et al., 2010). These results may be of relevance to human health in Greenland as an epidemiological study of 43 Inuit children in Greenland reported that mercury exposure in humans might be related to neurological deficits (Weihe et al., 2002).

PCBs

Most PCBs, particularly those with non-coplanar structures, have intrinsic neurotoxic properties (Mariussen & Fonnum, 2006), and can impede several neurological processes including dopaminergic signalling and calcium homeostasis. Whilst their action on the neurological system is clear, data regarding their accumulation in the brain are sparse. Where these data exist, liver to brain ratios range between 3-fold to more than 7-fold across mammals alone, making it difficult to derive exposure-response relationships (Giesy & Kannan, 1998; Kodavanti et al., 1998). As with mercury, the initial discoveries concerning neurotoxicological effects of PCBs were seen in wildlife. Several PCB mixtures and individual congeners at environmentally relevant levels (e.g. <1µg/g in the diet) could impair numerous health aspects including neuroendocrine function (reviewed in Basu et al., 2007b). PCB bans and restrictions have led to a decline in PCB concentrations in humans and wildlife over the past few decades, although geographic hotspots still exist where certain PCB congeners persist (Chapter 3.2.1 & 3.2.2). A few biomonitoring studies report PCBs in the brain tissues of mammalian wildlife and humans between 2-50 ng/g wet weight. In marine mammals, however, brain PCB levels are higher (up to 450 ng/g wet weight). Dominant congeners in the brain of mammalian wildlife are coplanar and are similar to those found in humans (CB153, 180, 170/190, 138 and 99).

As in humans and rodent models, the most commonly observed effects of PCB exposure is the disruption of thyroid hormone homeostasis. Laboratory studies with mink and with harbour seals have shown PCBs to decrease T3 and T4 (see Chapter 2.5). Moreover in numerous field studies of seals, sea lions and polar bears, decreased serum T4 was correlated with PCB exposure. There is, however, mixed evidence on the impacts of PCBs on brain neurochemistry in mammalian wildlife. In river otters, no significant correlations between brain PCB levels and several neurochemical markers were found (Basu et al., 2007c), whereas in captive female mink and in rodents and monkeys, changes in dopamine levels in the brain and hypothalamus were found following exposure to PCBs (reviewed in Seegal, 1996).

PBDEs

As already mentioned, the levels of PBDEs in the environment rapidly increased with the increasing popularity of PBDEs as flame retardants (Chapter 3.2). In the Baltic Sea, atmospheric deposition of PBDE still exceeds PCBs by a factor of 40X. Between 1981 and 2000, levels of PBDEs in the blubber of Arctic ringed seals and in the marine mammals of the temperate Asia-Pacific region increased about 9-fold (Ikonomou, Rayne & Addison, 2002; Tanabe et al., 2008). A single study reports levels of PBDEs in river otter brain at concentrations ranging from 1.1 to 6.6 ng/g wet weight, comprising only BDEs -99, -100 and -153 (Basu, Scheuhammer & O'Brien, 2007). Levels in avian species in Belgium are reported to be much higher (Voorspoels et al., 2006b): wild sparrows, 140-5800 ng/g; owls, 0.8-174 ng/g; and buzzards 0.2-1600 ng/g. Recent analyses of wildlife and human tissues for PBDEs show some declining concentrations due to restrictions and bans on their use, but levels in wildlife remain highest near urban centres (Voorspoels et al., 2006a) and vary considerably from one country to another as for humans (Chapter 3, sections 3.2.1 & 3.2.2).

There is still much to learn about the neurobehavioral toxicity of PBDEs in mammalian wildlife. In a recent review, Costa & Giordano (2007) concluded that subtle but lasting developmental neuroendocrine effects will occur at levels of PBDEs only marginally higher than currently found in animal tissues. Some of these effects are likely due to anti-thyroidogenic or brain cholinergic mechanisms. There are few if any studies examining this possibility. In a single ecological study on river otters, there were no correlations between cholinesterase activity and PBDE levels in the brain (Basu, Scheuhammer & O'Brien, 2007).

2.6.3.2 Non mammalian vertebrates

Methylmercury

Elevated exposure of fish and amphibians to methylmercury also impairs behaviours that are critical for successful reproduction, avoidance of predators and feeding (e.g. Weis, 2009). Laboratory studies have shown that, in general, the younger animals are more sensitive to the effects; for example, it takes 2-fold higher levels of methylmercury in adult than young fish to negatively affect behaviours (Beckvar, Dillon & Read, 2005). Similarly, in amphibians, maternal exposure negatively affected growth, duration of metamorphic climax, and swimming performance in a study of American toad

larvae. The duration of metamorphic climax is a period of increased vulnerability for immunological, energetic, and ecological reasons, and therefore mercury exposure at this time may increase mortality risk in exposed amphibian populations. It is interesting to note that the metamorphs from mercury-exposed mothers did not have elevated tissue concentrations due to dilution of maternally transferred mercury during growth.

PCBs

Over the last two decades Khan & Thomas (1997; 2001; & 2006) have accumulated substantial evidence on the involvement of PCBs in the disruption of the seratoninergic systems in fish brains. During gonadal recrudescence, PCBs reduce both dopamine and serotonin in various regions of the hypothalamus. This leads to an inhibition of the reproductive luteinizing hormone (LH) and impairment of gonadal growth. As in rats, this fall in dopamine and serotonin is thought to be caused by the inhibition of thyroid hormone induced by PCBs, and highlights the fact that adverse effects of endocrine disruptors on reproduction can also be due to their effects on neurohormones and thus indirectly on gonadal hormones.

PBDEs

In non-mammalian vertebrate wildlife, studies of neurodevelopmental disorders, occurring concomitantly with exposure to PBDEs, can be provided. There are multiple lines of evidence suggesting that PBDEs affect T_4 levels in developmentally exposed birds, fish and amphibians (e.g. Fernie et al., 2006; Lema et al., 2006; 2008; 2009) making it likely that these chemicals also affect neurodevelopment in these animals as in rodent models. The most dramatic effect reported in fish species is a hatching delay (Timme-Laragy, Levin & Di Giulio, 2006), which could be attributed to a T_4 mediated mechanism. Other possible T_4 mediated effects include those on tail curvature direction, hypo activity and elimination of the fright response, with important consequences for predator recognition and avoidance. Increases in thyroid hormone and its receptor occur just before hatching in zebra fish and can be altered by exposure to thyroid hormone receptor antagonists (Liu & Chan 2002).

2.6.4 Neuroendocrine effects of exposure to endocrine disrupting chemicals on courtship behaviour and mate choice in wildlife

2.6.4.1 Methylmercury

Studies of aquatic birds suggest that methyl mercury exposure at environmentally relevant levels can interfere with reproductive success- due either to overt neurotoxicity or more subtle neuroendocrine disruptive effects on courtship behaviour and mate choice. For example, common loons exposed to methylmercury showed increased lethargy, reduced time incubating the nest and foraging and feeding their young (Evers et al., 2008). As a result, adults in areas with higher exposure had decreased hatching success of eggs and production of chicks (Scheuhammer et al., 2007; Evers et al., 2008). High mercury levels in eggs have been suggested as a cause of declining ivory gulls in the Canadian Arctic (Braune, Mallory & Gilchrist, 2006). Moreover, methylmercury-exposed white ibises in the field and in the laboratory have shown altered testosterone and estradiol levels (Frederick & Jayasena, 2011), as well as altered courtship behaviour, altered song (Hallinger et al., 2010), high levels of male-male pairing and reduced reproductive success in successful pairs that did raise young (Frederick & Jayasena, 2011). Male to male pairing has been reported extensively in many animal species but it is most commonly associated with skewed sex ratios or limited mating opportunities. It is notable that this recent study did not report either of these conditions; mating opportunities and sex ratios were approximately equal. Moreover, male to male pairings do not normally occur in wild ibises. The exposure levels encountered in Frederick and Jayasena's study were environmentally relevant and are therefore of relevance to many bird populations. As reproductive output was decreased by both homosexual behaviours and as a result of a reduced number of fledglings raised by heterosexual pairs, mercury exposure could lead to altered demographic patterns in wild bird populations (Burgess & Meyer, 2008; Barr, 1986). Indeed, in the Frederick study, the breeding population size was inversely correlated with their annual methylmercury exposure in South Florida, USA (Frederick & Jayasena, 2011).

2.6.4.2 DDT

DDT is a persistent, widespread environmental contaminant found in most regions of the world and at high concentrations in countries where it is still used to control malaria mosquitoes (Chapter 3.2.1). The most well documented effects of DDT on neurobehaviour are those seen in birds, where DDT has been associated with decreased courtship behaviours (Zala & Penn, 2004), altered singing (in songbirds) and female to female pairing (in gulls). It is well known that administration of testosterone or estradiol to adult female or male birds, respectively, leads to mate attraction and courtship behaviour typical of the opposite sexes. In addition, early developmental exposures to estrogens or aromatase inhibitors has been shown to profoundly increase male and female sexual interest in the same sex and decrease male vigour. Sexual dimorphism of singing is also thought to be controlled not only by sexually dimorphic genes expressed in the brain but also by estrogen (reviewed in Adkins-Reagan, 2011); reducing the concentration of either 17β-estradiol or testosterone reaching the song system in the brain (a set of interconnected brain regions that mediate the learning, perception and vocalization of song) reduces the size of this area, concomitant with decreases in singing activity and song repertoire (Gulledge & Deviche, 1997; Metzdorf, Gahr & Fusani, 1999; Riters et al., 2000, Riters & Teague, 2003;). It is reasonable to hypothesise then that the effects of DDT on neurobehaviour in birds are at least partially due to its ability

to bind to and activate or inactivate the estrogen and androgen receptors, respectively, that are present at high concentrations in the song centre and two other areas of the brain (the ICo and the septum; Gahr et al., 2001). Both the size of the ICo and its neurons, thought to play a role in copulation, vocal displays and antagonistic behaviour, can also be affected by circulating hormone levels (Gurney & Konishi, 1980). Indeed, in a recent study, exposure to DDT (15-175 µg/g) during the embryonic and early post hatching period was shown to alter the structure of the American robin brain, such that relative forebrain size (male) and absolute song nuclei (male) and ICo volumes (male and female) were significantly reduced with increasing DDT exposure when the animals were examined 2 years post exposure (Iwaniuk et al., 2006). Although stress and direct neurotoxicity could not be ruled out as causes of these changes, it seems likely that endocrine disruption played a role, as the size of other adjacent AR and ER negative areas of the brain were not affected by the exposure.

Female-female pairing, as occurred when gulls, albatrosses and geese were exposed to DDT and other pesticides, could also have been due to feminization of the brain. In this case, however, these seemingly altered preferences could also have been opportunistic responses to a lack of availability of males (seen commonly in other species) and here caused by biased sex ratios in the gulls (also an effect of the DDT; see section 2.3 of this chapter).

2.6.4.3 Other EDCs

Feminizing effects of other xenoestrogens on sexually selected neurobehavioral traits have also been observed in wild mammals. A study of polygamous deer mice developmentally exposed to the endocrine disrupting chemicals ethinylestradiol (EE2) or bisphenol A (BPA) showed that although there were no changes in external phenotype, sensory development, or adult circulating concentrations of testosterone or corticosterone, spatial learning abilities and exploratory behaviours were compromised. Moreover, both BPA-exposed and control females preferred the control males in preference to the exposed males (Jasarevic et al., 2011). Males of this species compete for mates by expanding their territorial range during the breeding season, thereby increasing their prospects of locating mates that are widely dispersed. This adult male spatial ability and exploratory behaviour requires both a seasonal increase in testosterone and prenatal exposure to the same hormone (Galea, Karaliers & Ossenkopp, 1996).

The mechanism underlying the effects of ethinylestradiol (EE2) and BPA is not known, but could be either a direct effect of these EDCs on brain development in the male pups, and/or due to a decreased maternal investment in the pups by the dam (as has been observed also in rodents; Palanza et al., 2002; Della Seta et al., 2008). These changes could also occur as a result of effects on the expression of estrogen receptor genes during neurodevelopment or as a result of suppression of fetal testosterone production at the time when the androgens from the testes normally masculinize the developing brain.

The results of this study in a wild mammal are supported by numerous studies in rodents in the laboratory in which estrogenic chemicals have been shown to influence neurobiology. For example, several studies indicate that exposure of the developing brain to phytoestrogens affects early sexual differentiation of the brain, by mimicking effects of estrogens on the size and neurochemistry of sexually dimorphic regions causing alterations in reproductive behaviour. These effects are sensitive to the timing and duration of the exposure and inconsistent results are often noted due to differences in methodology. Moreover, increasing evidence also suggests PBDE exposure can also influence reproductive behaviours in birds at environmentally relevant concentrations (Fernie et al., 2008). It has been suggested that these studies are of relevance to human babies consuming soy formula during the early postnatal period (reviewed in Dickerson & Gore, 2007).

PCBs do not appear to influence adult volume of the sexually dimorphic areas of the brain but they can influence numbers of nuclear hormone receptors in these sexually dimorphic brain regions of exposed rodents during early development. It is not clear what these changes mean in terms of the function of the brain in this case. In addition, it is not yet clear whether the PCB congeners have equivalent potential for disruption of the sexual differentiation of the brain. Despite this, laboratory studies have consistently shown that PCB exposure during early development affects adult female reproductive behaviour and a limited number of studies also report effects on the male (reviewed in Dickerson & Gore, 2007).

Increasing evidence now indicates that xenoestrogens can affect sexualization of the brain in fish in a general manner, and not only sexually dimorphic features. Further study of this area is needed both in wild populations of fish and in biomedical toxicology where the fish brain could be considered a good model for brain sexualisation in humans. Unlike in mammals and birds, the brain of fish is not permanently sexualised during early development, but is instead, highly susceptible to hormones throughout its life. Despite this unique difference between fish and other vertebrates, most of the hormones sustaining the neurobehavioral controls of the reproductive process are similar, if not identical. Moreover, as recently discovered in mammals and birds estrogen, and not androgen, receptors in male and female fish play a role in differentiation of the neural circuits that control male-specific behaviour. The masculinizing effect of testosterone on the brain is through its conversion to estrogen by brain aromatase, a highly sensitive target for endocrine disruptors (reviewed in Le Page et al., 2010).

2.6.5 Evidence for a common EDC mechanism of neurodevelopmental disruption for humans and wildlife

In many cases, the neurotoxicological outcomes of chemical exposures are similar in wildlife and in humans, adding weight of evidence to relationships between chemical exposure and neurodevelopmental disorders in humans (Basu & Head, 2010).

Unlike in humans, pollutant levels in the brains of wildlife species can be easily sampled and measured and can reach levels that are associated with neurotoxic damage in humans. There are reports in the literature of damaged brains and spinal cords in wild bald eagles and great blue herons in the USA and a strong association between these anomalies and exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which was discovered at high levels in the birds. As early as 1915, lead-related neurological disorders were observed in horses and cattle living near industrial facilities. Moreover, before adverse neurological effects of mercury were seen in the human residents of the Great Lakes basin and in Minimata Bay (Japan), neurological effects were seen in local wildlife species through the 1950s and 1960s (Harada, 1995).

Wildlife can also provide important insights into mechanisms of neurotoxicity that may be important for human health. As one might expect from the high conservation of brain pituitary functions in vertebrates, neurohormones and neuropeptides controlling these tropic functions are well conserved and so the control of brain development by thyroid hormones and of reproductive behaviour by sex steroid hormones and GnRH neurons and neurotransmitters is similar amongst all vertebrates. Most of this work has been carried out in birds and fish, in which a diverse array of hormones have been shown to be involved in the stimulation of courtship and mating behaviour, including gonadal sex steroids produced locally within the brain, and neuropeptides.

2.6.6 Main messages

- Neurobehavioural disorders have increased in prevalence in human populations. The reasons for this are multiple and not understood.
- Despite this, the economic, societal and personal costs of this particular disease burden are high.
- There are some very strong datasets, for PCBs, showing that environmentally relevant exposures to these endocrine disrupting chemicals caused cognitive and behavioural deficits in humans.
- Studies of exposed wildlife provide important information on exposure levels, early and sub-clinical effects and clinical neurotoxicity of endocrine disrupting chemicals because the mechanisms underlying effects and the outcomes of exposures are often similar to those in humans. Wildlife data exist for some EDCs (e.g. PCBs) and potential EDCs (e.g. Mercury), but for other EDCs they are sparse or non-existent.
- Limited evidence shows that environmentally relevant exposures to some endocrine disrupting chemicals (mercury, bisphenol A, PCBs, PBDEs) could affect brain sexualisation, courtship behaviour and mate choice in some wildlife species, possibly leading to impacts at the population level.
- Chemical testing strategies do not routinely require evaluation of the ability of a chemical to produce developmental neurotoxic effects in a pre-market setting.
- New criteria for evidence are needed so that the scientific community and government agencies can focus their work and their funding on providing the most effective datasets required for regulation.

2.6.7 Scientific progress since 2002

Since the IPCS (2002) review on endocrine disruptors, the following advances have been made:

- Increased evidence for thyroid hormone mechanisms in brain disorders in humans and wildlife.
- Increased evidence of the great sensitivity of embryonic and postnatal development to EDCs when compared with adults.
- Increased number of studies showing a relationship between cognitive function and chemical exposures in humans. These studies however are often weakened by the nature of the study designs, and more prospective studies are warranted.
- Increased evidence for wildlife exposures to methylmercury and of effects on growth and development.
- First evidence of subtle effects of methylmercury and bisphenol A on reproductive behaviours of wildlife individuals that may be of relevance to populations.

2.6.8 Strength of evidence

There is sufficient evidence to conclude that published estimates of incidence and prevalence of some childhood neurobehavioural disorders have increased world wide over the past 10-20 years. Moreover, there is sufficient evidence to conclude that a number of factors, including environmental, contribute to the increases in autism spectrum disorders. There is also sufficient evidence to conclude that exposure to some industrial chemicals is plausibly related to the production of neurobehavioural disorders seen in both wildlife and humans. Exposures to lead, methylmercury, and PCBs represent strong cases in support of this, among others. There is sufficient evidence to conclude that PCBs can exert developmental neurotoxic effects in animals at doses that are similar to those of humans. More recent rodent studies of very low exposures to individual PCB congeners clearly make this point. There is sufficient evidence to conclude that specific PCB congeners and their metabolites can directly interfere with biological systems in rodents including thyroid hormone action and calcium regulation. There are limited data supporting an endocrine mechanism in the association of neurobehavioural disorders with some industrial chemicals. This is a challenging area that needs further focus. There are limited data to show that developmental exposure of some wildlife species to environmentally relevant concentrations of some chemicals can cause effects on brain sexualisation, leading to alterations in mate choice and courtship behaviours with outcomes that are relevant to populations. This area is important and needs further study.

2.6.9 References

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2.7 Endocrine disruptors and hormone-related cancers

2.7.1 Overview of hormon related cancer trends in humans and wildlife and evidence for endocrine disruption

This section deals with cancers of hormone-sensitive tissues such as the breast, uterus, ovaries, prostate and thyroid and considers the strength of the evidence for a contribution of endocrine disruptors to these diseases. Testis cancer is discussed in the section devoted to male reproductive health.

The role of steroidal hormones in various cancers has been a topic of intensive research from the early 1940s onwards. Although this work has established the biological plausibility of a strong involvement of endogenous estrogens and androgens in the disease processes, the possible contribution of foreign chemicals has only fairly recently received attention for two main reasons:

- Hormonal cancers of the breast, endometrium, ovary, testis prostate, and thyroid glands are continuing to rise among populations of "Western countries", and more recently also among Asian nations. Established risk factors alone cannot provide explanations for these unfavourable disease trends.
- The involvement of the synthetic estrogen, DES, in vaginal cancers and breast cancer has heightened concerns that a multitude of other hormonally active chemicals in everyday use are causing these diseases.

By far the most research into associations with endocrine disruptors has been carried out with breast, prostate and testis cancer, while other hormone-related cancers such as endometrial, ovarian and thyroid cancer have received very little attention.

Hormonal involvement in endocrine cancers

Despite a great deal of research, the etiology of most hormone-related cancers remains a mystery. It seems clear that hormones are necessary for the growth of cancerous tissues, but their involvement in the earlier steps of carcinogenesis is still unclear. The dominant theories of carcinogenesis invoke mutations as the ultimate cause of cancer, but most hormones are not strong mutagens (Soto & Sonnenschein, 2010). More recently, the field of epigenetics (Chapter 1.3.6) has begun to throw new light onto the processes that might contribute to hormonal cancers. It appears that mis-timed exposure of tissues to hormonally active agents can interfere with the subtle processes of gene-silencing, and that disruption of these processes might be one factor that predisposes towards cancer (see the review by Zhang & Ho, 2011). Other factors may include the disruption of tissue organization and differentiation during development (Soto & Sonnenschein, 2010).

2.7.2 Evidence for endocrine disruptor causation of hormonal cancers in humans and in rodent models

2.7.2.1 Breast cancer

Breast cancer incidence rates are increasing in almost all industrialized countries (WHO, 2010; Hery et al., 2008; **Figure 2.15**). These trends are not fully explained by improvements in diagnosis through mammographic screening (Coleman, 2000), nor in terms of changes in established risk factors, such as age at menarche or menopause, inherited susceptibility or increasing age of having babies. Twin studies have highlighted the importance of environmental factors, including chemical exposures (Lichtenstein et al., 2000; Luke et al., 2005).

Hormonal mechanisms of breast cancer

The cyclical secretion of estrogens during a woman's life is a key risk factor for breast cancer; the more estrogen one receives during life, the higher the overall risk (reviewed by Travis & Key, 2003). Neoplasms of the breast sequester estrogens which they require for their growth and hence, in order to demonstrate a link between breast cancer and exposure to estrogens, samples from women must be collected before they develop breast cancer. The lack of wide appreciation of this fact has led to many poorly designed and conflicting studies; the link between estrogen exposure and breast cancer was finally corroborated through nine prospective case control studies (The Endogenous Hormones and Breast Cancer Collaborative Group, 2002).

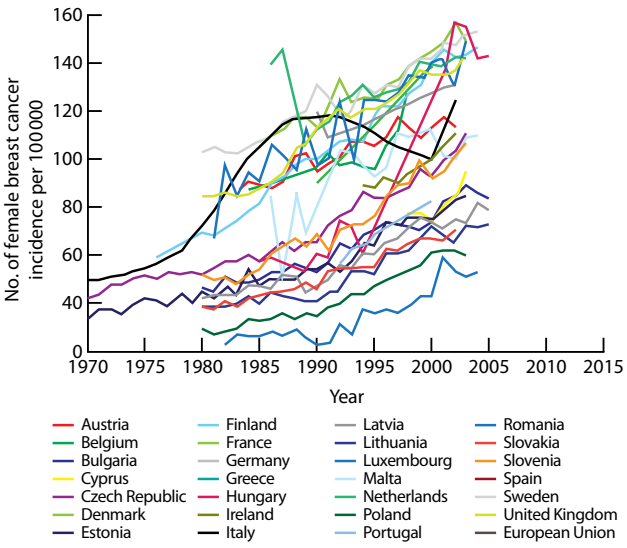

Figure 2.15. The rise in the number of new breast cancer age-standardized cases in several countries. All data from World Health Organisation (WHO), 20010, European health for all database (HFA-DB), World Health Organisation Regional Office for Europe. Database online at http://data.euro.who.int/hfadb/

The majority of breast cancers derive from the end buds of the breast, where the cells that contain estrogen receptors are the most responsive to estrogens (Russo & Russo, 2006). Ovarian estrogens signalling through estrogen receptors are also essential for the development of the breast during puberty, stimulating cell division in the "end buds" of the breast ducts leading to more "tree-like" branching and elongation of the ducts with every menstrual cycle. Although the exact mechanisms through which breast cancer is initiated by estrogens are still unclear today, one theory proposes that breast cancer cell populations arise from the well-established estrogen receptor-mediated proliferation of small numbers of incompletely differentiated cells in the end buds of the breast (Russo & Russo, 1998; Travis & Key, 2003). Other possibilities include direct genotoxicity (Liehr, 2001), aneuploidy induction (Russo & Russo, 2006; Liehr, 2001) and abnormal tissue remodelling through interactions between the stromal and epithelial tissues of the breast (Soto & Sonnenschein, 2010).

Epidemiological evidence that EDCs cause breast cancer

The breast is particularly vulnerable to cancer-causing influences during development in the womb and during puberty (Soto et al., 2008). Women whose mothers used the drug DES during pregnancy to avoid the risk of miscarriages (see section 2.1) have a high breast cancer risk (Palmer et al., 2006). Studies with laboratory animals also suggest that exposure to xenoestrogens during development can alter the development of the mammary tissue with possible consequences for breast cancer (Munoz-de-Toro et al., 2005; Maffini et al., 2006; Murray et al., 2007).

Natural and therapeutically used estrogens strongly contribute to breast cancer risks (Travis & Key, 2003). In particular, a meta-analysis of a large number of hormone replacement therapeutics (HRT) studies and trials done world wide concluded that estrogen-only HRT is associated with breast cancer (Grieser, Grieser & Doren, 2005). Moreover, the UK Million Women Study also showed that all forms of HRT, including estrogen only and estrogen-progesterone types increased breast cancer risk contributing to an extra 20 000 breast cancer cases in the preceding decade alone (Banks et al., 2003). A more recent USA study also corroborates the claims made by the Million Women Study for estrogen-progesterone combined HRT (Li et al., 2008). As a result of the publicity surrounding these results, there has been a steep decline in HRT use and concomitant steep declines in estrogen receptor positive breast tumours in European and US populations of women above the age of 50 (Verkooijen et al, 2009; Robbins & Clarke, 2007; Gompel & Plu-Bureau, 2010). Although this decline may be partially attributed to enhanced screening procedures for breast cancer, the collective evidence points very strongly towards HRT being one of multiple risk factors for breast cancer.

The findings related to HRT have fuelled concerns about chemical exposures, especially to estrogenic agents, and their role in breast cancer. However, most published human studies addressing the issue of estrogenic chemicals suffer from weaknesses that complicate their interpretation. Often, exposures were not measured during the periods of heightened vulnerability (during development in the womb or during puberty), and the effects of simultaneous exposures to multiple chemicals were not considered; there is now good experimental evidence that estrogenic chemicals with diverse features can act together to produce substantial combination effects (Kortenkamp, 2007). It is therefore not surprising that studies where single estrogenic pollutants (e.g. DDE, DDT or various estrogenic pesticides) were considered in isolation have failed to demonstrate significant breast cancer risks (reviewed by Snedeker, 2001; Mendez & Arab, 2003; Lopez-Cervantes et al., 2004). The importance of combined exposures was highlighted in a Spanish study, in which breast cancer risks increased with rising total estrogen load in adipose tissue (Ibarluzea et al., 2004). This observation supports the idea that estrogenic environmental chemicals in combination contribute to breast cancer risks, just as do natural and therapeutically used estrogens.

The most convincing evidence for associations between environmental pollutants (some with endocrine disrupting properties) and breast cancer comes from several epidemiological studies involving agents devoid of estrogenic activity (PCDD/F, PCBs, organic solvents; reviewed by Brody et al., 2007). Moreover, where DDT/DDE exposures during earlier life stages (puberty) could be reconstructed, breast cancer risks became apparent (Cohn et al., 2007; see Chapter 3.2.2.6 for DDT measurements in mothers' milk). This echoes insights from the DES epidemiology where the importance of periods of heightened vulnerability during development became obvious (Palmer et al., 2006; section 2.1 in this document). There are indications that exposure to cadmium, an estrogen mimic, is associated with breast cancer (reviewed by Kortenkamp, 2011). Epidemiological studies of more polar xenoestrogens, such as UV filter substances and phenolic agents, are missing altogether. An association between in vitro exposure to bisphenol A (exposure to this chemicals is reviewed in Chapter 3 and a pattern of gene expression related with higher tumour aggressiveness suggests a role of more polar xenestrogens in tumour progression and poorer patient outcome (Dairkee et al., 2008). By adopting targeted research strategies which take account of the origin of breast cancer early in life (prospective design) and which consider exposures to a multitude of chemicals, these issues should be pursued further.

Evidence from animal studies that EDCs play a role in breast cancer

Many experimental systems exist for the study of breast cancer, but the development of a coherent framework for the interpretation of all of the available evidence is severely hampered by a lack of fundamental knowledge about the mechanisms involved in breast cancer, and the extent to which observations in experimental models are relevant to the human situation.

Many assays sensitive to estrogen receptor activation are available, but a direct link between receptor activation and breast cancer causation cannot be assumed so that the interpretation of a positive result in such assays is not clear.

The utility and value of the two year chronic carcinogenicity bioassay as a tool for the identification of human breast carcinogens has been questioned (Rudel et al., 2007). Rudel and colleagues identified 216 chemicals as mammary gland carcinogens based on the outcome of this animal bioassay. However, the rodent strains used for these assays, the F344/N rat and the B6C3F1/N mouse, were not developed as models for the demonstration of mammary carcinogenesis; the assay aims to identify the ability of test chemicals to induce tumours, regardless of specific tissues. This complicates the interpretation of assay outcomes: an animal mammary carcinogen may be a human carcinogen but not necessarily with the breast as the target organ.

In a rat strain not routinely used for carcinogenicity testing, the ACI rat, DES, estradiol and other steroidal estrogens were found to induce mammary tumours (Shull et al., 1997; Ravoori et al., 2007). Equine estrogens used in hormone replacement therapy are also active (Okamoto et al., 2010). Evidence for an estrogen receptor-mediated mode of action in this model stems from the observation that estradiol-induced mammary neoplasms could be suppressed completely by co-treatment with the estrogen receptor antagonist tamoxifen (Li et al., 2002).

The ACI rat seems to be valuable tool for the identification of mammary tumours induced by estrogenic agents, yet, to our knowledge, other chemicals with estrogenic activity have not been tested in these models.

Various research models have been developed to explore the developmental anomalies that increase the susceptibility to mammary gland neoplasia later in life (summarized by Soto & Sonnenschein, 2010). The xenoestrogen bisphenol A has been used as a tool to explore these processes. It appears that exposure to bisphenol A during organogenesis induces profound alterations in the mammary gland that render it more susceptible to neoplasia. The accelerated maturation of the adipose tissue pad may be responsible for the epithelial changes and make the epithelium more sensitive to estrogens at later developmental stages. Consequently, increased sensitivity of the mammary glands to estradiol at puberty was observed in these animals, followed later by intraductal hyperplasia (a precancerous lesion) and carcinoma in situ (Durando et al., 2007; Murray et al., 2007; Vandenberg et al., 2008). Similarly, exposure to bisphenol A during nursing, followed by a challenge with DMBA produced increased numbers of tumours per rat and a shortened latency period (Jenkins et al., 2009).

2.7.2.2 Endometrial cancer

Endometrial cancer is the sixth most common cancer in women worldwide and, in industrialized countries, endometrial cancer is one of the most common cancers afflicting the female reproductive tract. The lowest rates were observed in South-Central Asia and Africa (excluding South Africa). Endometrial cancer was eight times more common in North America than in parts of Africa (**Figure 2.16**). In many countries, incidence has been increasing steadily over the past years (Kellert et al., 2009; Lindeman et al., 2010; Evans et al., 2011) such that around 288 000 cases of endometrial cancer were recorded in 2008.

There are two types of endometrial cancer, an estrogen-dependent variety, and one not dependent on estrogen. The increases in incidence seem to be limited to the estrogen-dependent type (Evans et al., 2011).

Endometriosis is most frequently diagnosed in post-menopausal women. As seen with breast cancer, elevated levels of endogenous sex hormones including total and free estradiol, estrone, and total and free testosterone are associated with increased risk (Allen et al., 2008). Not surprisingly, pharmaceutical estrogens used in combination with progestagen

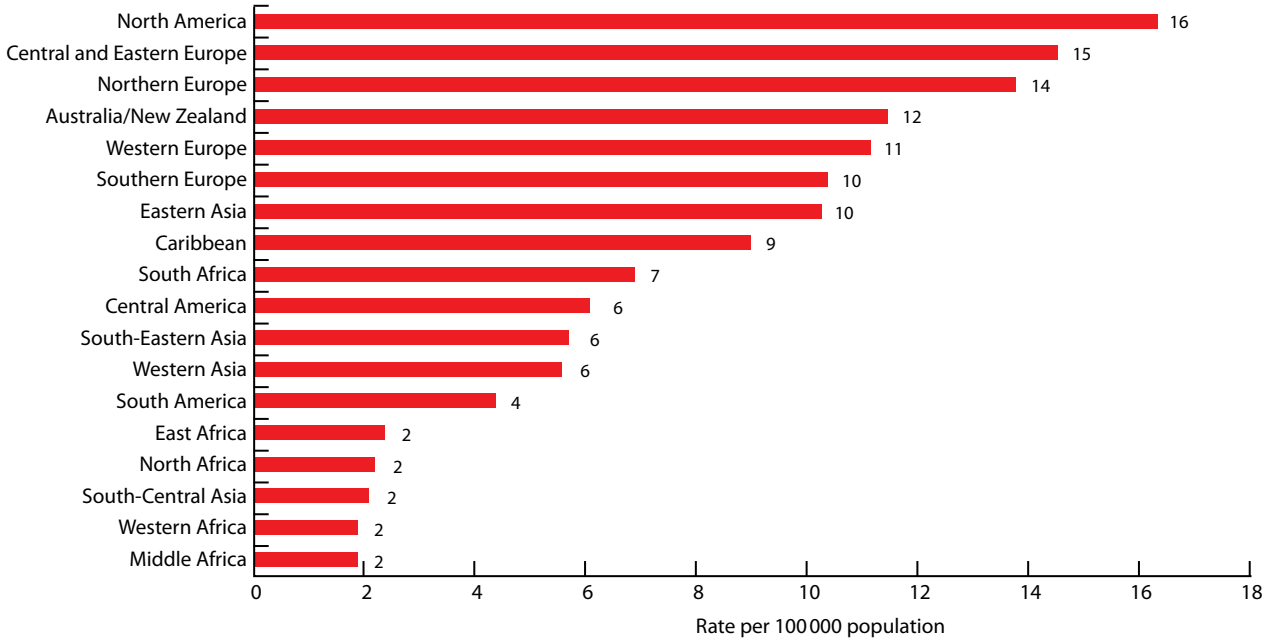

Figure 2.16. World wide age-standardized incidence rates for endometrial cancer in 2008 (Source: GLOBOCAN 2008, http://globocan.iarc.fr, Webpage:http://www.wcrf.org/cancer_facts/Endometrial_cancer_rates.php).

as hormone replacement therapy during menopause increase endometrial cancer risks (Jaakola et al., 2011).

Hormonal mechanisms of endometrial cancer

Most endometrial cancers are tumours that arise from tissues in the endometrium that peel off and regenerate repeatedly every month. Genetic alterations that cause endometrial cancers are, therefore, likely to arise in non-shedding cells, otherwise they would be lost with shedding during every menstruation cycle. It was recently hypothesized that these cells may be a type of stem cell (Kyo, Maida & Inoue, 2011).

The incidence of endometrial hyperplasia or adenocarcinoma is highly associated with prolonged unopposed estrogen action, often resulting from insufficient progesterone (Kim & Chapman-Davis, 2010). Indeed, a recent study found that the expression levels of over 100 genes known to be regulated by estrogen receptor α were also altered in the neoplastic uterus of mice, thus mimicking a hyper estrogenic environment. Emerging laboratory data also suggest that elevated levels of prostaglandin E_2 may underlie the transformation of normal endometrium to neoplastic tissue (e.g. Modugno et al., 2005). In both rodents and humans, deregulation of growth factor (IGFs) pathways and activation of phosphorylating enzymes are also characteristic of endometrial hyperplasia (McCampbell et al., 2006). Moreover, growth factors (e.g. IGF-II) are known to be targets of epigenetic gene silencing. Loss of imprinting of IGF-II resulting in its over expression, occurs in endometrial carcinosarcoma and may, therefore, contribute to abnormal endometrial proliferation characteristic of endometrial hyperplasia in both the rat and human. The complexity of mechanisms and risk factors for endometrial cancer are illustrated in **Figure 2.17**.

Epidemiological evidence that EDCs cause endometrial cancer

Although the involvement of estrogenic agents in the disease process of endometriosis would suggest risks also from estrogenic environmental chemicals, only very few investigations of that topic have been conducted. One of the earlier studies looked at possible associations with DDT serum levels, but produced inconclusive results (Sturgeon et al., 1998). In contrast, Hardell et al. (2004) found weak, but significant associations with serum DDE levels. Bisphenol A levels in patients with endometrial hyperplasia did not differ from those in healthy controls, but were lower in women suffering from hyperplasia with malignant potential (Hiroi et al., 2004). Some evidence also suggests that increased endometrial cancer risks could be linked to long-term cadmium intake (Akesson, Julin & Wolk, 2008).

Evidence from animal studies that EDCs cause endometrial cancer

Neonatal exposure of the developing rodent reproductive tract to xenoestrogens is a well-characterized model of hormone-dependent tumorigenesis in the uterus (Cook & Walker, 2004; Cook et al., 2005; Newbold, Bullock & Mclachlan, 1990; Walker, Hunter & Everitt, 2003) involving ERα-dependent mechanisms (Couse et al., 2001). Estrogen target genes induced in utero that persist into adulthood in DES exposed offspring, include c-fos and lactoferrin (Li et al., 1997; 2003). In the Eker rat model, neonatal DES exposure imparts a permanent estrogen imprint that alters reproductive tract morphology, increases susceptibility to develop uterine leiomyoma and induces endometrial hyperproliferative lesions in adult animals thought to be the precursors of endometrial cancer (Cook &

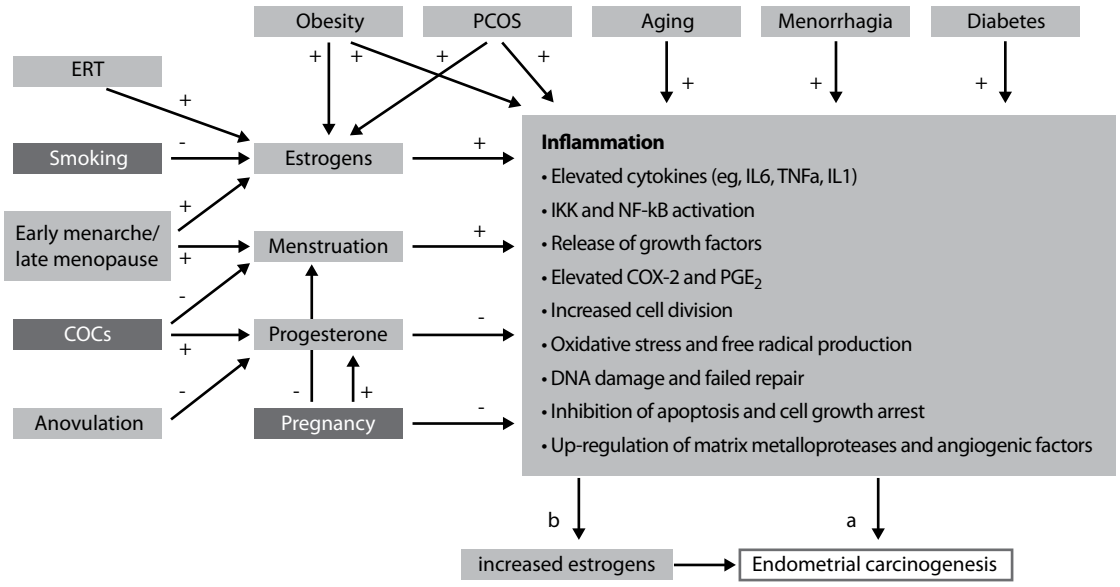

Figure 2.17. Proposed relationships among endometrial cancer risk/protective factors, inflammation, and endometrial carcinogenesis. Endometrial cancer risk factors either influence inflammation directly or influence factors that increase inflammation (e.g. estrogen, menstruation) or decrease inflammation (e.g. progesterone). Protective factors (in dark grey) exert the opposite effects. The effects of inflammation can cause mutagenesis, ultimately leading to endometrial carcinogenesis either directly (a) or indirectly (b) by increasing estrogen levels. ERT= unopposed estrogen therapy; COC=combined oral contraceptives; PCOS= polycystic ovary syndrome. (Figure from Modugno et al. (2005), redrawn; Used with publisher's permission)

Walker, 2004; Cook et al., 2005; 2007). Greater than 90% of CD-1 pups neonatally exposed to DES or the phytoestrogen genistein develop endometrial cancer by 18 months of age whilst C57BL/6 mice are resistant (Kabbarah, 2005). Few other xenoestrogens have been investigated for their ability to induce hyperplastic lesions of the endometrium. A recent study, however, showed that prenatal exposure of mice to bisphenol A elicited an endometriosis-like phenotype in the female offspring (Signorile et al., 2010).

2.7.2.3 Ovarian cancer

As with breast and endometrial cancer, the incidence trends for ovarian cancer are also pointing upwards (reviewed by Salehi et al., 2008). There are similarities with the risk factors important in breast cancer: increased age at menopause contributes to risks, while pregnancies are protective. Hormone replacement therapy increases the risks of developing ovarian cancer (Anderson et al., 2003; Beral et al., 2007).

The known role of estrogens in ovarian cancer indicates that endocrine disruptors might also unfavourably impact on risks, but very few studies of that issue have been conducted. An epidemiological association with exposure to triazine pesticides such as atrazine has been reported in one study (Young et al., 2005).

2.7.2.4 Prostate cancer

Prostate cancer is one of the most commonly diagnosed malignancies in European and USA men. Many countries, including all European countries, are experiencing dramatically increasing incidence trends, with the exception of high incidence countries such as The Netherlands and Austria (Karim-Kos et al., 2008; Jemal et al 2010; **Figure 2.18**).

Hormonal mechanisms of prostate cancer

Most prostate cancers derive from epithelial cells of the prostate gland, and androgens have long been established as playing a role in the causation of the disease (Huggins & Hodges, 2002). The involvement of estrogens has been recognised relatively late. Although estrogens, together with androgens, play a role in normal prostate development (Harkonen & Makela, 2004), estrogen exposure during fetal life can profoundly alter the developmental trajectory of the gland, sensitizing it to hyperplasia and cancer later in life (reviewed by Huang et al., 2004; Prins & Korach, 2008; Ellem & Risbridger, 2009).

Epidemiological evidence for EDCs causing prostate cancer

The upsurge in the incidence of prostate cancer in many countries has been attributed partly to changes in diagnostic methods, namely the introduction of prostate-specific antigen (PSA) screening, but this alone cannot explain the continuing rises. Changes in prostate cancer incidence among migrant populations and studies of twins show that environmental factors, including diet and chemical exposures, also contribute (Lichtenstein et al., 2000; Bostwick et al., 2004).

The spectrum of the environmental factors that may influence prostate cancer risks is, however, difficult to define; without a doubt dietary factors play an important role. In terms of chemical exposures, epidemiological studies have identified pesticide application in agriculture (Alavanja et al., 2003; Koutros et al., 2010), and pesticide manufacture (van Maele-Fabry et al., 2006) as issues of concern. Several current-use pesticides came to light as being associated with the disease,

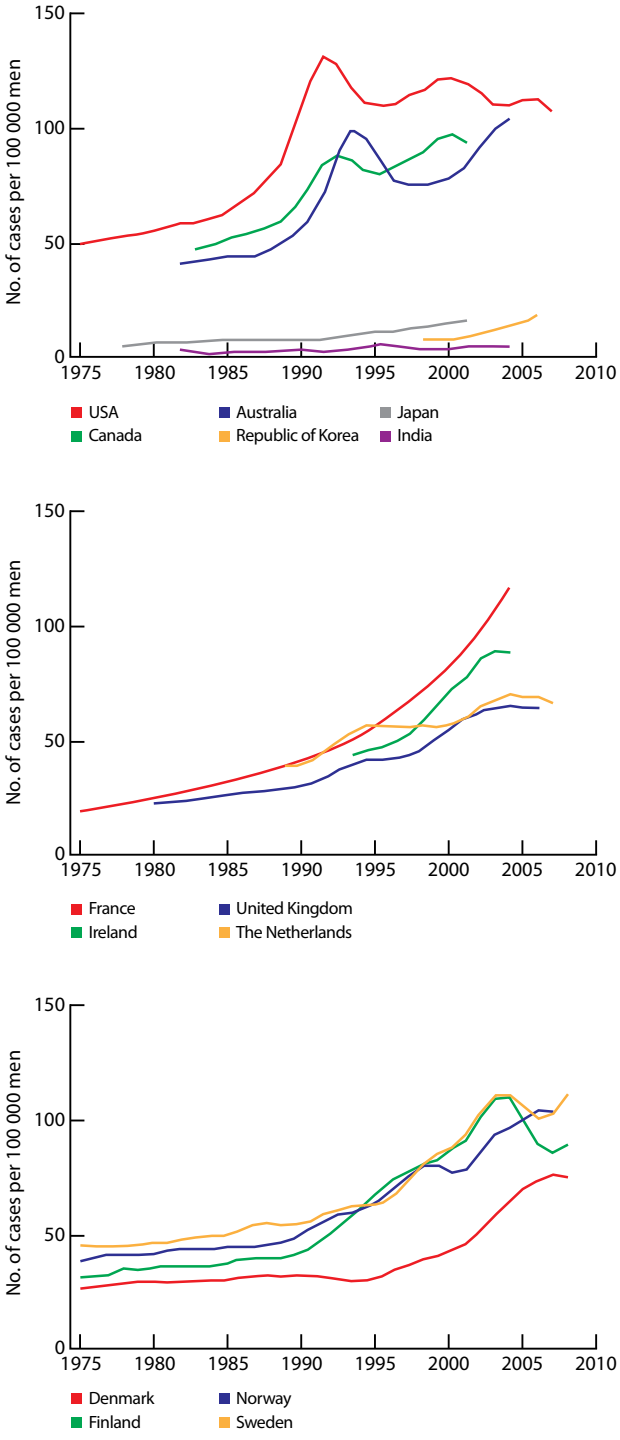

Figure 2.18. Trends in the incidence of prostate cancer in selected countries: age-standardized rate (W) per 100 000.
Source: http://globocan.iarc.fr/factsheets/cancers/prostate.asp

including methyl bromide, chlorpyrifos, fonofos, coumaphos, phorate, permethrin and butylate (see Chapter 3.1.1.6 for more information on these chemicals), the latter six only among applicators with a family history of the disease (Alavanja et al., 2003). Certain organochlorine pesticides, including oxychlordane (Ritchie et al., 2003), transchlordane (Hardell et al., 2006) and chlordecone (Multigner et al., 2010)(see Chapter 3.2.2 for review of human exposure to these POPs) were also found to be linked with increased prostate cancer risks.

Moderately chlorinated PCBs of the phenobarbital type, including CB -138, -153 and -180, could also be linked with prostate cancer, but there were no associations with PCB congeners of the co-planar, dioxin-like type (Ritchie et al., 2003; 2005). However, a Canadian case-control study among incident prostate cancer cases did not show any associations with PCB serum levels (Aronson et al., 2010).

Cadmium exposure (see Chapter 3.1.1.8 & 3.1.5.1 for a review of exposure and use) has been linked to prostate cancer in some, but not all epidemiological studies, and most positive studies indicate weak associations (Bostwick et al., 2004; Parent & Siemiatycki, 2001; Verougstraete, Lison & Hotz, 2003; Sahmoun et al., 2005). Arsenic exposure is strongly associated with prostate cancer (Benbrahim-Tallaa & Waalkes, 2008; Schuhmacher-Wolz et al., 2009).

The precise mechanisms by which the chemicals related to prostate cancer induce the carcinogenic process remain to be resolved. However, in the context of current understanding of the etiology of the disease, agents with androgenic, anti-androgenic and estrogenic activity are likely to be relevant. There is good evidence that the organochlorine pesticides shown to be associated with increased prostate cancer risks, including trans-chlordane, chlordecone, and trans-nonachlor, have estrogen-like activities (Soto et al., 1995). Cadmium also acts as an estrogen mimic, and arsenic seems capable of activating the estrogen receptor (Benbrahim-Tallaa & Waalkes, 2008).

Evidence from animal studies that EDCs cause prostate cancer

More than ten animal models for prostate carcinogenesis have been described (reviewed by Bostwick et al., 2004), but not one single model is able to re-capitulate the key features of the disease in men, which are 1) androgen dependence, 2) developing androgen-independence at more advanced stages, 3) slow growth, with long latency periods, and 4) able to metastasize to lymph nodes, bones and other organs.

In many rodent strains, including the F344 rat used for carcinogen testing, prostate tumours are not inducible by administration of androgens. Usually, tumours have to be "initiated" by exposure to genotoxic carcinogens such as nitrosoureas, followed by treatment with androgens in a "promotional" period.

The Noble rat is a good model for studying hormone-induced prostate cancers, but metastases are rare in this strain. This rat strain has not been widely used for the study of prostate cancers induced by chemicals.

Systematic screening exercises with endocrine disruptors for their ability to induce prostate cancers in animal models sensitive to hormonal prostate carcinogenesis have not been conducted, nor have international validation studies been initiated.

Many of the pesticides identified as being linked with prostate cancer are acetylcholine esterase inhibitors, and have not been shown to possess direct endocrine activity. However, they are capable of interfering with the metabolic conversion of steroid hormones and are thought to disturb the normal hormonal balance, with negative consequences for prostate cancer risks (Prins, 2008).

2.7.2.5 Testis cancer

Testis cancer is a relatively rare cancer, and the highest rates are reported in industrialized countries, particularly in western and northern Europe and Australia/New Zealand, (**Figure 2.19**). The incidence of testicular cancer is estimated to have doubled in the last 40 years, particularly in white Caucasians (also see **Figures 2.2, 2.3** and **2.4** of this Chapter 2.3). On average, the increases are 1-6% per annum and are reported for both seminomas and non-seminomas. The increasing trends appear to be influenced by birth cohort, with increasing risk for each generation of men born from the 1920s until the 1960s. For high risk countries there is evidence that the rate of increase has slowed over time and in several countries, including the UK, the most recent testicular cancer incidence rates have fallen slightly. Testicular cancer has an unusual age-distribution, occurring most commonly in young and middle-aged men, with its origin during fetal life. As such, it is discussed as part of the testicular dysgenesis syndrome in section 2.3.

2.7.2.6 Thyroid cancer

Although thyroid cancer is among the less common malignancies afflicting men and women, during the last few decades it has been increasing more rapidly than any other solid tumour. In most industrialized countries, the incidence of thyroid cancer has more than doubled since the early 1970s; for example 11 cases per 100 000 were diagnosed in the USA in 2006 (Sipos & Mazzaferri, 2008); Within the last two decades thyroid cancer has become the fastest rising neoplasm among women in North America (Holt, 2010). Similar trends have been observed in many other industrialized countries across the world (Cramer et al., 2010; Rego-Iraeta et al., 2009; Kilfoy et al., 2009; **Table 2.5**). Females, children and young adults are particularly vulnerable (Olaleye et al., 2011). Improvements in diagnostic histopathology are not regarded as the reason for the observed increases in thyroid cancer incidence (Cramer et al., 2010). There are several forms of thyroid cancer, defined in terms of their histology – follicular, papillary, and anaplastic. There is also medullary thyroid cancer. By far, anaplastic thyroid cancer is the most aggressive, with a mortality rate of nearly 100%.

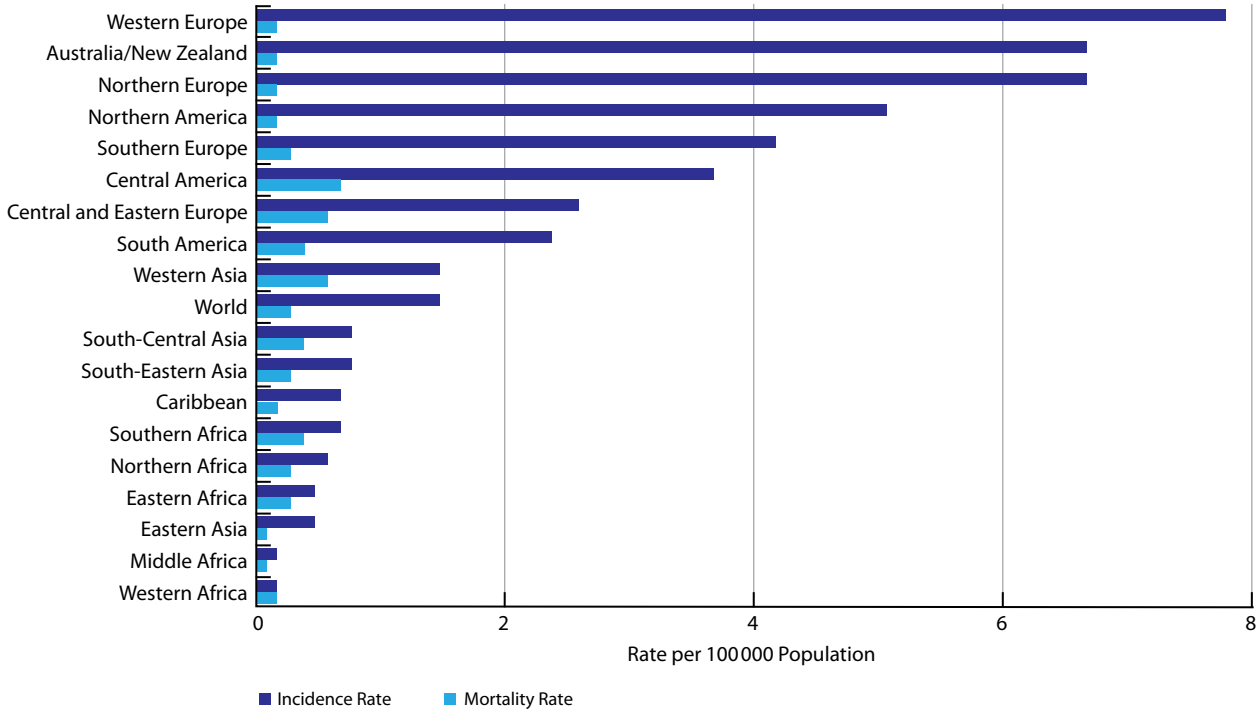

Figure 2.19. Testicular cancer, world age-standardized incidence and mortality rates, World Regions, 2008 (Figure from Ferlay et al. (2008), redrawn; Used with publisher's permission).

Table 2.5. International variation in thyroid cancer incidence rates, 1973-1977 to 1998-2002 (world age-standardized rates). (From: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2788231/table/T1/) Table printed with permission of the publisher.

	1973-1977				1998-2002				Males	Females
	Males		Females		Males		Females		%	%
	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate	Change	Change
Europe, Scandinavian Countries										
Denmark	168	1	330	1.6	210	1.2	524	2.9	20.0	81.3
Norway	182	1.4	558	4.4	247	1.6	649	4.2	14.3	-5.8
Sweden	463	1.6	1158	3.9	407	1.3	1031	3.3	-18.8	-18.2
Finland	221	1.7	684	4.3	384	2.2	1281	7.0	29.4	62.8
Europe, Other										
France, Bas-Rhin	25	0.9	85	2.8	75	2.3	198	5.8	155.6	107.1
Switzerland, Geneva	18	1.9	43	3.5	27	2.0	98	6.5	5.3	85.7
UK, England, Thames	134	0.6	391	1.5	433	0.9	1133	2.3	50.0	53.3
Italy, Varese	45	2.0	105	3.8	45	2.9	123	7.1	45.0	86.8
Spain, Zaragosa	28	1.2	134	5.4	37	1.4	123	4.0	16.7	-25.9
Ocenia										
New Zealand	108	1.2	285	3.1	181	1.6	598	5.1	33.3	64.5
Australia, New South Wales	116	0.9	315	2.3	506	2.5	1639	8.1	177.8	252.2
Americas										
USA SEER[a]: White	997	2.3	2491	5.4	2216	3.5	6306	10.0	52.2	85.2
Canada, BC	104	1.5	252	3.6	271	2.1	733	5.6	40.0	55.6
Colombia, Cali	20	1.5	104	6.1	85	2.2	450	9.4	46.7	54.1
	(1972-1976)		(1972-1976)							
USA SEER[b]: Black	47	1.2	173	3.8	121	1.6	494	5.2	33.3	36.8
Asia										
China, Hong Kong	126	1.6	352	4.2	447	2.2	1557	7.2	37.5	71.4
	(1974-1977)		(1974-1977)							
Japan, Osaka Prefecture	129	0.7	432	2.1	432	1.3	1194	3.2	85.7	52.4
Singapore	43	1.3	141	3.8	180	2.0	636	6.6	53.8	73.7
Israel:Jews	193	2.6	472	6.2	474	3.5	1747	12.1	34.6	95.2
Africa										
Algeria, Setif					32	1.4	88	3.6		
Egypt, Gharbiah					53	1.1	151	2.6		
Tunisia, Center, Sousse					14	1.3	34	3.1		
Uganda, Kyandondo County					11	0.5	26	1.5		
Zimbabwe, Harare					14	1.0	45	3.1		

[a] Surveillance, Epidemiology and End Results

Mechanisms of thyroid cancer

According to a widely held view, thyroid cancer derives from well-differentiated normal cells (thyrocytes) by multiple changes in the genome (Giusti et al., 2010). This is supported by the fact that radiation is the biggest risk factor for this cancer and by the fact that there are large familial associations. However, clinical and molecular findings in thyroid carcinoma raise questions regarding the multi-step carcinogenesis hypothesis of thyroid cancer (Takano & Amino, 2005). There is little evidence to prove the succession of genomic changes, which casts doubt on the idea that aggressive carcinomas are derived from thyrocytes by accumulation of genetic changes. The alternative hypothesis proposes that thyroid cancer originates from the remnants of fetal, poorly differentiated, thyroid cells, and not from thyrocytes (Reya et al., 2001; Takano & Amino, 2005). Fetal thyroid cells have the ability to move through other cells, which is similar to the ability to induce invasion or metastasis. The existence of stem cells in the thyroid gland had been discussed often but identification in humans has not been successful. This is a knowledge gap.

In rodents, there is a strong influence of thyroid stimulating hormone on thyroid cancers. Persistent output of thyroid stimulating hormone (TSH) by the pituitary forces the follicle cells of the thyroid to divide in an effort to keep up with the demand for thyroid hormone. This then leads to hyperplasia and increased risk of cancer. In humans, however, it is not clear whether persistent TSH is a cause or a consequence of thyroid cancer.

In women of childbearing age thyroid cancer incidence is about 3 times higher than in men of similar age, and this suggests the possible involvement of estrogens and estrogenic chemicals (McTiernan, Weiss & Daling, 1984). There are however contradictory findings regarding the presence of estrogen receptors (ER) in thyroid cancers. While Kavanagh et al., (2010) have found ERα and β in thyroid tumours, the presence of ERα is disputed and the significance of the β form for malignant growth is unclear (Vaiman et al., 2010). Nevertheless, the higher incidence of thyroid cancer in women is attributed to the presence of a functional ER that participates in cellular processes contributing to enhanced mitogenic, migratory, and invasive properties of thyroid cells. In *in vitro* studies estradiol caused a 50-150% enhancement of the proliferation of thyroid cells (Rajoria et al., 2010). In rodents both TSH and estrogens stimulated thyrocyte proliferation (Banu, Govindarajulu & Aruldhas, 2002).

Epidemiological evidence that EDCs cause thyroid cancer

Although there is abundant evidence that several key components of thyroid hormone homeostasis are susceptible to the action of endocrine disruptors (see section 2.5), it is not clear whether chemicals that affect thyroid cell growth can lead to human thyroid cancer. Epidemiological studies investigating exposure to EDCs and the occurrence of thyroid cancer are scarce, and little work has focused on the possibility that environmental chemicals may contribute to some of the increased incidence of thyroid cancer (reviewed by Leux & Guenel, 2010). Especially in children, ionizing radiation is recognized as a key risk factor in thyroid cancer.

There are indications that thyroid cancer is associated with occupational exposure to solvents, with excess risks among females employed in shoemaking (Lope et al., 2005; Wingren et al., 1995). Women who worked as a dentist/dental assistants, teachers, or warehouse workers also were at risk (Wingren et al., 1995). Benzene and formaldehyde have been implicated as contributing to thyroid cancer risk (Wong et al., 2006).

An excess risk of thyroid cancer was observed in Swiss agricultural workers exposed to pesticides (see Chapter 3.1.5 for a review of what humans are exposed to); however, these risks could also have stemmed from an iodine deficit in the agricultural regions studied (Bouchardy et al., 2002). In the American prospective cohort on 90 000 pesticide applicators and their wives (Agricultural Health Study, AHS), there was an increased incidence of thyroid cancers when compared to the general population (Blair et al., 2005). Pesticide applicators engaged in handling the herbicide alachlor showed a moderate, but statistically non-significant, increase in thyroid cancer risk (Lee et al., 2004).

Follow-up studies after the 1976 Seveso accident report a suggestive, almost significant increase for thyroid cancer associated with 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (Pesatori et al., 2003). Thyroid cancer risk was also increased in a large occupational cohort of pesticide sprayers with possible exposure to dioxin (Saracci et al., 1991).

Associations with other thyroid diseases

It was recently documented that individuals with autoimmune thyroid diseases (discussed in sections 2.5, 2.11) such as Graves' disease or Hashimoto thyroididtis tend to have a much higher risk of developing cancer of the thyroid gland (Shih et al., 2008). A total of 50% of the 474 patients evaluated in this study had thyroid cancer, many more than the 28% who went into the surgery with a thyroid cancer diagnosis. The prevalence of thyroid cancer in the Hashimoto patients was 35.6%, and twice that found in the patients with Graves' disease. Likewise, participants with Hashimoto's thyroiditis were more likely to have benign thyroid adenomas, with advanced age being an especially strong risk factor. It is unclear why there is a link between Hashimoto thyroiditis and cancer, but it warrants further study.

Evidence from animal studies that EDCs cause thyroid cancer

A number of pesticides have been shown to induce thyroid follicular cell tumours in rodents, which according to the US Environmental Protection Agency (EPA) are relevant for the assessment of carcinogenicity in humans. Of 240 pesticides screened, at least 24 (10%) produced thyroid follicular cell tumours in rodents. Of the studied chemicals, only bromacil lacked antithyroid activity. Intrathyroidal and extrathyroidal sites of action were found for amitrole, ethylene thiourea,

and mancozeb, which are thyroid peroxidase inhibitors; and acetochlor, clofentezine, fenbuconazole, fipronil, pendimethalin, pentachloronitrobenzene, prodiamine, pyrimethanil, and thiazopyr, which seemed to enhance the hepatic metabolism and excretion of thyroid hormone (Hurley, Hill & Whiting, 1998).

The current understanding of the etiology of thyroid cancer does not clearly link it to an endocrine mechanism. However, chemicals disrupting the hypothalamic pituitary thyroid (HPT) axis and xenestrogens seem to be of importance at least in the progression of the disease. Therefore, the precise mechanisms of cancer causing action of the chemicals demonstrated in epidemiological studies to be related to thyroid cancer remain to be resolved. There is plenty of evidence that EDCs interfere with thyroid homeostasis through numerous mechanisms of action. Many substances exert a direct and/or indirect effect on the thyroid gland by disrupting certain steps in the biosynthesis, secretion, and peripheral metabolism of thyroid hormones (Boas et al., 2006). However, it is not clearly established whether chemicals that affect thyroid cell growth lead to human thyroid cancer. Investigations that compare the susceptibility to disruptors associated with thyroid cancer between rodents and humans would be useful. A recent review by Mastorakos (2007) summarizes substances that have been found to act as EDCs via the HPT axis in different species. Ten of the listed chemicals have been shown to cause an increased risk of thyroid neoplasms and tumours in rodents and the possible mechanisms are explained.

Mechanisms of thyroid cancer are heterogeneous and include gene mutations as well as the potential for a resident population of stem cells to become tumorigenic, or invasion of brain marrow-derived stem cells. This later process has been speculated to be sensitive to estrogen action which may explain the gender difference.

Several rodent two-step carcinogenesis models have been developed (Kitahori et al., 1984; Son et al., 2000; Takagi et al., 2002). These rodent carcinogenesis models are useful, as little is known about the development of thyroid cancer in general. However, thyroid cancer development in humans seems to differ from that in rodents in that the latter are more sensitive to increased TSH levels. There are also differences in the normal physiological thyroid hormone processes between rats and humans. In rats, 40% of T3 is secreted directly from the thyroid, compared to 20% in humans and the structure of the deiodinase enzyme in rats is different from that of humans (Takser et al., 2005). In humans, circulating thyroid hormones are primarily bound to thyroxine-binding globulin (TBG), with smaller amounts bound to albumin and transthyretin. In developing rats, TBG is not present in the circulation between months two and seven and adult rat thyroid hormones are primarily bound to transthyretin, and, to a lesser extent, albumin. These proteins have a lower affinity for thyroid hormones than TBG resulting in a shorter half-life of thyroid hormones in adult rats (Lans et al., 1994).

2.7.3 Evidence for endocrine disruptor causation of hormonal cancers in wildlife

Until relatively recently, cancer in wildlife species was not of particular concern, because it appeared to occur at lower rates in most wildlife species than in humans. However, with increased monitoring of particularly endangered wildlife species, the identification of Tasmanian devil facial tumour disease (a neuroendocrine cancer), sea turtle fibropapillomatosis and sea lion genital carcinoma, it has become apparent that neoplasias (including endocrine neoplasias) can be highly prevalent in wildlife and have considerable effects on populations of some species (McAloose & Newton, 2009).

2.7.3.1 Vertebrate wildlife

Little has been written specifically about hormonal cancers in animal species other than humans. The available literature suggests that most of the endocrine cancers of humans are known to occur as similar entities in dogs, cats and wildlife species. The rates of endocrine cancers in both domestic and wild animal species are generally lower than those observed in humans, but there are reports of increased rates of these and other tumours in some populations. In a review of cetacean tumours, for example, the reproductive tract was cited as one of the more common organ systems to be affected by neoplasia. If the reproductive tract is affected, this can interfere with successful breeding and parturition. In one study, benign genital papillomas were present in 66.7% of dusky dolphins and 48.5% of Burmeister's porpoises, a rate considered to be high enough to interfere with copulation in some cases (Van Bressem et al., 1996; Van Bressem, Van Waerebeek & Raga, 1999). Genital tract carcinomas were also reported to be increasing in California sea lions between 1979 and 1994, only rarely reported in any type of seal/sea lion prior to 1980 (Gulland, Lowenstine & Spraker, 2001; Sweeney & Gilmartin, 1974; Newman & Smith 2006); 18% of sexually mature sea lions found stranded on the California coast in 1994 had aggressive genital carcinomas (Gulland et al., 1996), a rate that is unprecedented in any pinnepid species.

Associations between exposure to anthropogenic contaminants and the development of neoplasia (but not particular endocrine neoplasias) in wildlife populations is difficult to study, but in certain monitored populations such as beluga whales of the St Lawrence estuary in Canada (reviewed in McAloose & Newton, 2009) and bottom-dwelling fish of the same region (Malins et al., 1985a; 1985b; Smith, Ferguson & Hayes, 1989; Bauman, Smith & Metcalf, 1996), cancers are quite well documented. In the St. Lawrence estuary beluga whales, monitored for a period of 17 years, the estimated annual rate of all cancers, including hormonal cancers (163/100 000 animals) is much higher than reported for any other cetacean population and similar to that reported in humans and hospitalized cats and cattle (Martineau et al., 2002). Endocrine

cancers including leiomyomas of the vagina, cervix and uterus, mammary adenocarcinomas and adrenal and thyroid tumours were found (Mikaelian et al., 2000). For all types of cancer, the annual incidence rate was higher than or equal to that found in any other animal population and highest for intestinal cancer. There is no evidence that cancer is normally frequent in beluga from less polluted environments, or that it is caused by old age (Martineu et al., 2002). Carcinogenic PAHs from aluminium smelters are, however, present in the environment of the St. Lawrence beluga and are likely ingested by these animals when feeding on benthic species of invertebrates. Systematic studies to assess the direct or potential roles of these contaminants have not been done, although most of the published examples strongly suggest that carcinogenesis in the beluga whale is a result of the combined effects of multiple factors, including exposure to PAHs present in their local environment (DeGuise, Lagace & Beland, 1994; DeGuise et al., 1995; Mikaelian et al., 1999; Martineau et al., 1988; 1995; Muir 1996a; 1996b; Hobbs et al., 2003; Chapter 3.2.1 contains a review of chemicals exposures in wildlife generally).

There are several other instances where the interplay between chemical contaminants and other factors in causing cancer in wildlife is clearly visible. In many of these cases, viruses are known causal factors (King et al., 2002; Buckles et al., 2006), but in some instances there is also evidence of a co-causal role played by chemical contaminants. These include increased incidence of fibropapillomatosis in sea turtles living in polluted bodies of water (Herbst & Klein, 1995; Foley et al., 2005; Work et al., 2004), an 85% higher level of PCBs found in the blubber of sea lions with genital carcinoma compared to those without this disease (Ylitalo et al., 2005), and an increased rate of epizootics of the liver and skin cancer in fish living in industrial waterways (Malins et al., 1985a; 1985b; 1987; Black & Baumann, 1991; Blazer, 2006; Sakamoto & White, 2002; Williams et al., 1994). Indeed, epidemics of liver cancer have been found in 16 species of fish in 25 different polluted locations, both fresh and salt water. The same tumours have been found in bottom-feeding fish in industrialized and urbanized areas along Canada's Atlantic and Pacific coasts, whereas in Canada's less polluted waters cancer in fish is reported to be almost non-existent.

Experimental support for relationships between environmental pollutant exposure and cancer in fish and mammals also exists. For example, laboratory models have demonstrated that exposure to the PAH, benzo(a)pyrene, produces liver and/or skin tumours in fish, depending on the route of exposure (Hendricks et al., 1985; Black, 1984). In rodents, a relationship between intestinal cancer and PAHs is supported by observations in mice, whereby chronic ingestion of coal tar mixtures (containing benzo(a)pyrene) causes small intestinal carcinomas. Moreover, associations have been made in fish populations where environmental contamination decreased concomitant with decreases in the cancer rates (Bauman, Harshbarger & Hartman, 1990; Bauman & Harshbarger, 1995).

2.7.3.2 Invertebrate wildlife

Little information is available on endocrine neoplasias in invertebrate species and even less information links any incidence of invertebrate neoplasia with contaminant exposure. Nonetheless, a field survey carried out in three geographically distinct populations of soft-shell clams in eastern Maine, USA identified a high incidence of gonadal tumours (Gardner et al., 1991) and at all three locations, exposure to significant concentrations of the herbicide, Tordon 101 (picloram), 2,4-D and 2,4,5-T had occurred. Although 2,4-D and 2,4,5-T are not known to be potent carcinogens, TCDD, a by-product contaminant from the synthesis of 2,4,5-T is (Schmidt, 1992). Other types of neoplasias (in the respiratory system and hemolymph) are commonly found in bivalves; at least 22 species of estuarine bivalve molluscs show neoplasias (sometimes in >90% of individuals) on both coasts of North America, in Australia and in several countries in South America, Asia, and Europe (Wolowicz, Smolarz & Sokolowski, 2005). Incidence of these neoplasias tends to be highest in molluscs living in more polluted sediments (Wolowicz, Smolarz & Sokolowski, 2005).

2.7.4 Evidence for a common mechanism of hormonal cancers in human and wildlife

In many cases, hormonal cancers in vertebrate wildlife and domestic animal closely resemble the corresponding human carcinoma in terms of clinical behaviour, pattern of circulating hormone levels and expression of hormone receptors in primary tumours. For example, the role of steroidal hormones in wildlife cancers has been best described in domestic and zoo animals, where the use of the progestin, melengestrol acetate, as a contraceptive has been strongly associated with both ovarian and mammary carcinomas, as well as endometrial hyperplasias in domestic dogs and cats, tigers, lions and jaguars (McAloose, Munson & Naydan, 2007; Harrenstien et al., 1996). This contraceptive prevents the animals from breeding, resulting in their being exposed to recurrent estrogen peaks followed by high persistent levels of progesterone. As for women, estrogen (ER) and progesterone receptor (PR) expression varies in canine and feline mammary cancers. In general, ER expression is low, but PR expression persists in most cancers. Alterations in molecular controls of cell proliferation or survival in breast cancer, as seen in humans, have been identified in dog and cat mammary cancers, making them excellent models for human breast cancer.

There is no evidence available to show that endocrine disrupting environmental contaminants that are hypothesized to play a role in the causation of specific human endocrine cancers also play a role in wildlife and domestic animal endocrine cancers. Notwithstanding this, the links between animal and human health are long-standing. Viral and chemical-induced oncogenesis is a familiar concept in both human and wildlife studies and the study of animal viruses has led to new insights

into the molecular mechanisms of human cancers. Moreover, differences in mammary cancer prevalence between carnivores and herbivores and between captive and wild carnivores are striking and support the hypotheses that diet and reproductive history are major risk factors for these cancers. In wildlife, relationships between tumour development and environmental contamination are strongly suggested by scientific data in some contaminated regions of the world. Similarities of high cancer incidence and tumour type between species would support the conclusion of common risk factors in shared environments and show the value of wildlife as important environmental sentinels. In the St. Lawrence estuary, for example, the human population is also affected by higher rates of cancer than populations in other parts of Quebec and Canada, and some of these cancers have been epidemiologically related to exposure to PAHs, as seen in the beluga whale. In another example, a study of more than 8 000 dogs showed that canine bladder cancer was associated with their living in industrialized countries, mimicking the distribution of bladder cancer among their human owners (Hayes, Hoover & Tarone, 1981). Further investigations of the role of pesticides in canine bladder cancer showed that the risk of bladder cancer was significantly higher among dogs exposed to lawns or gardens treated with herbicides or insecticides, including peony herbicides, but not among dogs exposed to lawns or gardens treated with insecticides alone. Moreover, risk of bladder cancer was higher if the dogs were obese and lived near another source of pesticides and lower if the diet contained green leafy vegetables (Glickman et al., 1989; 2004). A final example from a survey of cancer mortality rates in the United States indicated that the mortality rates due to ovarian and other reproductive organ cancers in human females from Washington County, Maine, and from Indian River, Florida were significantly higher than the national average (Riggan et al., 1987), coinciding with geographic areas in which tumour-bearing clam populations were also located (Gardner et al., 1991; Hesselman, Blake & Peters, 1988). It is also of interest that DNA from mollusc tumours is able to transform mammalian cells into cancerous cells in vitro (Van Beneden, 1994).

Taken together these observations suggest that human and animal populations may be affected by specific types of cancer because they share the same habitat and are exposed to similar types of contaminants. Many of the molecular mechanisms governing cancer are evolutionarily conserved between wildlife and humans and so this is a mechanistically plausible hypothesis. Although none of these examples particularly highlight endocrine cancers, there has been very little if any study of this topic.

2.7.5 Main messages

- The incidences of all endocrine-related cancers (breast, endometrial, ovarian, prostate, testis and thyroid) in humans are rising in many countries, or are levelling off at a high plateau.
- The increase in incidence of endocrine-related cancers in humans cannot be completely explained by genetic factors; environmental factors, including chemical exposures, are involved, but very few of these factors have been pinpointed.
- For breast, endometrial and ovarian cancer, the role of endogenous and therapeutical estrogens is well documented; this makes it biologically plausible that xenoestrogens might also contribute to risks. However, the EDCs shown to be associated with breast cancer risks (PCDD, PCBs and solvents) do not have strong estrogenic potential. There are indications that endometrial cancer is linked to the xenoestrogens DDE and cadmium.
- For prostate cancer in humans, weak associations with exposures to pesticides (occupational), PCBs, cadmium and arsenic have come to light. There is good biological plausibility that androgens and estrogens are involved in the disease process.
- For human thyroid cancer, there are indications of weak associations with pesticides and TCDD.
- For most of the hormonal cancers in humans, valid animal models are not available. This makes the identification of hormonal carcinogens very difficult, and forces researchers to rely on human epidemiological studies. However, epidemiological studies cannot easily pinpoint specific chemicals, and can identify carcinogenic risks only after the disease has occurred.
- A general weakness of the environmental epidemiology of hormone-related cancers has been a lack of focus on holistic exposure scenarios. So far, epidemiology in this area has explored quite narrow hypotheses about a few priority pollutants, without taking account of combined exposures to a broader range of pollutants.
- Cancers of endocrine organs, particularly reproductive organs, are also found in wildlife species (several species of marine mammals and invertebrates) and tend to be more common in animals living in polluted regions than in more pristine environments.
- Wildlife populations and domestic pets may be affected by the same types of cancers as humans because they share the same habitat and are exposed to similar types of contaminants. Greater study of this wildlife and domestic pets as environmental sentinels for hormonal cancers in humans is needed.

2.7.6 Scientific progress since 2002

Significant advances in our knowledge of hormonal cancers have occurred since the 2002 IPCS *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002). These include:

- In breast cancer, the vulnerability of breast tissue to cancer-causing influences during fetal life and puberty has been recognized.

- Where exposures during these life stages could be re-constructed, as was the case with DES and DDT, associations with elevated breast cancer risk later in life could be demonstrated. Combined internal exposures to non-steroidal estrogens are also risk factors in breast cancer. Taken together, this evidence strengthens the biological plausibility that other estrogenic chemicals are also contributors to risks, but adequate studies to prove this point have not been conducted.
- Steroidal estrogens are also risk factors in endometrial and ovarian cancer, but the involvement of other estrogenic chemicals remains to be elucidated.
- In prostate cancer, the importance of exposures to estrogens as risk factors has received attention. There is evidence that cadmium, arsenic and non-coplanar PCBs contribute to prostate cancer risks, as do exposures to unspecified pesticides among pesticide applicators. Whether there are hormonal mechanisms at work remains to be clarified.

2.7.7 Strength of evidence

There is sufficient evidence that the incidence of most hormonal cancers has increased or remains at a high level, and that environmental exposures play a role in these unfavourable trends. However, the nature of these environmental factors is poorly defined in terms of contributing chemicals.

Several independent studies have shown associations between PCDD/F exposures and elevated breast cancer risks (reviewed by Brody et al., 2007; Dai & Oyana, 2008). There is therefore sufficient evidence linking breast cancer with dioxins and furans (see Chapter 3.2.2 for a review of what humans are exposed to).

There is also sufficient evidence for increased breast cancer risks among women with elevated PCB exposures and a Cyp polymorphism (Brody et al., 2007).

Sufficient evidence exists for a link between pesticide exposures during application (Alavanja et al., 2003; Koutros et al., 2010) and manufacture (van Maele-Fabry et al., 2006) and prostate cancer. However, the nature of the implicated agents remains to be pinpointed.

One epidemiological study (Ibarluzea et al., 2004) has demonstrated a link between internal estrogen burden from lipophilic chemicals and breast cancer. Single epidemiological studies have shown associations between DDT and endometrial cancer, and between triazine pesticides and ovarian cancer. Because these observations have thus far not been replicated by others, the evidence is limited.

There is thus far no evidence linking thyroid cancer with any endocrine disrupting chemicals.

In general, the study of endocrine disrupting environmental pollutants and hormonal cancers is characterized by epidemiological studies that have pursued very narrow hypotheses about the contributing chemical substances. In many cases, investigations were driven by the availability of chemical analytical techniques, rather than by biologically plausible ideas about etiological factors. If epidemiology is to make a larger contribution to this field of study, the effects of combined exposures will have to be considered.

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2.8 Endocrine disrupting chemicals and adrenal disorders in humans and wildlife

2.8.1 Overview of adrenal function and dysfunction in humans and wildlife and evidence of endocrine disruption

2.8.1.1 Adrenal dysfunction caused by exposure to EDCs

Disorders relating to hyper-secretion of the hormone cortisol from the adrenal glands are classically referred to as Cushing disease/syndrome. Normally, adrenocorticotropic hormone (ACTH) is released from the pituitary gland in the brain to stimulate the release of the stress hormone cortisol from the adrenals. In Cushing disease, either a tumour in the pituitary secretes ACTH or excess cortisol is produced by adrenal gland tumours, or by hyperplastic adrenal tissues. Along with a suite of metabolic disturbances, including high blood sugar levels (hyperglycemia), redistribution of body fat and an increased protein catabolism, gross enlargement of the adrenal cortex is frequently observed in this disease. The reversed situation resulting from adrenal hypotrophy and hypo-secretion of adrenal steroid hormones may result in Addison disease, a potentially fatal condition depending on the extent of tissue destruction in the adrenal cortex.

The involvement of EDCs in adrenal health problems came into focus when frequent cases of massive adrenocortical hyperplasia and a suite of pathological lesions characteristic of Cushing disease were identified in all three species of seals inhabiting the Baltic Sea (Bergman & Olsson, 1985). The syndrome, also involving lesions in the female reproductive tract, the intestines, skin and skeleton, was associated with high body burdens of PCBs and DDT compounds in Baltic wildlife at the time (see Chapter 3.1.4 & 3.2.1 for a review of exposure of wildlife to DDT and PCBs).

Persistent aryl methyl sulfone metabolites derived from PCBs and DDT were originally demonstrated in the blubber of Baltic grey seals (Jensen & Jansson, 1976). Moreover, these compounds are now known to be widely present in the tissues of other marine animals as well as in human milk (Bergman et al., 1994; Haraguchi et al., 1992; Letcher, Norström & Bergman, 1995; Newsome & Davis, 1996; Noren et al., 1996; Troisi et al., 2000; Weistrand & Noren, 1997; Chapter 3.2.2). Subsequent research on this new class of POPs revealed that the DDT metabolite methylsulfonyl-DDE ($MeSO_2$-DDE) was a highly potent and tissue-specific toxicant that induced degeneration and necrosis in the adrenal cortex of laboratory mice following a single dose (Lund, Bergman & Brandt, 1988; Jönsson et al., 1991; Lund & Lund, 1995; Lindhe et al., 2001). Reduced plasma corticosterone levels were subsequently recorded in suckling mice following administration of $MeSO_2$-DDE to the lactating dam (Jönsson, Lund & Brandt, 1993). Representing a completely different mechanism of action, methylsulfonyl metabolites formed from PCBs ($MeSO_2$-PCBs) were found to act as antagonists following binding to the human glucocorticoid receptor (GR) *in vitro* (Johansson, Nilsson & Lund, 1998).

The exceptional adrenocorticolytic potency of $MeSO_2$--DDE *in vivo* in mice and the antagonistic binding of PCB-metabolites to the human GR *in vitro* triggered interest in other environmental contaminants with a capacity to perturb hormone secretion in the adrenal cortex of humans and wildlife species. Since adrenal steroid hormone synthesis is regulated through the feed-back system of the hypothalamic pituitary adrenal (HPA) axis, it is highly responsive to various types of stress, including handling. It is therefore difficult to accurately measure plasma levels of the stress hormones cortisol and corticosterone in free-ranging wildlife as well as in experimental animals and relate them solely to exposure to chemical exposures. In current research efforts, all components of the HPA axis, rather than only the glucocorticoid-secreting adrenal cells, have come into focus.

Numerous chemicals affecting adrenal structure and function have been described (Hinson & Raven, 2006). Due to its high blood supply and lipid content, a variety of persistent environmental contaminants and other chemicals are selectively taken up and retained in the adrenal cortex, both in adults and fetuses (Brandt, 1977). Due to a high expression of cytochrome P450 (CYPs), the cortex also has a pronounced ability to metabolize specific chemicals to reactive and toxic intermediates that become covalently bound to cellular macromolecules and subsequently degenerate or kill the cells in which they were formed. Since adrenal CYPs are expressed also in fetuses and neonates, a high toxicity in the fetal and neonatal adrenal cortex may occur following trans-placental transport and exposure via milk (Jönsson, Rodriguez-Martinez & Brandt, 1995; Jönsson et al., 1992).

2.8.1.2 The adrenal gland and its hormones

The mammalian adrenal glands are small, pyramid-shaped organs, situated on top of the kidneys. They consist of an inner medulla and an outer cortex. The adrenal medulla secretes catecholamines like adrenalin and noradrenalin, while the three histologically and functionally distinct layers (zonae) of the adrenal cortex secrete a variety of steroid hormones (corticosteroids), many of which are essential for survival: the outer *zona glomerulosa* synthesizes mineralocorticoids, the large middle *zona fasciculata* synthesizes glucocorticoids, and the inner *zona reticularis*, which does not fully develop until puberty, produces androgens. The functional zonation of the adrenal cortex is a consequence of layer-specific expression of steroidogenic genes: the resulting enzymatic repertoire determines the fate of cholesterol in a given *zona*.

Steroidogenesis

Steroid hormones of the adrenal glands are synthesized from cholesterol via activation of a number of enzymes and regulatory proteins in both the mitochondria and

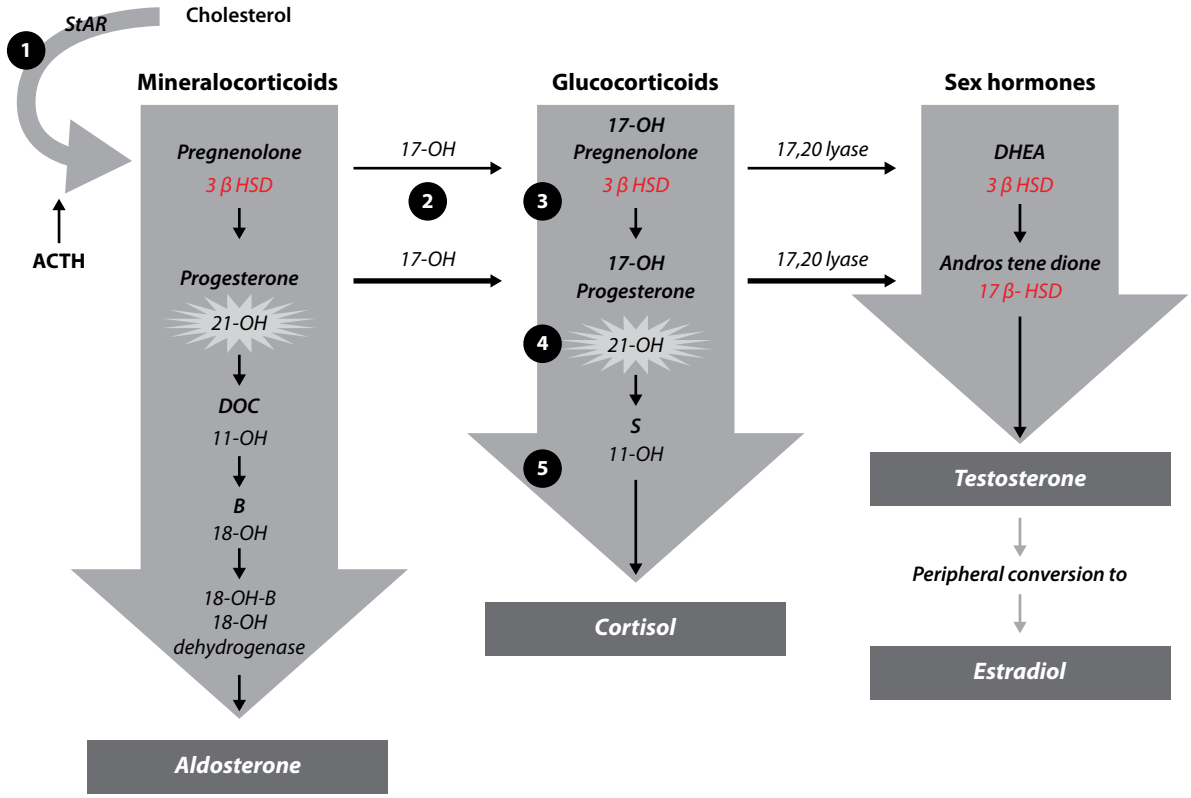

Figure 2.20. Adrenal steroidogenesis: Five enzymatic steps necessary for cortisol production are shown in numbers. 1= 20, 22 desmolase, 2= 17 hydroxylase (17-OH), 3=3ß-hydroxysteroid dehydrogenase (3ß HSD), 4=21 hydroxylase (21-OH), 5=11ß hydroxylase (11-OH). In the first step of adrenal steroidogenesis, cholesterol enters mitochondria via a carrier protein called StAR. ACTH stimulates cholesterol cleavage, the rate limiting step of adrenal steroidogenesis. (Figure from Lekarev et al. (2012); Used with publisher's permission).

the endoplasmic reticulum, most of which belong to the cytochrome P450 (CYP) family (**Figure 2.20**). The transfer of cholesterol across the mitochondrial membranes is facilitated by steroidogenic acute regulatory (StAR) protein. At the inner mitochondrial membrane, the cholesterol side chain is cleaved off, yielding pregnenolone. Pregnenolone is further converted to deoxycortisol, deoxycorticosterone, dehydroepiandrosterone (DHEA), and androstenedione. DHEA and androstenedione are weak androgens. Deoxycortisol and deoxycorticosterone are hydroxylated by the enzyme CYP11B1 to form the glucocorticoids cortisol and corticosterone. Deoxycorticosterone may also be transformed to the mineralocorticoid aldosterone.

In humans, cortisol and aldosterone are the physiologically most important hormones, while the androgen DHEA is the most abundant.

Glucocorticoids

Cortisol and corticosterone act to degrade protein and fat for use in gluconeogenesis with the aim to maintain blood glucose levels. The glucocorticoids are also involved in regulating blood volume and blood pressure and mediate anti-inflammatory and immunosuppressive effects. Glucocorticoids produced during embryogenesis by an adrenal progenitor influence the development of the neural crest-cells into what will become the adrenal medulla (Hammer, Parker & Schimmer, 2005).

Cortisol production is regulated through the hypothalamic-pituitary-adrenal (HPA) axis. In response to physical and/or emotional stress, the hypothalamus secretes corticotropin-releasing hormone (CRH), which causes the release of ACTH from the pituitary gland. ACTH stimulates transcription of several steroidogenic genes.

Mineralocorticoids

Aldosterone regulates water and electrolyte balance in the body by reducing excretion of sodium ions from the body, mainly by stimulating their reabsorption in the kidney. Hyper-secretion of aldosterone causes hypertension and retention of body fluid (edema) due to excess sodium and water retention. It also induces neuromuscular dysfunction due to loss of potassium.

Sex steroids

The hormone DHEA is produced in early fetal life (Hammer, Parker & Schimmer, 2005) by an adrenal gonadal progenitor. This hormone is essential for sustaining pregnancy before placental estrogens take over. The role of sex steroids produced by the adrenal cortex after birth is unclear. They are produced as early as 3 years of age in humans (adrenarche) and then gradually decrease after 30 years of age (adrenopause) (Kempná and Fluck, 2008; Idkowiak et al., 2011). They may cause pre-pubertal growth of pubic and axillary hair, and are possibly involved in the onset of puberty.

Steroidogenesis in the fetal adrenals

In humans, the fetal adrenal cortex is arranged differently than in adults (Coulter, 2005). The inner fetal zone comprises 85-90% of the entire gland and is producing DHEA throughout gestation. The outer definitive zone is inactive until late gestation, while the transitional zone is capable of producing glucocorticoids at the beginning of the third trimester. After birth, the fetal zone regresses. Fetal adrenal growth is independent of ACTH before 15 weeks of gestation, while after 15 weeks, ACTH and other factors are required for normal development (Langlois, Li & Sacz, 2002). Sensitive windows of human adrenal development are found between weeks 4 and 16 of pregnancy and comprise organogenesis from weeks 4 to 8 and establishment of steroidogenesis between weeks 12 and 16. The size of the new born adrenal is similar to its adult size (Langlois, Li & Sacz, 2002).

In the fetus, cortisol and corticosterone play an important role in the maturation of the organs, preparing them for an extra-uterine life and in induction of birth (Challis et al., 2001). In many mammals, such as ruminants and humans, there is a steep increase in cortisol that induces labour through activating enzymes such as CYP17 in the placenta (Wood, 1999).

Towards term, the HPA axis becomes progressively more responsive to stress stimuli (Fowden, Giussani & Forhead, 2005). Since glucocorticoids are responsible for maturation of tissues essential for neonatal survival, disruption of normal HPA axis activity may have widespread consequences. In humans, low birth weight is associated with elevated cortisol and aldosterone levels, suggesting fetal mis-programming of the HPA axis (Martinez-Aguayo et al., 2011)

2.8.2 Evidence for endocrine disruptor causation of adrenal hormone signalling in humans and in rodent models

EDCs may disrupt regulation of adrenal hormone secretion and function at different levels of the (HPA) axis. In addition to a targeted local toxicity in the hormone-secreting cells leading to mitochondrial degeneration and cell death, reduced hormone secretion may result from inhibition of steroidogenic (CYP) enzyme catalytic activity. Hormone secretion may also be up-regulated, both as a compensatory response from the HPA axis (increased ACTH secretion) or as a local response in the steroid-producing cells. Several persistent environmental pollutants are further known to reduce hormone secretion by inhibiting steroidogenic enzymes in vitro, while others induce mRNA synthesis and hormone secretion. Despite emerging evidence that adrenal function may be an important target for EDCs, only a limited number of studies have been published on EDC-induced disruption of adrenal hormone secretion in humans. Likewise, the numbers of experimental in vivo studies are few. However, a comparatively large number of in vitro studies based on the human adrenocortical cell line H295R have been published.

2.8.2.1 Humans

The information on EDC-induced effects on adrenal function in humans is restricted. No evidence is available to show that adrenocorticolytic POPs can affect the human adrenal cortex and HPA axis at environmentally relevant concentrations in vivo. However, there is evidence in the literature that environmental factors, including tobacco smoking and intake of alcohol, may affect the HPA axis and the way individuals respond to stress stimuli. For example, it has been reported that urban psychosocial stress may affect plasma cortisol concentrations (Rosati et al., 2011). Lessened activity of the HPA axis at the onset of and during a stress procedure was reported in adolescents who began drinking alcohol at an early age (Evans et al., 2012). Recent evidence also suggests that tobacco smoke exposure affects the activity of the HPA axis (Granger et al., 2007, Soldin et al., 2011). However, there is conflicting evidence as to what extent exposure to products of tobacco in utero can cause neurobiological and behavioural changes in exposed offspring (Huijbregts et al., 2011, Granger et al., 2007). Current evidence also suggests that reactivity to stress is reduced in cannabis users relative to abstainers (van Leeuwen et al., 2011).

2.8.2.2 In vitro studies

POPs present in human milk and adipose tissue may alter steroidogenesis and gene expression in the human H295R adrenal cell line in vitro. Both non-ortho and ortho-PCBs exert effects on steroidogenesis in H295R cells (Li & Wang, 2005; Xu et al., 2006). Both congener groups stimulated the steroidogenic machinery, although the potency of the different ortho-PCBs varied (Xu et al., 2006). Only small effects on hormone levels were measured after exposure to various hydroxylated PBDE congeners (Song et al., 2008), whereas in another study on PBDE metabolites greater increases in both estradiol and testosterone were observed, and expression of most steroidogenic genes were up-regulated (He et al., 2008). In contrast, the organohalogen pesticide γ-HCH (Lindane) decreased cortisol production in both stimulated and non-stimulated H295R cells (Oskarsson et al., 2006; Ullerås, Ohlsson & Oskarsson, 2008). Following prolonged exposure, $MeSO_2$-DDE decreases H295R cell viability in vitro (Asp et al., 2010b).

Complex mixtures of chemicals extracted from sediments, coastal waters, fresh water and sewage effluents have also been examined and reported to interfere with expression of steroidogenic genes in H295R cells (Blaha et al., 2006; Gracia et al., 2008, Zimmer et al., 2011a; Montano et al., 2011). There was not a uniform pattern of up- or down-regulation, but the CYP11B2 gene, encoding the enzyme that catalyzes the last step of aldosterone synthesis, was up-regulated by the majority of extracts tested. Data analysis performed on extracts from a Norwegian freshwater system suggested that PCBs, and to a lesser extent DDTs, were responsible for the cortisol responses, whereas estradiol and testosterone alterations were best explained by HCB and PCBs, respectively. Brominated flame

retardants were less important contributors to the steroidogenic responses (Zimmer et al., 2011a).

Recently, there has been a concern regarding the potential risk linked to intake of marine fisheries products and whether this risk is outweighed by the potential benefits. Exposure to extracts from crude cod liver oil was done in the H295R-model, resulting in effects on gene expression and hormone production similar to those induced by PCBs, which were major contaminants in the extracts (Montano et al., 2011). Observed effects after exposure to pharmaceutical oil extract, from which POPs had been removed, were considerably lower.

There is also increasing concern about exposure to and potential harmful effects of perfluorinated compounds on humans and the environment (see Chapter 3.1, 3.2.1 & 3.2.2 for exposure). These compounds are used in a huge number of industrial products and have attracted interest because of their persistence in the environment (Chapter 3.1.1). Recently published data using the H295R model provide some evidence that the adrenal cortex is a potential target for perfluorononanoic acid (PFNA) (Kraugerud et al., 2011). In a follow-up study PFNA reduced concentrations of testosterone, progesterone and cortisol.

2.8.2.3 In vivo studies with mammalian models

Some in vivo studies on adrenocorticolytic DDT metabolites and other compounds have been discussed in section 2.8.1 above. Other laboratory studies also give support to the contention that PCBs can also perturb adrenocortical function in mammals.

Some early studies in laboratory strains of mice and rats revealed effects of technical PCB mixtures on the HPA axis and cortisol homeostasis (Miller et al., 1993; Sanders & Kirkpatrick, 1975). Later $MeSO_2$-PCBs were found to be glucocorticoid receptor antagonists (Johansson, Nilsson & Lund, 1998), but so far no studies to explore the toxicological significance of this observation have been carried out. However, a recent developmental study in sheep does give support to the hypothesis that mono - and diortho PCBs are able to interfere with fetal adrenal development and cortisol production in mammals (Zimmer et al., 2011b). The results indicated that both CB-153 and CB-118 were associated with a decrease in fetal adrenal cortex thickness and decreased fetal plasma cortisol concentrations, whereas CB-118 also increased fetal ACTH. In adrenal cortex tissue, the expression of several genes encoding enzymes and receptors related to steroid hormone synthesis were down-regulated by PCBs. In addition, persistent effects of perinatal exposure to PCBs (CB-153 and CB-126) were indicated by the fact that intrauterine and lactational exposure affected the cortisol response to short-term stress in adulthood (Zimmer et al., 2009). Male goat kids exposed to either PCB congener showed a greater and more prolonged rise in plasma cortisol levels than controls when animals were subjected to mild stress at 9 months of age. However, neither the basal cortisol plasma level nor the adrenal mass were affected by PCB exposure.

2.8.3 Evidence for endocrine disruptor causation of adrenal hormone signalling in wildlife

2.8.3.1 Marine mammals

The most conclusive evidence for a causal link between adrenocortical disorders and exposure to persistent EDCs comes from studies in grey and ringed seals in the Baltic Sea (Bergman & Olsson, 1985). The high body burdens of PCBs and DDT compounds determined in the tissues of Baltic seals were associated with dramatic population declines that seemed to be most severe in the mid 1970s. The finding of a high incidence of occlusions/strictures in the uterine horns suggested that pregnancies were discontinued by early abortion of fetuses and that the subsequent development of strictures prevented new pregnancies. A high incidence of benign smooth muscle uterine tumours (leiomyoma) could also contribute to reproductive failure. In addition to these alterations in the female reproductive tract, a massive hyperplasia of the adrenal cortex was recorded in both sexes. Adrenocortical hyperplasia and a suite of alterations characteristic for Cushing disease were considered a cardinal finding in the disease syndrome (**Figure 2.21**). These alterations included decreased epidermal thickness, intestinal ulcers, and an osteoporosis-like condition (Bergman & Olsson, 1985; Lind et al., 2003). Although these alterations are compatible with adrenocortical hyper-secretion, no information on the plasma cortisol levels in these animals is available, and the exact mechanisms behind the Cushing-like condition remain unclear. Likewise, the individual compounds producing these adrenal lesions remain unknown. Although it cannot be ruled out that a component of stress was playing a role, it seems likely that persistent exposure to organohalogens were involved. Several adrenocorticolytic compounds are

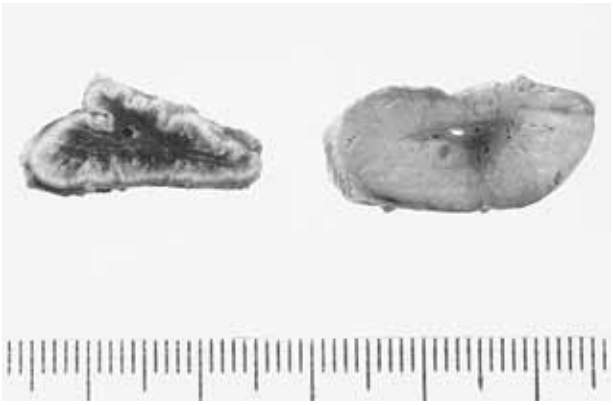

Figure 2.21. Adrenocortical hyperplasia in grey seal (Bergman & Olsson, 1985). Transected adrenals from seals collected at Svalbard (left) and in the Baltic Sea (right). Note massive hyperplasia in the adrenal cortex (light area) of the Baltic seal. Dark area in both adrenals represents the adrenal medulla. (Photo: Anders Bergman).

known to accumulate in the adrenals and other tissues in seals (see Chapter 3.2.1). The temporal trends for reduced exposure to PCBs and DDT, and for recovery of the seals, support the conclusion that these POPs and their persistent metabolites were the causative agents. Along with the dramatic reduction of POPs in Baltic biota that has occurred during the last decades (Chapter 3.2.1.4), the Baltic seals have gradually recovered, supporting a role for classical legacy POPs in the etiology of this syndrome.

It is noteworthy that adrenocortical hyperplasia and Cushing-like alterations have not been reported in seal populations outside the Baltic Sea. Similar symptoms were, however, found in beluga whales. The adrenocortical lesions might have been caused by stress or by adrenocorticolytic xenobiotics, although such lesions could also be part of a normal ageing process (Lair et al., 1997).

In polar bears, which are among the most highly organochlorine-contaminated species of Arctic mammals, there is growing concern that several organochlorines (OCs) may be able to change basic endocrine pathways. Epidemiological data from 151 free ranging polar bears in the Svalbard region of the Arctic showed that alteration in the HPA axis, as indicated by plasma cortisol upon capture, was associated with plasma concentrations of OCs (Oskam et al., 2004). After correction for other factors, the overall contribution of OCs to plasma cortisol was negative, explaining around 25% of the variation in cortisol concentration. The plasma concentrations measured were comparable with plasma cortisol concentrations reported in experimental studies, in which ACTH was used to stimulate the adrenal cortex in order to produce a maximal cortisol response, indicating an activation of the HPA axis as a result of stress. In view of the complexity and lack of knowledge on stress responses in polar bears and their interactions with environmental factors, including OCs, it is difficult to predict the biological implications of such findings. However, these results, and the increasing evidence of EDC exposure effects during perinatal life stages, justify further attention to the effects of environmental contaminants on the HPA axis in polar bears and other highly exposed mammals.

2.8.3.2 Birds and amphibians

Both the p,p'-DDT metabolite, $MeSO_2$-DDE, and o,p′-DDD are known to be activated to toxic metabolites in the adrenal inter-renal cells of embryos and newly hatched chicks, implying that wild bird species should also be sensitive to adrenocorticolytic DDT metabolites (Jönsson el al., 1994). Although no information on such effects in wild birds is available as yet, several reports indicate that stress-induced responses of the HPA axis may be modulated by various environmental contaminants. High concentrations of organochlorines, brominated flame retardants and metabolism-derived products in blood plasma have been associated with high baseline corticosterone concentrations, and a reduced stress response, in arctic birds (Verboven et al., 2010). In a study on free-living, nestling white storks, Baos et al. (2006) reported that stress-induced corticosterone levels were positively correlated with the levels of lead in blood. In another study in mercury-contaminated tree swallow nestlings, stress-induced corticosterone levels were suppressed at the end of the nestling period (Wada et al., 2009). Studying pesticide-induced responses on the HPA axis in tree swallows and Eastern bluebirds, Mayne et al. (2004) observed a significant negative correlation between DDE levels and the capacity of bluebird chicks to elevate blood corticosterone levels in response to ACTH. Disruption of the responsiveness of the HPA axis has also been demonstrated in the common mudpuppy, an amphibian species, exposed to persistent organochlorines in the St Lawrence and Ottawa Rivers in Canada (Gendron et al., 1997). Altogether, these observations imply complex interactions of environmental pollutants with the HPA axis.

2.8.3.3 Fish

As is the case in mammals and birds, *o,p′*-DDD and $MeSO_2$-DDE are activated to toxic metabolites in the cortisol-producing adrenal inter-renal cells in various fish species (e.g. Lacroix & Hontela, 2003; Lindhe et al., 2003). Furthermore, deviation in cortisol levels and cortisol response to stress has been demonstrated in wild fish from polluted environments (Hontela, 1998) as well as in experimentally exposed fish (e.g. Jörgensen et al., 2002). Yellow perch and northern pike collected in PAH, PCB and heavy metal contaminated waters showed a reduced cortisol secretion in response to acute handling stress (Hontela et al., 1992; 1995). Combined with the observation that the ACTH-secreting cells in the pituitary were atrophic, it was proposed that life long exposure to contaminants resulted in exhaustion of the cortisol regulatory system in these fish. Studying the combined effect of PCB exposure and nutritional status in Arctic char, Jörgensen et al. (2002) observed that cortisol levels were elevated in food-deprived control fish compared with control fish given food during the winter period. The basal cortisol levels were decreased by PCBs in food-deprived fish but elevated in fed fish. It was proposed that the stress response in Arctic char is compromised by PCBs and that long term fasting, typical of high-latitude fish, make these fish particularly sensitive to persistent organochlorines.

The Norwegian Lake Mjøsa has been subject to major pollution from industry and agriculture in the past decades. High levels of PBDEs, predominately BDE -47 and -99 (approximately 450 times higher than concentrations measured in fish from un polluted lakes) have been found in fish from Lake Mjøsa together with significant amounts of PCBs, DDT and its metabolites. High concentrations of PBDEs were also reported in a group of high consumers of fish from Lake Mjøsa (Thomsen et al., 2008). Little evidence is available on effects caused by these compounds on wild fish or humans consuming fish from the lake. However, developmental and reproductive effects of lifelong exposure to environmentaly relevant concentrations of two natural mixtures of persistent

organic pollutants found in the lake were investigated using classical and molecular methods in a controlled zebrafish model (Nourizadeh-Lillabadi et al., 2009; Lyche et al., 2010; Berg et al., 2011; Lyche et al., 2011). Phenotypic effects observed in both exposure groups included earlier onset of puberty, increased male/female sex ratio, and differences in body weight at 5 months of age. Interestingly, genome-wide transcription profiling identified functional networks of genes, in which key regulators of weight homeostasis (PPARs, glucocorticoids, CEBPs, estradiol), steroid hormone functions (glucocorticoids, estradiol, NCOA3) and insulin signaling (HNF4A, CEBPs, PPARG) occupied central positions. The exposure effects found during development in zebrafish and the regulation of genes associated with weight homeostasis and insulin signaling suggest that environmental pollution may affect the endocrine regulation of body functions influenced by hormones and that the adrenal axis may play an important role.

2.8.4 Evidence for a common mechanism for adrenal disruption in humans and wildlife

A species comparison of the adrenocorticolytic DDT metabolites DDD and methylsulfonyl-DDE characterized in experimental systems suggests that these compounds should be considered as adrenal EDCs also in humans:

o,p'-DDD and *p,p'*-DDD

o,p´-DDD (Mitotane) and the *o,p´*-DDT as well as the environmentally more prevalent *p,p´*-DDD are formed by dechlorination of the corresponding DDT isomers. *o,p´*-DDD is a registered human pharmaceutical (Lysodren) for treatment of adrenocortical carcinoma (ACC) and Cushing disease in Europe and the USA. At therapeutic doses, which are high compared to environmental exposures, *o,p´*-DDD treatment results in reduced cortisol plasma concentrations and a reduction of adrenal tissue mass. The mechanism of adrenocorticolytic action has been worked out using adrenal tissue homogenate from several species including humans. *o,p´*-DDD is bioactivated to a reactive metabolite in a CYP-catalysed reaction which subsequently generates irreversibly bound protein adducts, mainly in the mitochondrial fraction of adrenal homogenate (Cai et al., 1995; Martz & Straw 1977; 1980). In dogs and mink, as in humans, *o,p'*-DDD induces cell death in the adrenal cortex in vivo and inhibits steroid hormone secretion in vitro (Hart, Reagan & Adamson, 1973; Jönsson, Lund & Brandt, 1993; Nelson & Woodard, 1949). The adrenocorticolytic activity is not restricted to the *o,p*'-DDD isomer but also resides in the environmentally more abundant *p,p´*-DDD isomer. The toxic potency of *p,p´*-DDD is roughly similar to that of *o,p´*-DDD in mink adrenal cortex in vivo and in human H295R cells in vitro (Asp et al., 2010a; Jönsson, Lund & Brandt, 1993).

Methylsulfonyl-DDE

As already mentioned, the second adrenocorticolytic DDT metabolite, $MeSO_2$-DDE, was originally identified in blubber of Baltic grey seal (Jensen & Jansson, 1976), a species suffering from adrenocortical hyperplasia. There is strong evidence to suggest that humans are sensitive to this compound, demonstrated to be a highly potent and completely tissue-specific adrenal toxicant activated by the enzyme CYP11B1 (11ß-hydroxylase) in the *zona fasciculata* of fetal (**Figure 2.22**) and adult mice (Lund, Bergman & Brandt, 1988; Jönsson et al., 1992; Jönsson, 1994; Jönsson, Rodriguez-Martinez & Brandt, 1995). CYP-catalysed irreversible binding of $MeSO_2$-DDE has been confirmed both in human adrenal homogenate (Jönsson & Lund, 1994), and in human adrenal tissue slice culture (Lindhe, Skogseid & Brandt, 2002). Moreover, $MeSO_2$-DDE alters glucocorticoid synthesis in human adrenal tissue slices ex vivo (Lindhe, Skogseid & Brandt, 2002) and in the human adrenal cell line H295R in vitro (Asp et al., 2010b).

2.8.5 Main messages

- The adrenal cortex has been identified as the most commonly affected and vulnerable endocrine organ in toxicology. Experimental data and data from exposed wildlife populations suggest that both the HPA axis and the adrenal glands are targets for endocrine disruption caused by pollutants at environmentally relevant exposure concentrations. Despite this fact, and compared with other

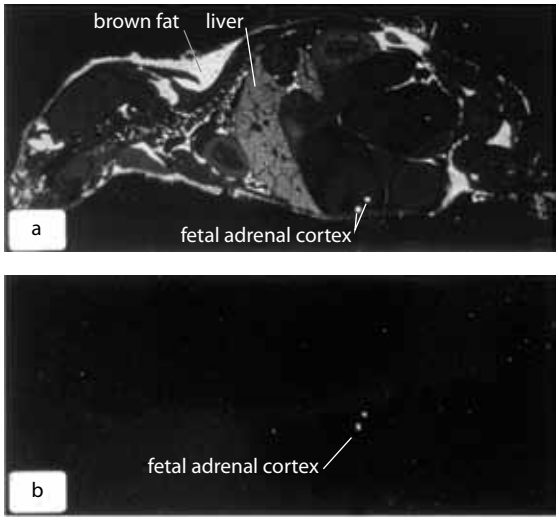

Figure 2.22. Autoradiograms showing the tissue-specific irreversible binding of ^{14}C-labelled $MeSO_2$-DDE in the fetal adrenal cortex of a pregnant mouse (Jönsson et al., 1992). The autoradiogram in the lower panel reveals that an irreversibly bound adduct is confined to the fetal adrenal cortex (maternal adrenals not present), while all other tissues are devoid of bound adducts. The upper panel shows the distribution of both the unmetabolized $MeSO_2$-DDE (high concentrations in fat and liver) and its covalently bound adduct (fetal adrenal cortex). White areas in the autoradiograms correspond to high levels of radioactive compound. (Figure reproduced with publisher's permission)

endocrine axes, the HPA axis has so far gained relatively little attention in endocrine disruptor research.

- The behavioural and physiological traits of an individual are strongly influenced by events occurring during embryonic/fetal life. The HPA axis is one of the major systems implicated in the responses to environmental manipulations and stress. Glucocorticoid hormones represent the final step in the activation of the HPA axis and play an important role in effects induced by the perinatal environment.
- Developing organs are particularly sensitive to alterations in hormone levels, and exposure to chemicals during critical windows of development may cause effects on the adrenal function that persist until adulthood. Recent experimental data suggest that environmentally relevant exposures to pollutants (PCBs) affect development of the fetal adrenal cortex, the function of the HPA axis, and induce delayed effects in the response to stress in animal models.
- A disrupted HPA axis may lead to altered stress responses and changes in cognitive functions. A topic for further research would be to investigate whether selected POPs and their mixtures will affect endocrine cells in the HPA axis following relevant exposures during early life-stages, and whether these changes will result in long-term effects on cognitive functions and stress responses.

2.8.6 Scientific progress since 2002

Adrenal dysfunction was not fully covered in the 2002 *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002).

2.8.7 Strength of evidence

There is sufficient evidence to show that adrenocortical hyperplasia and a suite of pathological changes characteristic of Cushing disease in Baltic seals were caused by exposure to a mixture of DDT, PCBs and their methylsulfonyl metabolites; along with the drastic reduction of DDT and PCBs in Baltic biota, the seal populations have gradually recovered.

There is sufficient evidence to show that adrenocorticolytic DDT metabolites have a similar mechanism/mode of action in a variety of vertebrate species including in human adrenal tissues and cells.

There is some evidence to show that altered responsiveness of the HPA axis is associated with tobacco smoking and alcohol consumption in human subjects, and with exposure to persistent environmental pollutants and toxic metals in wild mammals, birds and fish and that PCBs may disrupt the feedback system regulating cortisol levels in young and adult sheep and goats.

For the great majority of chemicals in modern commerce, there is no evidence to show effects of exposures on adrenal function. Nor have there been any studies to test this hypothesis. There is sufficient evidence to suggest that a variety of chemicals and mixtures alter gene expression and hormone synthesis in the human adrenal H295R cell line in vitro.

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2.9 Endocrine disrupting chemicals and bone disorders

2.9.1 Overview of bone disorders in humans and wildlife and evidence for endocrine disruption

Since World War II, there has been an increase in age-standardized incidence rates of osteoporotic bone fractures in industrialized countries, with the Nordic countries taking the lead (Ismail et al., 2002). The reason for this increase is unknown, but the idea that exposure to EDCs could be involved has been suggested (Lind, 2000) with the hypothesis that EDCs affect bone, as well as reproductive tissues, and that these effects are sex-dependent. If this hypothesis is proven, exposure to EDCs could have contributed to the increase in the observed age-standardized incidence rates of osteoporotic fractures observed in most industrialized countries, mainly in women (Kanis et al., 2012). The literature on this topic is, however, rather small.

2.9.1.1 The role of hormones in bone formation

It is well established that bone is a target tissue for estrogens; both ERα and ERß have been identified in bone cells (Eriksen et al., 1988; Onoe et al., 1997; Migliaccio et al., 1992a) and several studies have revealed that estrogens can influence bone cells in vitro by a direct ER-mediated mechanism (e.g. Migliaccio et al., 1992b; Oursler et al., 1993). Moreover, intrauterine exposure to the endocrine disruptor diethylstilbestrol increased bone mass, but decreased bone length in adult female offspring (Migliaccio et al., 1992b).

In women, a marked decrease in bone mineral density is seen following oophorectomy and bone loss is markedly increased also in the years following menopause, when the circulatory levels of estrogen are reduced (for reviews see Kanis 1996 and Francucci et al., 2010). Hormone replacement therapy with estrogens decreases the rate of further bone loss with age, thus providing corroboration of the relationship between bone mineral density and estrogen (Lindsay et al., 1980). Furthermore, mutations in genes encoding for the estrogen receptors in a young man were reported to result in tall stature, delayed bone maturation and development of severe osteoporosis. The authors concluded, therefore, that estrogen is important for bone maturation and mineralization in men as well as in women (Smith et al., 1994). This observation was subsequently supported by the finding that estrogen deficiency is involved in the pathogenesis of osteoporosis in men (Khosla et al., 1998).

2.9.2 Evidence for endocrine disruptor causation of bone disorders in humans and in rodent models

Epidemiology

At the end of the 1950s, in Turkey, more than 4000 people were accidentally poisoned due to ingestion of hexachlorobenzene (HCB) that had been added to wheat seedlings as a fungicide. The exposed individuals displayed a huge spectrum of symptoms, such as dermatological, neurological and orthopedic defects. In observations performed 20-30 years after the accident, it was revealed that as many as 64.7% of the victims had resorption of digits due to osteoporosis (Cripps et al., 1984).

In another food poisoning accident, several thousands of people in Japan (Yusho) ingested rice-oil contaminated with PCBs and polychlorinated dibenzofurans (PCDFs). In this accident too, the victims exhibited an extensive range of symptoms: children born to exposed mothers were shorter and skeletal lesions, such as irregular calcification of the skull bones and dentition at birth, were also found (Miller, 1985; Yamashita & Hayashi, 1985).

Two rather small cross-sectional population-based studies also demonstrated a negative relationship between the levels of the DDT metabolite, *p,p'*-DDE, and bone density (Beard et al., 2000; Glynn et al., 2000). Furthermore, it has been shown that Swedish fishermen's wives on the east coast who ingested fatty fish from the Baltic Sea had a significantly increased incidence of osteoporotic and hospitalized vertebral fractures when compared to fishermen's wives eating fish from the Swedish west coast (Wallin, Rylander & Hagmar, 2004); the high dietary intake of POPs through fatty fish might be a risk factor for vertebral fractures, because the levels of POPs have been shown to be much higher in fatty fish from the Baltic Sea compared to the Swedish west coast.

Evidence from laboratory studies that EDCs could impair bone structure and function

Experimental studies with laboratory rodents in vivo and with bone tissues in vitro suggest that bone tissue could be an important target for a number of endocrine disrupting persistent organohalogen pollutants, including hexachlorobenzene (Andrews et al., 1989), PCBs (Lind et al., 1999; Lind, 2000; 2004a; Lundberg et al., 2006; Alvarez-Lloret et al., 2009; Gutleb et al., 2010), and high affinity AhR-ligands such as TCDD (Jämsa et al., 2001; Miettinen et al., 2005; Wejheden et al., 2006; Hermsen et al., 2008), since exposure to those compounds impaired both structure and function of bone tissue in all of these studies.

2.9.3 Evidence for endocrine disruptor causation of bone disorders in wildlife

The possibility that EDCs might contribute to bone disorders in humans is supported by a range of studies in wildlife, including those in East Greenland polar bears (Sonne et al., 2004), Baltic grey seals (Lind et al., 2003) (See **Figure 2.23**), American alligators (Lind et al., 2004b), clapper rails (Rodrigues-Navarro et al., 2006), and herring gulls (Fox et al., 2008). In all cases, bone tissue abnormalities were correlated with exposure to mixtures of pollutants.

Figure 2.23. Skull of 14 year old male Baltic grey seal showing loss of bone and several teeth but also exostoses (outgrowth, to the right in the picture) on maxillary bone, found 1990 at the Baltic coast. (Photo: Hans Lind)

2.9.4 Main messages

- Very limited studies, published mostly within the last few decades, indicate that bone tissues of experimental and wild animals are negatively affected by exposure to persistent EDCs.
- Epidemiological studies on humans also support this hypothesis since they show a relationship between exposure to endocrine disrupting POPs and decreased bone mineral density or increased risk of bone fractures.
- Prospective studies on *in utero* or early EDC exposure and future osteoporotic bone fractures are missing and the mechanisms behind the deleterious effects of EDCs on bone tissue also need to be further studied.

2.9.5 Scientific progress since 2002

Bone disorders were not covered in the 2002 *Global Assessment of the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002).

2.9.6 Strength of evidence

There is some evidence to show that accidental poisoning of humans with persistent organic pollutants HCB, PCBs and DDT that are also endocrine disruptors caused bone disorders, and a plausible, although not proven, endocrine mechanism of action through which these disorders may have occurred.

For the great majority of chemicals in modern commerce, there is no evidence to show effects of exposures on bone function. Nor have there been any studies to test this hypothesis.

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2.10 Endocrine disruptors and metabolic disorders

2.10.1 Overview of metabolic disorders in humans and wildlife and evidence for endocrine disruption

The role of hormones and of endocrine disruptors in metabolic disorders such as obesity and diabetes has been a topic of recent research. There are multiple reasons for this:

- Metabolic disorders are continuing to rise in human populations, including in children and adolescents. Established risk factors alone cannot account for these disease trends.
- Some endocrine disrupting chemicals can affect the function of the insulin producing beta cells in the pancreas (e.g. Cooper et al., 2009) and are also immunotoxic.
- There are now animal data suggesting that exposure to some endocrine disrupting chemicals during pregnancy can lead to altered cholesterol metabolism, weight gain and type 2 diabetes in the offspring later in life.

This section of our review considers the evidence for the involvement of endocrine disrupting chemicals in causing metabolic disorders.

2.10.2 Evidence for endocrine disruptor causation of metabolic disorders in humans and in rodent and primate models

2.10.2.1 Obesity

Secular trends

The prevalence of obesity has risen dramatically in many parts of the world over the past two decades, as in the OECD countries (Sassi et al., 2009; OECD 2010) graphically presented in **Figure 2.24**. In the United States, for example, some reports cite 30% of adults as clinically obese and 65% as overweight (Ogden et al., 2007; Cunningham, 2010). Perhaps more important is that the rate of obesity and its related diseases, such as diabetes, are rising dramatically in children: estimates from the USA suggest that more than 60% of children 10 years of age and older either are currently obese or will become obese later in life (Oken & Gillman, 2003).

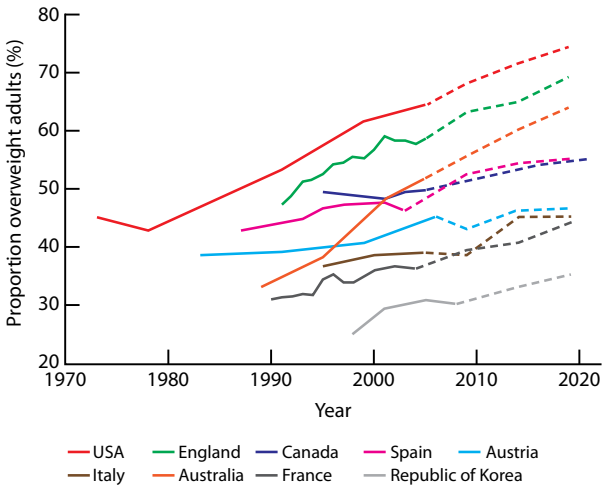

Figure 2.24. Past (solid lines) and projected (dashed lines) overweight rates in selected OECD countries (OECD, 2010). Used with publisher's permission.

There is evidence that the obesity risk may begin early in life, during pregnancy, and in early childhood and that rapid weight gain, in the first few months of life, is associated with obesity later in life (Ong et al., 2000; McAllister et al., 2009)

Endocrine role

Obesity is a complex endocrine-related disease caused by the interaction between genetic, behavioural, and environmental factors (Stanley et al., 2005). The control of weight gain is governed by many components of the endocrine system, including the adipose tissue, brain, skeletal muscle, liver, pancreas and gastrointestinal (GI) tract. Thus, there are multiple endocrine and paracrine factors that need to integrate to control this multi-functioning system and to regulate adipose development, number and function of fat cells, food intake, satiety, pleasure-related (hedonic) reward mechanisms, insulin sensitivity, lipid metabolism and, ultimately, body weight (Newbold et al., 2008; Grun & Blumberg, 2009). Estrogens, androgens, glucocorticoids, and thyroid hormones play important roles in controlling adipose tissue development, metabolism, and satiety.

Evidence for endocrine disruption

Because obesity is an endocrine-related disease/dysfunction, it is potentially sensitive to endocrine disrupting chemicals (Ropero et al., 2008; Sargis et al., 2010). In addition to the well-established modern societal influences of over-nutrition and lack of exercise, it has been hypothesized that exposures to chemicals are also contributing to the rapid rise in cases of obesity (Newbold et al., 2008; Newbold, 2010; Keith et al., 2006). Indeed, there are now data in animal studies indicating that chemical exposures during vulnerable windows of development may affect adult weight (Newbold, Padilla-Banks & Jefferson, 2006; Baillie-Hamilton, 2002). For instance, there are animal data suggesting that developmental exposure to chemicals including tributyltin, bisphenol A, organochlorine and organophosphate pesticides, air pollution, lead, diethylstilbestrol, perfluorooctanoic acid, monosodium glutamate and nicotine can lead to altered cholesterol metabolism and weight gain later in life (Newbold, Jefferson & Padilla Banks, 2007; Newbold et al., 2008; Grun et al., 2006; La Merrill & Birnbaum, 2011; Heindel & vom Saal, 2008; Li, Ycaza & Blumberg, 2011; Slotkin, 2011; Dirinck et al., 2011; Janesick & Blumberg, 2011; **Table 2.6**). Chapter 3.2.2) provides an overview of exposure to some of these chemicals. There are, however, limited data in humans (Tang-Peronard et al., 2011) supporting the notion that exposure to

such chemicals during development can affect weight gain in infants and in children (La Merrill & Birnbaum, 2011; Verhulst et al., 2009; Heindel, 2011). These epidemiology studies suffer from limited numbers of subjects, lack of long term follow up and examination of only single chemicals and single time points. The strongest epidemiology data show that smoking during pregnancy leads to weight gain later in life in the offspring. Indeed, a meta-analysis of over 15 studies showed a consistent effect of this factor (Oken, Levitan & Gillman, 2008).

Chemicals with endocrine disrupting properties may potentially act either on specific or multiple sites to:

- Alter endocrine pathways responsible for control of adipose tissue development
- Increase the number of fat cells
- Alter food intake and metabolism via effects on sexually dimorphic and appetite and reward centers in the brain
- Alter insulin sensitivity and lipid metabolism via effects on endocrine (and endocrine-related) tissues such as the pancreas, adipose tissue, liver, GI tract, brain and muscle

The net result of these changes is an alteration or deregulation of the "endocrine set point" or changes in homeostatic sensitivity that predisposes individuals to obesity later in life. Chemicals that have been shown to cause increased weight gain in animal models have been termed obesogens (**Table 2.7**). Chemicals that cause both weight gain and alter lipid metabolism and glucose sensitivity have also been called

Table 2.6. List of known and suspected environmental obesogens (A=Animal study, C=Cell culture study, H=Human study). Janesick & Blumberg (2011) provide more detailed information about obesogens.

Chemical	Commercial use	Relevant EDC action	Obesogenic activity
Tributyltin	Pesticide, wood preservation	Binds PPARγ	Changes identity of adipose precursors, increases triglycerides in adipose tissue (A)
Phthalates	Plasticizer	Binds PPARγ	Induce adipocyte differentiation (C), men's waist size (H)
PFOA	Non-stick coatings	Weakly activates PPARγ	Induce adipocyte differentiation (C))
Flavanone	Natural plant products used as flavourings	Binds PPARγ	Induce adipocyte differentiation (C))
PCBs	Electronics	Binds AhR in adipocytes	CB-77 promotes adipocyte differentiation, obesity (C,A)
Bisphenol A	Plastics	Binds ER, ERRγ	Induces adipogenesis (C), obesity (A)
Hexachlorobenzene	Fungicide	Alters TH signaling	Gestational exposure levels influence BMI (H)
Bisphenol A diglycid ether	Epoxy resins	Unknown	Induces adipogenesis (C)
PBDEs	Fire retardants	Reduces thyroid function	Stimulate fat production (C)
Diethylstilbestrol	Pharmaceutical estrogen	Binds ER	Perinatal exposures cause obesity (A). BMI in young children (H)
Genistein	Natural component in soy	Binds ER	Perinatal exposures cause obesity (A).
Perfluoroalkyl sulfonate	Non-stick coatings	Binds ER	Perinatal exposures cause obesity, alter insulin & leptin levels (A).
Nicotine	Found in tobacco products		Alters development of pancreas & adipose tissue, increases adipose cell size (A)
DDE	DDT metabolite	Binds ER	Concentrations in mothers associated with weight and BMI in female offspring (H)

The mechanisms by which most of these chemicals affect weight gain are largely unclear. Tributyltin, one of the few chemicals studied in detail, activates the combined peroxisome proliferator -activated receptor gamma (PPAR-γ)/ retinoid-X-receptor (RXR) pathway, the main pathway for fat cell differentiation (Janesick & Blumberg, 2011) and thereby stimulates fat cell differentiation in vitro and increases adipose tissue in vivo in mice. Similarly, chemicals with estrogenic activity like DES, genistein and BPA appear to act via estrogen receptors on fat cells, and cells of the brain and other tissues to regulate adipose tissue and food intake (Janesick & Blumberg, 2011).

Table 2.7. The obesogen hypothesis

During development: Environmental chemicals with endocrine activity
• Alter programming of components of the homeostatic or • Hedonic, reward, pathway and or • Number of fat cells and/or • Energy expenditure and/or • Inflammation/inflammatory responses and/or • Emotional and/or stress responses
Throughout life: Continued stress on an already abnormal metabolic system
• Increased access to high fat and sugar foods leading to "food addiction" • Lifestyle with reduced activity • Continued exposure to "obesogenic" chemicals leading to more fat cells, altered homeostatic and reward pathways

metabolic disruptors but also fit under the term obesogens. (Casals-Casas & Desvergne, 2011).

2.10.2.2 Type 2 diabetes

Type 2 diabetes (formerly called non-insulin-dependent or adult-onset) occurs when the body cannot effectively use the insulin produced by the pancreas. Insulin is a hormone that regulates blood sugar. Hyperglycemia, or raised blood sugar, is a common consequence of uncontrolled diabetes which can cause serious damage to many of the body's systems, especially the nerves and the blood vessels. Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity.

Secular trends

Diabetes (including type 2 diabetes) poses a serious burden on public health systems around the world and is recognized as a global epidemic (Wild et al., 2004). Similar to obesity, estimates of the global prevalence of diabetes suggest it has more than doubled over the past three decades from 153 (127-182) million people in 1980 to 347 (314-382) million in 2008 (Danaei et al., 2011). This recent study of diabetes prevalence for adults aged 25 years and older in 199 countries and territories revealed that the worldwide age-standardized adult diabetes prevalence had risen to 9.8% (8.6-11.2) in men and 9.2% (8.0-10.5) in women, up from 8.3% (6.5-10.4) and 7.5% (5.8-9.6) in 1980. As important as the global rise are the trends in different countries, ranging from nearly flat in some regions to the steepest rise in age-standardized prevalence of 5.9 percentage points for men and 7.8 percentage points for women of Oceania (Marshall Islands, Samoa, Kiribati), to reach 21-25% of adult men and 21-32% of women in this region. Large increases were also recorded in southern and tropical Latin America, south and central Asia, North Africa, and the Middle East (Saudi Arabia). No change in diabetes prevalence was recorded in central and eastern Europe during these 28 years, although ageing and population growth led to an increase in the number of people with diabetes in these regions. Of the high-income countries studied, diabetes prevalence in 2008 was highest in the USA, Greenland, Malta, New Zealand, and Spain and lowest in The Netherlands and Austria. Over the preceding three decades, diabetes increased the least and the most in Western Europe and North America, respectively. Notwithstanding these comprehensive statistics, global estimates of diabetes are still far from complete even today; no population-based data were identified for 92 countries of the world, especially from some low-income and middle-income countries.

Seventy percent of risk associated with type 2 diabetes is attributed to excess weight gain, suggesting a direct metabolic link between increased adiposity and type 2 diabetes. For example, American statistics suggest that obesity is the leading cause of this disease (CDC, 2008). Of particular concern is the fact that the incidence of type 2 diabetes is increasing in children and adolescents at similar rates to the increases in obesity; data from North America show that one in four overweight children have impaired glucose tolerance. Type 2 diabetes is more common in girls than boys and girls are less insulin sensitive as early as 5 years of age (Pleis, Lucas & Ward, 2009). Notwithstanding the relationship between diabetes and obesity, global and regional trends in diabetes differ from those of other metabolic risks such as high blood pressure, which decreased globally and total cholesterol which decreased in Australasia, Europe, and North America, but rose in east and southeast Asia and Asia-Pacific, leading to relatively unchanged global means (discussed in Danaei et al., 2011). Because high BMI is a risk factor for all three metabolic indicators, the differences in diabetes trends are probably due to other factors; chief amongst these are diet and medical treatment.

Endocrine role

Maintenance of blood glucose homeostasis involves a series of complex gene-environment interactions between different tissues, including the liver, skeletal muscle, adipose tissue, brain and the pancreas. Altered glucose homeostasis leads to type 2 diabetes, which consists of defects in both insulin secretion and insulin action (insulin resistance) (Leng, Karlsson & Zierath, 2004), both of which are controlled by the endocrine system. Since the beta cells of the pancreas are central to controlling glucose homeostasis, an endocrine disrupting chemical that can initiate, facilitate and/or accelerate the loss of beta cell function can play an important role in type 2 diabetes. As already mentioned, obesity is the leading comorbidity factor in type 2 diabetes. Other contributing factors include increased tissue inflammation, reduced adiponectin secretion (a protein hormone produced exclusively by the fat cells that regulates metabolism of lipids and glucose), impaired inhibition of the breakdown of fat by the hormone insulin and altered liver function (Hevener & Febbraio, 2010). Type 2 diabetes is also closely related to thyroid function (see section 2.5) due to common signalling pathways and genetic susceptibility.

Evidence for endocrine disruption

There are limited data showing that, like obesity, sensitivity to develop type 2 diabetes is programmed during development and that both altered nutrition and exposures to environmental chemicals may be important in its etiology. Indeed numerous endocrine disrupting chemicals (EDCs) have been implicated in the development of type 2 diabetes, in both animal and epidemiological studies, including bisphenol A, phthalates, flame retardants, arsenic, POPs, and pesticides (reviewed by Alonso-Magdalena, Quesada & Nadal, 2011)

In humans, there is growing epidemiological evidence that adult exposures to EDCs may contribute to the development of type 2 diabetes: studies report an increased risk of type 2 diabetes after exposure to POPs (including PCBs, DDE, dioxin, organochlorine pesticides, hexachlorobenzene), arsenic and some flame retardants (e.g. Neel & Sargis, 2011; Everett, Frithsen & Player, 2011; Reilly et al., 2011; IPCS, 2011). For example, a 2006 study found a relationship between six POPs and diabetes in USA adults: the risk of type 2 diabetes was 37.7 times higher in people with the highest exposure than in

people with the lowest levels of exposure. The POPs included the dioxins HpCDD and OCDD, DDE, CB-153, oxychlordane, and trans-nonachlor, with the latter three showing the most significant relationships (Lee et al., 2006). Moreover, a nested case control study and a prospective study in adults both showed that exposure to a variety of POPs (PCBs and organochlorine pesticides) was associated with type 2 diabetes (Lee et al., 2011). A mechanism that links exposure to POPs with type 2 diabetes is unclear currently, but many of the POPs have endocrine disrupting activity, including effects on thyroid (see Chapter 3.1.1, 3.2.2.2 & 3.2.2.3 for an overview of human exposure to some of these chemicals).

Of the chemicals in modern commerce, Lang et al. (2008) reported that higher urinary concentrations of bisphenol A were associated with diabetes and altered liver function in USA adults in 2003- 2004 using the National Health and Nutrition Examination Survey (NHANES) dataset. In addition, some epidemiological studies have linked arsenic exposure to diabetes, especially in areas with high arsenic levels in drinking water, although whether lower exposure levels are also diabetogenic is unclear (Huang et al., 2011; Saldana et al., 2007). Arsenic exposure has been associated with impaired glucose tolerance in women with relatively low levels of arsenic exposure during pregnancy (Ettinger et al., 2009; see Chapter 3.1.1.8 & 3.1.5 for information on arsenic exposure).

Since gestational diabetes (a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy) increases the risk of later diabetes in the mother and the risk of later glucose abnormalities in the child, the role of endocrine disrupting environmental chemicals in gestational diabetes should be further investigated. The lack of longitudinal data, or developmental exposure data, on less persistent chemicals in modern commerce make it difficult to form conclusions from the existing data, without developmental exposure information.

In animal studies, exposures to environmental chemicals during critical developmental periods, such as in utero, have been linked to the later development of glucose intolerance and insulin resistance. For example, one study showed that in utero exposure to bisphenol A caused increased insulin resistance and secretion and glucose intolerance in adult male mice. Bisphenol A exposure to the pregnant mothers also decreased glucose tolerance and increased insulin resistance to the mothers during pregnancy, and increased insulin resistance 4 months post-partum (Alonso-Magdalena et al., 2010). Developmental exposure to arsenic and organophosphorous pesticides have also been linked to later diabetogenic effects in the offspring. Low level exposure to arsenic, from prenatal development until adulthood, leads to beta cell damage, impaired glucose tolerance, hyperglycaemia, increased insulin resistance, and altered insulin secretion in rats (Davila-Esqueda et al., 2011). Moreover, early post-natal exposure to organophosphorous pesticides produces lasting effects on metabolism that are consistent with pre-diabetes. Some effects intensified between adolescence and adulthood, while others waned (Adigun et al., 2010). Moreover, the pre-diabetic effects of exposure to these pesticides during critical windows of development have been shown to be exacerbated by a high-fat diet in adulthood (Slotkin, 2011). Taken together, these effects raise the possibility that exposure to chemicals during pregnancy may contribute not only to type 2 diabetes in the offspring, but also gestational diabetes and later type 2 diabetes in the mothers. The effect on insulin secretion has been specifically shown to involve estrogen receptors and a role for estrogen receptors in all of the effects of endocrine disruptors on the pancreas and adipose tissue is suspected (Toschke et al., 2003; Salsberry & Reagan, 2007; Karmaus, et al., 2009; Richter et al., 2007; Lim et al., 2009).

In adult animals, some chemicals can cause diabetes or insulin resistance following adult exposures. One analysis followed primates who were fed the pesticide DDT for 130 months and analysed at the age of 18-24 years. Two of the 24 exposed monkeys developed diabetes, and two developed hypoglycemia, compared to none in the control group. The exposed monkeys also developed fatty changes in the liver, central nervous system abnormalities, and tumours more often than controls (Takayama et al., 1999). In rodents also, pesticides and air pollutants have been shown to contribute to diabetes-related effects following adult exposures. For example, long term, low dose exposure to the current-use herbicide, atrazine (see Chapter 3.1.1.6) caused insulin resistance in adult rats, an effect worsened by a high-fat diet (Lim et al., 2009). Similarly, adult male rats exposed to fine particulate matter (PM2.5) in conjunction with a high fat diet developed insulin resistance (Yan et al., 2011). Adult male rats exposed to the POPs present in crude farmed Atlantic salmon oil developed insulin resistance and hepatosteatosis. In another study, the same POPs, especially the organochlorine pesticides, were shown to inhibit insulin action in cultured adipocytes (Ruzzin et al., 2010).

Some metals also have diabetogenic effects in adult rodents. For example, exposures to cadmium have high blood glucose levels and impaired glucose tolerance (Edwards & Prozialeck, 2009). It can also impair insulin secretion by beta cells as well as reduce glucose uptake by peripheral tissues, processes that likely contribute to diabetogenicity. Arsenic can also lead to higher blood glucose levels, higher insulin levels, and impaired glucose tolerance, depending on the dose, form of arsenic, and duration of exposure (Huang et al., 2011).

2.10.2.3 Type 1 diabetes

Secular trends

Type 1 diabetes (T1DM, IDDM, or formerly, juvenile diabetes) is a form of diabetes that results from selective autoimmune destruction of insulin-producing beta cells of the pancreas (for a full discussion of autoimmune diseases, see section 2.11). The consequent lack of insulin leads to increased blood and urine glucose. At clinical diagnosis there are usually only 10-20% remaining active beta cells. This clinical presentation is preceded by an asymptomatic period of high variability from months to many years or decades. Type 1 diabetes is the most common autoimmune-related disease in children. In recent decades, there has been a progressive decrease in the age of onset of type 1

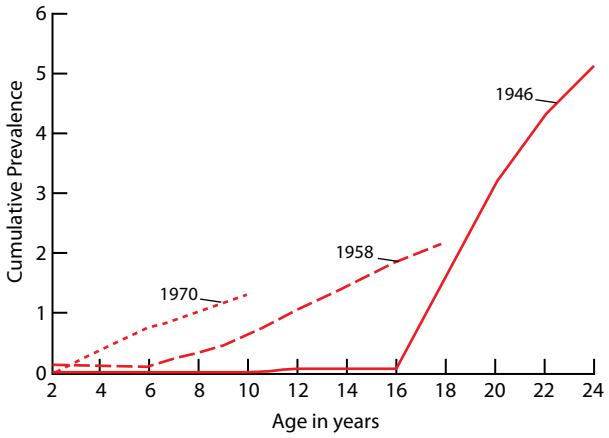

Figure 2.25. A progressive leftward shift in age of onset of childhood type 1 diabetes has been and continues to be observed in the United Kingdom. This trend in a progressively earlier onset of diabetes is consistent with data from other countries. In 1946 no diabetes was shown in children until 16 years of age; in 1970, at 2 years of age. Source: Diabetes, 2002 American Diabetes Association, Inc. www.medscape.com (Used with publisher's permission).

diabetes; the incidence has increased among children under the age of 15 yrs, with the biggest increase in children under 5 yrs old (Ziegler et al., 2011; **Figure 2.25**).

Evidence for endocrine disruption

Preliminary epidemiology studies support a role for exposure to chemicals in type 1 diabetes. While limited, there are epidemiological data linking adult exposures to nitrate/nitrite/nitroso compounds, air pollutants ozone and sulfate, and PCBs with increased incidence of type 1 diabetes (Howard & Lee, 2012)

It seems likely that type 1 diabetes, like obesity and type 2 diabetes, has its origins during development; in utero and/or the first few years of life. While there are no compelling animal data linking exposure to endocrine disruptors to the development of type 1 diabetes, there are chemicals noted above for type 2 diabetes that can affect beta cell function, including bisphenol A, PCBs, dioxin, arsenic, and phthalate plasticizers (see Chapter 3.1 and 3.2.2). It is interesting that many of these same chemicals (e.g. the estrogens bisphenol A, DES, chlordecone as well as dioxin, phthalates, and trichloroethylene) are also immunotoxic in animal models (e.g. Cooper et al., 2009 and see section 2.11). Thus a hypothesis that needs testing is that chemicals with both immunotoxic/autoimmune activity and also endocrine disrupting activity could act via both mechanisms to cause type 1 diabetes. The endocrine disrupting effects could alter the development of beta cells, and the immunotoxic component could lead to the production of auto-antibodies, resulting in the destruction of the altered and susceptible beta cells leading to type 1 diabetes.

2.10.2.4 Metabolic syndrome

Metabolic syndrome is also associated with the rise in obesity and may contribute to the progression of type 2 diabetes (Abrams & Levitt Katz 2011). Metabolic syndrome is defined clinically as a combination of at least three of the following five dysfunctions: hypertension, abdominal (central) adiposity, increased serum triglycerides and low serum high density lipoproteins (HDL) and high blood sugar, even after fasting (Steinbeck 2004).

There are significant data supporting the notion that disruptions of developmental programming cause metabolic syndrome and that there is a role for maternal diet in its aetiology (Ng et al., 2010). While there are currently no data linking developmental programming and environmental chemical exposure to metabolic syndrome *per se*, there are studies that have shown effects of chemical exposure on the progression of obesity and type-2 diabetes, noted above (Newbold, 2010; La Merrill & Birnbaum 2011) and a possible role of exposures to endocrine disrupting chemicals as a causative factor of metabolic syndrome, obesity and diabetes has recently been reviewed (Tang-Peronard et al., 2011). Thus, there is clearly a need for experimental evidence that elucidates the effects of environmental exposures on metabolic syndrome *per se* and not just diabetes and obesity. Specifically, studies are needed to determine the impact of chemical exposures on lipid profiles, blood pressure, altered glucose tolerance, insulin resistance and liver function in addition to weight gain.

2.10.3 Metabolic disorders in other vertebrate wildlife species

Recent efforts have been made to explore how endocrine disrupting chemicals may alter endocrine signalling and lipid homeostasis in other vertebrates, such as fish and amphbians. Tributyltin is a good example of a chemical that causes weight gain across species: It has been shown to be a potent inducer of adipogenesis in vitro and in vivo in developing toads and mice, by acting as a novel, high-affinity xenobiotic ligand for retinoic X receptor alpha (RXRα) and peroxisome proliferator-activator receptor gamma (PPARγ) (Janesick & Blumberg, 2011; Grun et al., 2006) that interacts, at least partially, with the same receptor-binding sites as other high-affinity ligands for these receptors and promotes the necessary cofactor interactions required for agonist activation. TBT binds to RXRs and activates signaling in gastropod snails, amphibians, and mammals. In all species exceopt the snails (in which masculinization occurs), TBT promotes long-term changes in adipocyte number or lipid homeostasis following developmental or chronic lifetime exposure. The fact that wildlife species are sensitive to an obesogen like TBT has led to the development of the "zebrafish obesogenic test" as a tool for screening compounds that target adiposity (Tingaud-Sequeira, Ouadah & Babin, 2011). This short-term assay, in which adipocyte droplets are visualised by fluorescence, has been used to demonstrate that zebrafish larvae treated at an environmentally relevant concentration of TBT exhibit a marked increase in adiposity. Similarly, research into the effects of long-term, dietary exposure to TBT on juvenile Chinook salmon revealed an increase in whole-body lipid content, although the analysis of associated physiological parameters revealed some inconsistencies with

metabolic syndrome (Meador et al., 2011). Probably the most environmentally-relevant studies to date, from the aquatic ecotoxicology literature, are those of Lyche et al. (2010; 2011), in which the effects of lifelong exposure to natural mixtures of POPs were analysed in zebrafish. The exposure mixtures were extracted from the livers of wild fish and contained PBDEs, PCBs and DDTs. The phenotypic effects reported included increased weight gain, advanced puberty and skewed sex ratios. Changes in the expression of genes associated with weight, homeostasis, steroid hormone functions and insulin signalling were also reported. These increased weight and gene expression changes reported suggest that environmental pollutants may affect the endocrine regulation of metabolism, possibly leading to increased weight gain and obesity in wild fish. These data are particularly pertinent given that the concentrations of POPs measured in these fish were comparable with the levels found in other species in the wild (see Chapter 3.2.1 for a review of exposure of wildlife to these chemicals). Thus, although there are still no data demonstrating that EDCs are associated with metabolic disorders in wildlife, the results of laboratory-based studies on both mammalian and non-mammalian vertebrates have revealed remarkable consistencies with the patterns reported in the human literature.

Finally, it is important to note that the discovery of "obesogens" and "metabolic disruptors" greatly expands the list of chemicals classed as EDCs, but also expands the list of receptors and hormone systems impacted by these chemicals.

2.10.4 Main messages

- Obesity, diabetes and metabolic syndrome are due to disruption of the energy storage-energy balance endocrine system and thus are potentially sensitive to endocrine disrupting chemicals.
- Obesity, diabetes and metabolic syndrome have their origins during development and are influenced by the environment during development and throughout life.
- Exposures of animal models to a variety of chemicals have been shown to result in weight gain. Because they are disrupting many components of the endocrine system involved in controlling weight gain (adipose tissue, brain, skeletal muscle, liver, pancreas and gastrointestinal (GI) tract), these chemicals constitute a new class of endocrine disruptors called "obesogens".
- Some studies report that developmental exposures of rodents to a variety of chemicals with estrogenic activity result in increased weight gain, while in adults, estrogenic activity is protective against weight gain, highlighting the importance of timing in assessment of the effects of endocrine disrupting chemicals on these diseases.
- Human epidemiological studies have shown an association between in utero exposures to several POPs, and subsequent increased weight gain in the first few years of life and that smoking during pregnancy is consistently and strongly associated with increased weight gain in infants.
- Obesity is also correlated with type 2 diabetes and chemicals that have been shown to cause obesity, in animal models, also result in altered glucose tolerance and reduced insulin resistance.
- There are strong and consistent epidemiological data linking adult exposure to dioxin and other POPs to type 2 diabetes in both cross sectional and prospective studies.
- In animal models, there are some preliminary studies showing a relationship between developmental exposures to some chemicals and glucose intolerance and insulin insensitivity later in life.
- The findings that show that developmental exposures to some environmental chemicals can lead to obesity and/or type 2 diabetes expands the likely list of chemicals and the list of hormone receptor systems, as yet not tested, that are responsive to endocrine disruptors.

2.10.5 Scientific progress since 2002

- Obesity and type 1 and type 2 diabetes have increased greatly across the globe, especially in developed countries and in children.
- Animal studies have shown that obesity, diabetes and metabolic syndrome have their origins during development and can be influenced by environmental chemical exposures, amongst other factors, during development and throughout life.
- There are human epidemiological data linking adult exposure to POPs and diabetes and data showing developmental exposures to a variety of chemicals can lead to weight gain in children.
- A new class of endocrine disrupting chemicals that cause obesity, called "obesogens", has arisen.

2.10.6 Strength of evidence

There is sufficient evidence to show that over the last three decades, the global prevalence of obesity, diabetes and metabolic syndrome have increased based on comprehensive global surveys. There is also sufficient evidence that these diseases have an endocrine origin and thus are susceptible to disruption by environmental chemicals with endocrine function. The hypothesis that environmental chemical exposures are playing a significant role in the etiology of obesity is really less than a decade old and there is currently a lack of human literature for many of the chemicals shown to cause effects in animal studies. In addition, many of the animal studies are descriptive and have not shown specific endocrine mechanisms of action. The "obesogen" hypothesis, while now encompassing close to 20 environmental chemicals and classes, is still considered

an emerging hypothesis. The data on tributyltin and bisphenol A and obesity are sufficient in animal studies but there are no human studies. The data linking environmental chemical exposures to diabetes or metabolic syndrome are insufficent, due to lack of both animal and human data. The strongest data are for POPs and type 2 diabetes, as the human data are consistent, but there is a lack of mechanistic insight from animal studies. Thus the potential for the "obesogen" hypothesis as a mechanistic explanation for various metabolic diseases is great. However, the field needs more data in both animal and human studies, including stronger linkages to endocrine mechanisms of action.

2.10.7 References

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2.11 Endocrine disruptors and immune function, immune diseases, and disorders in humans and wildlife

2.11.1 Overview of immune diseases in humans and wildlife and evidence for endocrine disruption

Alterations in the immune system such as immune modulation, hypersensitivity, and autoimmunity can lead to a decreased quality of life. Laboratory studies with animals show that many chemicals introduced into the environment have the potential to disturb the immune system of wildlife and humans. The consequences of such interference on the developing immune system are not, however, well understood. Nonetheless, there are certain situations where human and wildlife populations are experiencing immune alterations that are consistent with those produced by synthetic chemicals identified as immunotoxic in studies with laboratory animals:

- **Human allergic diseases constitute the most common causes of chronic illness** in developed countries and incidences are rising in developing countries.
- **Human autoimmune diseases are also rising**. To date, more than 80 systemic and organ-specific autoimmune diseases have been defined, and their cumulative burden is substantial, both medically and financially. In developed countries around the world, 5 to 7% of the population is affected and rates are rising, making these diseases a ubiquitous global phenomenon that is predicted to further increase in the coming decades (an expert panel reported at the "Global State of Autoimmunity" briefing for international health ministers hosted by the United Nations' NGO Health Committee and the American Autoimmune Related Disease Association (AAARD) on 25 September 2010).
- **Rising prevalence rates of autoimmune disease in humans are most evident in type 1 diabetes.** Data from Finland, tracked by that country's national health system, show type 1 diabetes rates more than doubled from 31 cases per 100 000 people in 1980 to 64 cases per 100 000 in 2005 (Harjutsalo, Sjoberg &Tuomilehto, 2008) Increases were also detected in 17 other European countries, at an average annual rise of 3.9% from 1989 to 2003 (Patterson et al., 2009). The authors of that study predicted the number of new cases in children younger than 5 years in Europe will double by 2020 compared with 2005, while the number of cases among those under age 15 will rise by 70%.
- **High rates of Chytridomycosis and Rana virus in amphibian populations have raised concern about the sustainability of affected populations**, especially those that are threatened or endangered.
- **Fibropapillomas have become an important emerging disease of sea turtles** since the early 1980s.
- **Marine mammals worldwide have been affected by emerging disease during recent years.** At least 20 species of cetaceans (whales, dolphins and porpoises) and 15 species of pinnepids (seals, sea lions and walruses) have been affected by more than 30 different emerging and re-emerging disease conditions.
- **Disseminated neoplasia (DN) emerged during the 1980s and was first described in New England (USA) in soft-shelled clams.** DN has been compared to vertebrate leukemia as a disease process.

The causes of these disease trends are unknown. It is clear that autoimmunity is a multifactorial process in which genetics, immunological, environmental and hormonal factors act in concert, together representing what was termed years ago the 'mosaic of autoimmunity'. Genetics plays a key role in susceptibility to autoimmune disease (among identical twins, for example, if one has an autoimmune disease, there is a 30 % chance the other twin will also develop one, though not always the same disease). Whilst genetics may "load the gun", the environment appears to pull the trigger, explaining up to 70% of the risk of some diseases (Ramos & Olden, 2008). For example, lupus (an autoimmune disease commonly affecting the joints, skin, kidneys, blood cells, heart, and lungs) is four times more likely to strike people of African descent who live in London than those who live in sub-Saharan Africa. Globally, a similar trend applies to other autoimmune diseases, since autoimmune disease remains uncommon in developing countries, while increasing in developed nations. Human evidence for other environment-disease links is more tenuous, however, in part because of inherent limitations in environmental epidemiology. By contrast, effects of chemicals on immune function in free-ranging marine mammals have been inferred from a number of studies (Beckmen et al., 2003; Lahvis et al., 1995; Beineke et al., 2005), the most comprehensive of which are studies on the immunotoxic effects of contaminants in harbour seals (*Phoca vitulina*) (De Swart et al., 1994; 1995a; 1995b; 1996a; 1996b; Ross et al., 1995; 1996).

Many immune disorders are deeply rooted in the endocrine system and, therefore, inappropriate activation or inactivation of select endocrine pathways may aberrantly disturb the balance of the immune response. This is due to the fact that the immune and the endocrine systems are intricately connected, ensuring that the body can simultaneously handle infections, stress, the immune response, and hormonal signalling. Cytokines, small cell signalling molecules, traditionally thought to have an immunomodulatory role, may also be crucial regulators of autocrine-paracrine effects of hormones and may send and receive signals (Khardori, Adamski & Khardori, 2007). EDCs have been linked with disorders of metabolism, energy balance, thyroid function and reproduction, as well as an increased risk of endocrine cancers (Walker & Gore, 2011).

This chapter discusses the current data linking EDC exposure to immune disorders and stresses the need for further research to deepen our understanding of the effect of EDCs on the immune system.

2.11.2 Evidence for endocrine disruptor causation of endocrine-immune diseases and disorders in humans and in rodent models

2.11.2.1 Interaction between the immune and endocrine systems

The interaction between the immune and endocrine systems has been well documented, as have the immunomodulatory effects of various hormones (Elenkov & Chrousos, 2002), particularly glucocorticoids secreted by the adrenal cortex (Oberbeck, 2004; see section 2.8). Other compounds and neurotransmitters known to affect immune function include the adrenal androgen dehydroepiandrosterone (DHEA; section 2.8), the catecholamines adrenalin, noradrenalin, and dopamine, and the pituitary hormone prolactin (Oberbeck, 2004). Catecholamines, prolactin, and DHEA levels become elevated following major surgery and during systemic inflammation (Oberbeck, 2004), and prolactin may both stimulate the immune response and reduce the release of glucocorticoids following a stress (Davis, 1998). Growth hormones (GH), insulin-like growth factor-I (IGF-I), glucocorticoids, and thyroid hormones also have established interactions with the immune system; stress, nutrition, and environment may mediate their function (Davis, 1998). Growth hormone has been implicated as being involved in leukemia and lymphoma (Hooghe, et al., 1998), and it may stimulate proliferation of beta cell-derived tumours (Baglia, Cruz & Shaw, 1992). Glucocorticoids are immunomodulatory (specifically, immunosuppressive), and are frequently prescribed following organ transplantation, for serious allergic reactions or for autoimmune incidents. Together with other select chemotherapies, glucocorticoids affect both the innate (immediate) and the adaptive (long-lasting) arms of the immune response (Flammer & Rogatsky, 2011). Thyroid-stimulating hormone (TSH) is produced by a variety of immune cells, including B and T lymphocytes, splenic dendritic cells, hematopoietic cells in the bone marrow, and other lymphocytes (Klein, 2006).

The hypothalamic-pituitary-adrenal (HPA) axis is known to regulate a variety of immune functions (Marx et al., 1998; see also section 2.8). Activation of this axis generates a stress response, which consequently affects the immune response. Interactions between the HPA axis and the immune system may result from a cytokine-regulated feedback mechanism. The adrenal gland (the major effector organ of the HPA axis) also produces a variety of cytokines, implicating the adrenals as crucial players in the immuno-endocrine intersection (Marx et al., 1998). Cytokines may also be produced by activated immune cells, and these cytokines can subsequently direct hormone secretion from the HPA axis or the hypothalamic-pituitary-gonadal (HPG) axis. In the other direction, hormones (both adrenal and sex steroids) may direct the production of cytokines by immune cells (Marx et al., 1998). Macrophages in the ovary can also produce cytokines that affect the follicle and corpus luteum and, as a result, may affect ovarian steroidogenesis. In turn, these ovarian steroids may also affect the cytokines produced by the macrophages. Estrogen receptors have been detected in immune cells such as dendritic cells, macrophages, and B cells (Nalbandian & Kovats, 2005), suggesting that estrogens (or xenoestrogens) may act on these cells (Chryssikopoulos, 1997). In general, testosterone is viewed as largely anti-inflammatory, whereas estrogen is thought to be more pro-inflammatory (Klein, 2004; McClelland & Smith, 2011). Testosterone also suppresses the immune system and increases susceptibility to infection (Schuurs & Verheul, 1990). These data are in accord with the fact that autoimmune and inflammatory disorders are found in a disproportionately higher incidence in females versus males (Whitacre, 2001). Finally, mucosal immunity (which protects an organism's various mucous membranes from invasion) and immunosenescence are thought to be under the control of steroid hormones via their effects on cytokine production (Daynes et al., 1995). Together, these data support a role for steroid and sex hormones in immunity and it is likely that disruption of normal steroid signalling by exogenous chemicals may affect immune function.

2.11.2.2 Mechanisms via which EDCs might influence the immune system

Nuclear hormone receptors and inflammation

Although nuclear receptors are best known for their direct effects of activating transcription of target genes by binding to hormone response elements in the DNA, nuclear receptors are also well known for their signaling crosstalk with the immune system, particularly through NF-κB and AP-1 (De Bosscher, Vanden Berghe & Haegeman, 2003; Valledor & Ricote, 2004; Pascual & Glass, 2006). Nuclear Factor Kappa B (NF-κB) is a family of transcription factors that controls the transcription of DNA and is activated in response to stress, bacterial or viral antigens or an inflammatory stimulus. Upon activation of either the T- or B-cell receptor, NF-κB family members become activated through distinct signalling components and play a key role in regulating inflammation and the immune response to infection (Baeuerle & Baltimore, 1996). In a resting cell, NF-κB proteins are in an inactive state in the cytoplasm, but upon an inflammatory signal, the proteins are released from inhibition and migrate to the nucleus to activate transcription of a variety of target genes (Baeuerle & Baltimore, 1996) that control cell proliferation and cell survival to protect the cell from conditions that would otherwise cause it to die via apoptosis. As such, many different types of human tumours have misregulated NF-κB.

NF-κB is known to crosstalk with a variety of nuclear receptors, among them, the glucocorticoid receptor (GR) that mediates the actions of glucocorticoids, a subset of steroid hormones produced by the adrenal cortex after the HPA axis is activated by inflammation (Elenkov & Chrousos, 2006; Sternberg, 2006; Bowers et al., 2008). Glucocorticoids may inhibit NF-κB through a variety of mechanisms and this process is likely cell type-specific (Nissen & Yamamoto, 2000; Liberman et al., 2007; Bowers et al., 2008) and mediated by the glucocorticoid receptor (Heck et al., 1994; Liden et al., 1997; Tao, Williams-Skipp & Scheinman, 2001). Estrogen receptor (ER) function has also been linked with constitutively active NF-κB, and this may have profound consequences for aggressive hormone-resistant cancers (De Bosscher, Vanden Berghe & Haegeman, 2006). It has also been shown that binding of NF-κB to target genes can be blocked by estrogens in vitro (Stein & Yang, 1995) and that activation of the progesterone receptor (PR) can reduce NF-κB-driven gene expression (Kalkhoven et al., 1996). Reciprocal negative cross-talk has also been documented between NF-κB and the androgen receptor (AR) (Palvimo et al., 1996). All of these pathways offer possible mechanisms for EDCs to impact immune function through receptor-mediated crosstalk with NF-κB. Additionally, "classical" ARs can activate the Map Kinase (MAPK) pathway through the epidermal growth factor (EGF) receptor (Cheng Watkins & Walker, 2007), and androgens influence T cell development and cytokine development (Bebo et al., 1999). This identifies a role for AR in inflammation and provides an additional immune signalling pathway through which EDCs may act.

Several groups have demonstrated that activation of the Steroid and Xenobiotic Receptor (SXR; also known as PXR and NR1I2) by commonly used pharmaceuticals inhibits the activity of NF-κB (Gu et al., 2006; Zhou et al., 2006). NF-κB target genes are up regulated in cells and tissues lacking SXR and inhibition of NF-κB function boosts SXR activity. Activation of NF-κB inhibits SXR activity while activation of SXR inhibits NF-κB activity (Gu et al., 2006; Zhou et al., 2006). The SXR/NF-κB axis provides a potential molecular explanation for suppression of cytochrome P450 genes by inflammation and infection and clarifies the immunosuppressant role of pharmaceuticals that activate SXR. Consequently, many xenobiotics or EDCs that activate or inactivate SXR could be responsible for regulating inflammation through NF-κB.

Lastly, it has been shown that peroxisome proliferator-activated receptor gamma (PPARγ) mediates repression of NF-κB (Delerive et al., 1999) and that PPARα activation suppresses inflammation (Yu, et al., 1995; Devchand et al., 1996; Lehmann et al., 1997). Since EDCs such as phthalate monoester plasticizers are known to activate both rodent and human PPARα and PPARγ (Hurst & Waxman, 2003), biologically plausible that environmental chemicals acting through PPARα may have strong negative impacts on human immune function. Additionally, other receptors such as the Liver X Receptors may cross-talk with NF-κB (Joseph et al., 2003; Wu et al., 2009). Liver X Receptors (LXRs) are known regulators of lipid-inducible gene expression that negatively regulate inflammatory gene expression in macrophages (cells known to have important roles in lipid metabolism and in inflammation). Stimulation of macrophages with lipopolysaccharide demonstrated that NF-κB target genes such as interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) were inhibited by LXR ligands in vitro. LXR agonists reduced inflammation in both the skin and aorta of in vivo models, securing a role for LXR as a key mediator of lipid metabolism and inflammation (Joseph et al., 2003). It was recently shown that EDCs such as bisphenol A affect the expression of LXR (Marmugi et al., 2011) and that LXR affects estrogen metabolism through expression of estrogen sulfotransferase, a transcriptional target of LXR (Gong et al., 2007). Therefore, it is plausible that EDCs, particularly xenestrogens, may exert at least a fraction of their effects via LXR. Further research is needed to fully understand the role of LXR in endocrine diseases that could be partially or wholly caused by exposure to endocrine disrupting chemicals.

2.11.2.3 Endocrine-immune diseases and disorders in humans

Allergies

Allergies are becoming increasingly common, particularly in younger individuals. Data suggest that the incidence of childhood allergy may be connected to environmental aspects such as stress, but a growing amount of work indicates that disturbances of the fashion in which the HPA axis responds to stress may be to blame. It is possible that an over reactive or under reactive HPA axis during the initiation and continuation of childhood allergy may contribute to the allergic response (Buske-Kirschbaum, 2009). Since select nuclear receptors (such as ER) interact with CRH-binding protein (CRH-BP), which can activate the HPA axis by binding and inhibiting CRH, it is plausible that EDCs could ultimately lead to an under reactive or over reactive HPA axis (van de Stolpe et al., 2004).

Endometriosis

Endometriosis is a common gynaecological disorder that is characterized by the presence of endometrial cells or tissue outside of the uterine cavity, usually in the ovaries. It is commonly thought that retrograde menstruation is to blame for endometriosis (Sampson's theory) (Seli & Arici, 2003; Jensen & Coddington, 2010), but because the rate of endometriosis is far lower than the incidence of retrograde menstruation (over 75% of women), it is likely that other factors contribute to susceptibility. The disease is linked with changes in both cell-mediated and humoral immunity; dysfunctional natural killer cells may not properly remove refluxed menstruation debris, and this may lead to endometriotic implants (Seli & Arici, 2003). Additionally, larger numbers of immune cells are found in the peritoneal cavity fluid of women with the disease, but these cells appear to be positively correlated with disease progression. Cytokines and growth factors secreted by

macrophages (which usually help in clearing up tissue debris) may foster the growth of endometrial cells and it is possible that they contribute to endometriosis-associated infertility and pelvic pain (Seli & Arici, 2003). Danazol (a derivative of the synthetic steroid ethisterone, a modified testosterone) and gonadotrophin-releasing hormone (GnRH) activators are often used in clinics for treatment of endometriosis; they dampen the cellular and humoral immune responses (in addition to their effects on endometrial cells outside the uterine cavity) (Seli & Arici, 2003). It is likely that the immunosuppressant activity of these compounds contributes greatly to their therapeutic value, implicating the immune system in the disease. It has been suggested (McLachlan, Simpson & Martin, 2006) that endometriosis may be at least partially attributed to exposure to EDCs (also discussed in section 2.2).

Autoimmune thyroid disease

Autoimmune thyroid diseases (AITD) are the most frequently occurring organ-specific autoimmune disease; approximately 5% of the population is thought to be affected (Klecha et al., 2008). Autoimmune thyroid disease is caused by abnormal interactions between atypical thyrocytes (cells of the thyroid gland), aberrant antigen-presenting immune cells, and abnormal T lymphocytes, with the end phenotype being an immune reaction against "self" that attacks antigens in the thyroid (Klecha et al., 2008). It is possible that environmental or hormonal influences may affect the etiology of this disease, perhaps by changing the normal immune-endocrine interaction. Alterations of the healthy immune-endocrine axis may tip the delicate balance between the two types of immune response, which ultimately leads to either Hashimoto thyroiditis (hypothyroidism and destruction of thyrocytes) or Graves disease (an immune response against the TSH receptor, leading to hyperthyroidism) (Klecha et al., 2008). Stress, environment, immune function, and thyroid hormones may all be affected by EDCs (see also section 2.5) and, consequently, influence the development of autoimmune thyroid disease (Diamanti-Kandarakis et al., 2009; Zoeller, 2010).

Bone disorders

The immune system plays an important role in osteoporosis, which often arises from estrogen deficiency and secondary hyperparathyroidism (excessive production of parathyroid hormone (PTH) by the parathyroid glands situated at the back of the thyroid gland). Current research suggests that the remodelling of bone is a very tightly controlled process that is easily perturbed by small fluctuations in pro-inflammatory and inhibitory cytokines, NF-κB, together with hormones and their corresponding receptors (Clowes, Riggs & Khosla, 2005; see also section 2.9). An imbalance in this interplay, due to infection or inflammation, could tip the bone creation/bone destruction scale in favour of bone loss, which subsequently increases the risk of fracture. Furthermore, age-related fluctuations in the immune and endocrine systems add to the risk for decreased bone density. It is possible that exposure to EDCs may influence the development of osteopenia and osteoporosis.

2.11.2.4 Evidence that exposure to EDCs causes immune system disorders in humans

EDCs and endometriosis

There are data that correlate phthalate plasticizer (Chapter 3.1.1.3) levels in plasma and endometriosis. Cobellis et al. found high plasma concentrations of di-(2-ethylhexyl)-phthalate in women with endometriosis (Cobellis et al., 2003) and the concentration of phthalate esters was also linked with endometriosis in a study of Indian women (Reddy et al., 2006). Human exposure to phthalates is extensive and is reviewed in Chapter 3.2.2 PCBs (Chapter 3.1.1.1) CB-138, CB-153, and CB-180 (Gerhard & Runnebaum, 1992), as well as dioxins (Koninckx et al., 1994), have also been loosely associated with the disease. Mayani et al. (1997) reported that dioxin exposure (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) was positively correlated with endometriosis, although it is not possible to conclude from these data that TCDD was the sole cause of the disease. Data collected from accidental dioxin exposure after an industrial accident in Seveso, Italy, revealed an increased risk of endometriosis (Eskenazi et al., 2000), but this study was limited by difficulties in disease classification and statistical power. Dioxin-like compounds have been found in elevated levels in the blood of women with endometriosis (Heilier et al., 2005), as have PCBs (Porpora et al., 2006) in a study of women who had not had children (thereby controlling for fluctuation of chemicals during breastfeeding; see Chapter 3.2.2.6 for exposure through mothers' milk). Although evidence is mounting that correlates EDCs found in the bloodstream of women with endometriosis, data that suggest a mechanism, or that establish causal links, are scant. Dioxins activate the aryl hydrocarbon receptor (AhR) (Yoshioka, Peterson & Tohyama, 2011) and dioxin bound to AhR interacts with estradiol, leading to cell growth in the mouse uterus (Ohtake et al., 2003). More recently, it was shown that AhR mediates non-genomic actions of AhR-ligands and promotes the degradation of ERα and AR (Ohtake et al., 2011). Taken together, these findings may provide insight for future epidemiological studies, and indicate that additional work on EDC exposure and endometriosis is needed.

Asthma

A variety of EDCs, including estrogenic compounds, are known developmental immunotoxicants (Fenaux, Gogal & Ahmed, 2004; Guo et al., 2006). The developing and neonatal immune response is easily affected by EDCs, and disruption during critical windows of development may have detrimental long-term consequences. Developmental immunotoxicity (DIT) caused by EDC exposure may be one early-life immune insult that could cause lifelong effects on immunity and the overall health of exposed individuals. Evidence from studies with mice suggests that exposure to certain EDCs, such as diethylstilbestrol (Fenaux, Gogal & Ahmed, 2004) and genistein (Guo et al., 2006), may lead to postnatal immune disorders such as asthma. Epidemiological studies further suggest that exposure to

chemicals may be involved in the etiology of childhood asthma or cancers such as childhood leukemia (Dietert, 2009). The incidence of childhood allergic disease (including asthma) has increased since the mid-1900s, and this increase is thought to be linked with changes in the environment. Predisposition toward allergic disease is one of many adverse outcomes that could be caused by developmental immunotoxicity. Environmental estrogens and other EDCs have been identified as contributors to developmental immunotoxicity-related immune disorders (Dietert & Zelikoff, 2008).

Phthalates and asthma

Phthalates are used in soft polyvinylchloride (PVC) material and are present in many household materials and personal care products. The most common phthalate indoors is DEHP, which can be found in cosmetics, toys, construction material, and cleaning solutions (Schettler, 2006). An epidemiological study examining the association between PVC products in the home and the incidence of airway symptoms determined that the presence of PVC materials increased the risk for bronchial obstruction in young children (Oie et al., 1999; Chalubinski & Kowalski, 2006). Additional studies from Sweden, Russia and Finland supported this finding and showed that exposure to PVC flooring and/or PVC wall covering material was correlated with airway symptoms in children (Jaakkola, Verkasalo & Jaakkola, 2000; Bornehag et al., 2005). Moreover, two case-control prevalence studies from Sweden and Bulgaria describe an association between the concentration of DEHP in indoor dust and asthma and wheezing in children (Bornehag et al., 2004; Kolarik et al., 2008). Taken together, these studies are in accord with published animal studies (Bornehag & Nanberg, 2010).

Incubating peripheral blood mononuclear cells from allergic individuals with mono-n-butyl phthalate (MnBP) elevated the production of interleukin-4 (IL-4; Glue et al., 2002). Moreover, mono-2-ethylhexyl phthalate (MEHP) was shown to potentiate the allergic reaction to ovalbumin (OVA; a commonly-used allergen) exposure in a mouse inhalation model (Hansen et al., 2007). Increased levels of OVA-specific immunoglobulins in the serum, as well as an increase in eosinophilic (acid loving) white blood cells, were observed. When cells harvested from the draining lymph nodes were cultured, cytokine expression was indicative of a activation of a helper cell (Th2) immune pathway (Hansen et al., 2007); the helper cells activate and direct other immune cells.

Triclosan and asthma

Triclosan, an antimicrobial agent found in many household soaps, toothpastes, and disinfectants, was investigated for links to allergies and hay fever in the United States. Triclosan levels in children under 18 years of age were tightly correlated with development of these diseases in data taken from the 2003-2006 USA NHANES database (Clayton et al., 2011). These findings suggest that further work on triclosan and its correlation with immune and respiratory diseases is needed (for more information on exposure pathways for triclosan see Chapter 3.1.1.5).

Autoimmune thyroid disease

Many have hypothesized that diet during early life may influence immune disorders that develop later in life (Fort et al., 1990). Since thyroid dysfunction is one of the most common autoimmune diseases early in life, it is possible that EDCs play an important role in the development of these diseases (see also section 2.5). For example, soy-based milk formulas given to children are linked with a higher incidence of autoimmune thyroid disease as compared to their breast-fed siblings (Fort et al., 1990). On the other hand, environmental chemicals may also be linked with autoimmune thyroid disease. While iodine is the most well-known environmental factor associated with this disease, limited epidemiological data also show that methylcholanthrene, furan, polybrominated biphenyls, PCBs (Guarneri & Benvenga, 2007), and polyaromatic hydrocarbons (Burek and Talor, 2009) may be associated with autoimmune thyroid disease. PCBs were associated with thyroid disease in data collected from a heavily polluted area in Slovakia; of particular epidemiological note in this study was that autoimmune antibodies were found in the offspring of exposed individuals (Langer et al., 2008) even though their levels of organochlorines were far lower than those of their parents, suggesting that the effect of EDCs on autoimmune thyroiditis may be multigenerational. It is unclear whether the effect is due to direct PCB transfer in utero or in breast milk, or whether this effect is due to an epigenetic phenomenon (for a review of human exposure to POPs see Chapter 3.2.2 and for a description of epigenetics see Chapter 1.3.6).

EDCs, dendritic cells, and cytokines

Dendritic cells are antigen-presenting cells that act as messengers between the innate and adaptive immune systems. They are present in tissues in contact with the external environment, such as the skin and the inner lining of the nose, lungs, stomach and intestines. Dendritic cells can also be found in an immature state in the blood. Once activated, they migrate to the lymph nodes where they interact with T cells and B cells to initiate and shape the adaptive immune response. At certain development stages they grow branched projections, the dendrites that give the cell its name.

Cultured human circulating dendritic cells (mDCs) treated with two common EDCs, nonylphenol (NP) or 4-octylphenol (4-OP), demonstrated changes in anti-inflammatory cytokine production that was partially reversible by an estrogen receptor antagonist. Overall, the findings suggested that NP and 4-OP affected the immune response of dendritic cells through the estrogen receptor (ER) (Hung et al., 2010). Some EDCs also boost STAT3 mediated signaling through ER (Sekine et al., 2004). STAT3 plays a key role in many cellular processes such as cell growth and apoptosis and is mostly activated by interleukin-6 (IL-6), also a target for NF-kB (Kishimoto, Taga & Akira, 1994). The strong STAT3 activation by EDCs through ER can be reversed by the anti-estrogen tamoxifen (Sekine et al., 2004). EDCs can also activate MAP kinase (MAPK) signalling, which suggests that EDCs can interfere with

normal endocrine and immune homeostasis by influencing the cytokine signalling pathways as well as through direct binding to estrogen receptors (Sekine et al., 2004).

Organotins and immune dysfunction

Triphenyltin (TPT) is a well-known EDC, but little is known about its effects in the immune system. TPT increased superoxide production by approximately 45% and increased expression of crucial neutrophil proteins that endow the cell with its capacity to migrate towards and eliminate microbial pathogens, by about 90% (Watanabe et al., 2003). Other EDCs tested (e.g. parathion, vinclozolin, and bisphenol A) did not have these effects. Microarray data also supported the conclusion that TPT elevated the expression of crucial neutrophil proteins, thus enhancing the neutrophilic maturation of leukocytes. Dibutyltin (DBT) has been found to inhibit binding of ligands to the glucocorticoid receptor (GR) and, subsequently, its activation (Gumy et al., 2008). DBT also inhibited the action of the enzyme 11-β hydroxysteroid dehydrogenase, thereby increasing circulating glucocorticoid levels (Odermatt & Gumy, 2008). DBT inhibited glucocorticoid-mediated inhibition of inflammatory cytokines in stimulated macrophages, suggesting that DBT could have potent effects on immune processes (Gumy et al., 2008). TBT and TPT are also strong inhibitors of human natural killer (NK) cell function as measured by tumour cell lyses (Whalen, Loganathan & Kannan, 1999; Whalen, Hariharan & Loganathan, 2000; Wilson et al., 2004; Gomez, et al., 2007). In summary, these findings imply that organotins may play a key role in immune disruption, can inappropriately stimulate cytokine signaling, and might reduce the ability of natural killer cells to effectively eliminate their targets. Furthermore, exposure to these compounds could be associated with an increased risk of cancer, as the immune system may not be able to lyse tumour cells.

Childhood lymphomas and leukemias

It has been suggested that occupational exposure to hydrocarbons in parents is associated with an increased risk of childhood leukemia (Fabia & Thuy, 1974; Shu et al., 1999). A single study from the Children's Cancer Group, that examined self-reported occupational exposure to a variety of hydrocarbons, revealed that exposure to solvents and paints or thinners during a preconception window and during pregnancy and exposure to plastics within the postnatal window were correlated with an elevated childhood risk of acute lymphocytic leukemia (Shu et al., 1999). Additionally, exposure to plastics pre-conception was also linked to acute lymphocytic leukemia (Shu et al., 1999).

Nuclear hormone receptors and lymphomas in rodent models

The steroid and xenobiotic receptor (SXR) is a broad-specificity nuclear hormone receptor that is highly expressed in the liver and intestine, where its primary function is to regulate drug and xenobiotic metabolism (Zhou, Verma & Blumberg, 2009). Mice lacking SXR demonstrate aberrantly high NF-κB activity and over expression of NF-κB target genes and ultimately develop B cell lymphoma in an age-dependent manner. SXR knockout mice develop lymphocytes with cell surface and molecular characteristics of either chronic lymphocytic leukemia or non-Hodgkin lymphoma (Casey et al., 2011), providing a link between metabolism of xenobiotic compounds and the initiation of lymphoma (Casey et al., 2011). It is plausible that chemical antagonists of SXR would be associated with the development of lymphoma, and inhibition of the xenobiotic response may be a key step in the development of certain lymphoid tumours (Casey et al., 2011).

In addition to SXR, rodent studies also show that loss-of-function in several other nuclear receptors has been linked with proliferation of lymphoid cells. Loss of the nuclear receptor LXR resulted in enhanced proliferation of T cells, connecting sterol metabolism to the acquired immune response (Bensinger et al., 2008). B cell lymphomas also developed in aged steroid receptor coactivator-3 null mice (Coste et al., 2006) and retinoid-related orphan receptor ROR gamma knockout animals displayed lymphocyte accumulations in the spleen (Zhang, Guo & He et al., 2003) and T cell lymphoma (Ueda et al., 2002). Loss-of-function in the nuclear receptors Nr4a3 and Nr4a1 also leads to acute myeloid leukemia (Mullican et al., 2007). These data support a role for nuclear hormone receptors in the regulation of lymphocyte proliferation and in lymphomas and leukemias.

Links between PCBs, POPs and hematopoietic disorders and malignancies

Xenobiotic chemicals and environmental pollutants, particularly PCBs, have long been associated with increased risk of non-Hodgkin lymphoma (Bertrand et al., 2010 ; Maifredi et al. 2011; Rothman et al., 1997; De Roos et al., 2005; Engel et al., 2007; Spinelli et al., 2007). PCBs were commonly produced in North America for over half a century, but now banned worldwide. PCBs remain persistent environmental contaminants (see Chapter 3.2 for a review of exposure to PCBs in humans and wildlife). Chang et al. (1981) found that children exposed to PCBs in utero displayed decreased immunoglobulin levels, and had fewer total T cells. Moreover, a study of Flemish 17 and 18 year olds indicated that elevated serum PCBs correlated with decreases in immune cells such as eosinophils and natural killer cells, decreases in serum immunoglobulins IgE and IgG, and an increase in serum immunoglobulin IgA (Van Den Heuvel et al., 2002).

Persistent organic pollutants (POPs), particularly organochlorine pesticides, have been associated with other immune-based disorders. For example, they were positively correlated with periodontal disease, which is likely due to immunomodulation. Moreover, neutrophil counts in peridontal disease patients were negatively correlated with organochlorine levels (Lee, Jacobs & Kocher, 2008).

Pesticides and childhood leukemias

Several studies have connected pesticide exposure and the development of leukemia in children (Birnbaum & Fenton, 2003). Unfortunately, many of these studies do not have

detailed exposure information, only studied small numbers, and are limited by a recall bias of the parents (Belson, Kingsley & Holmes, 2007; Infante-Rivard & Weichenthal, 2007). In one study, the California Department of Health Services found that in utero exposure to metham sodium (odds ratio of 2.05; 95% confidence interval=1.01-4.17) and dicofol (odds ratio of 1.83; CI=1.05-3.22) were associated with a higher incidence of early-childhood leukemia; dicofol has been found to have estrogenic activity (Guillette et al., 1994; Okubo et al., 2004) and may function as an EDC. More work is needed to uncover the role of endocrine disrupting pesticides and childhood leukemias and lymphomas. In summary, it is feasible that selected pollutants may have strong effects on immune alteration, immune disorders, and hematopoietic cancers.

EDCs and prostate inflammation in rodent models

Gilleran et al. (2003) found that exposure to estrogens in the embryonic rat ultimately leads to disrupted development of the prostate gland. In the resulting adult offspring, the prostate gland developed epithelial dysplasia and chronic inflammation comprised of T lymphocytes and macrophages, a phenotype seen in chronic prostatitis (Gilleran et al., 2003). Furthermore, males that received estradiol after birth displayed larger spleens and thymuses, and smaller prostates and testes, with prostate inflammation and immune infiltration that was both prolactin dependent and independent. Since benign prostatic hyperplasia (BPH) often precedes prostate cancer in older male humans, these findings are of particular interest for the study of the relationship between exposure to xenoestrogens and the ultimate development of prostate cancer.

Bisphenol A and inflammation

Bisphenol A is used in food packaging and is also found in dental sealants, a variety of plastics, resins, and cosmetics (Chapter 3.1.1.5). In comparison to phthalates, there is less literature demonstrating the associations between human bisphenol A exposure and development of asthma or allergy, but available studies suggest that bisphenol A exposure is pro-inflammatory. Ex vivo exposure to bisphenol A resulted in elevated interleukin-4 (IL-4) in lymph node helper (Th2) cells in mice (Tian, Takamoto & Sugane, 2003). Prenatal (Yan, Takamoto & Sugane, 2008) and early postnatal (Sawai, Anderson, & Walser-Kuntz, 2003) exposure of mice generated a greater Th2 response (Kwak et al., 2009). Perinatal bisphenol A exposure enhanced allergic sensitization and bronchial inflammation and responsiveness in vivo (Midoro-Horiuti et al., 2010). Moreover, human monocyte-derived dendritic cells exposed to bisphenol A in vitro produce cytokine signals that favour development of the Th2-dominated responses in allergic reactions (Guo et al., 2010). Lastly, BPA enhanced the production of immunoglobulin M (IgM) from certain types of lymphocytes (which often produce autoantibodies) (Yurino et al., 2004), further implicating bisphenol A in inflammatory diseases. Taken together, these data indicate that exposure to bisphenol A could be a factor in the development of inflammatory and autoimmune diseases.

Atrazine and immune function

Atrazine, as well as its main metabolite, desethylatrazine, are the most prevalent groundwater contaminants in agricultural, undeveloped, and urban-use areas in the United States (Barbash et al. 2001; see Chapter 3.1.1.6). When human peripheral blood mononuclear cells from healthy donors were treated with atrazine in vitro, production of interferon gamma (a type 1 cytokine), interleukin-5 (a type 2 cytokine) and TNFα (an inflammatory cytokine) were reduced to a level comparable to the positive control - the known immunosuppressant dexamethasone (Hooghe Devos & Hooghe-Peters, 2000). When purified natural killer cells were exposed to 10 μM atrazine, a significant decrease in lytic function was observed, suggesting that atrazine has potent immunomodulatory effects on both T cells and natural killer cells (Whalen et al., 2003). It is thought that atrazine exposure blocks the ability of natural killer cells to lyse their target cells by inhibiting lytic granule release without affecting the ability of these cells to form interactions with target cells (Rowe, Brundage & Barnett, 2007).

In a rodent model, exposure during the prenatal period and during lactation resulted in a sex- and age-dependant depression of immune function in the adult offspring (Rowe, Brundage & Barnett, 2007). Atrazine treatment was also shown to reduce the number of naïve T helper and T cytotoxic cells in the spleen of treated animals, while it increased the percentage of activated cytotoxic T cells, and inhibited dendritic cell maturation in the spleen (Filipov et al., 2005). In a separate study using a Balb/c mouse model, prenatal/lactational exposure to atrazine altered adult immune function and led to an increase in both T cell proliferation and cytolytic activity in male offspring, and the humoral immune response was also significantly increased (Rowe et al., 2006). Another study showed that atrazine directly targets maturation of dendritic cells and that EDCs (such as atrazine) that remove MHC-I molecules on the surface of dendritic cells are likely to have a potent role in immune evasion (Pinchuk, Lee & Filipor, 2007). Together, these data indicate that atrazine has convincing effects on the immune system, and further investigation is warranted.

Arsenic

Millions of people worldwide are exposed to arsenic, a toxicant that is associated with increased risk for a variety of cancers and cardiovascular disease. Arsenic treatment of an in vitro mast cell model inhibited antigen-stimulated degranulation at environmentally-relevant, non-toxic concentrations, suggesting that arsenic may reduce overactive immune responses, and, additionally, may inhibit normal immune responses against immune insults such as parasitic disease (Hutchinson et al., 2011). Furthermore, these data imply that different EDCs work in different ways on mast cell degranulation and may have unique responses on the immune response in asthma, and in allergies.

2.11.3 Evidence for endocrine disruptor causation of endocrine-immune diseases and disorders in wildlife

The potential for widespread effects of endocrine disrupting chemicals on the immune systems of wildlife is significant, but less understood than their potential for disruption of reproductive health and development. As EDCs are typically found as complex mixtures in a changing environment, wherein additive, antagonistic or synergistic interactions may be expected, the science of immunotoxicology must overcome a number of challenges including:

- chronic exposures to EDCs in diet and/or environment.
- exposures to complex mixtures where determining causation associated with any given contaminant or class of contaminants is problematic.
- constantly changing profiles of contaminants as new chemicals emerge and legacy chemicals decline.
- confounding factors such as age, sex, and condition that can affect EDC concentration as well as the health of the individual.
- the interacting effects of multiple stressors in addition to EDCs, such as climate change, habitat alteration, invasive species, and/or eutrophication.
- legal, technical and ethical constraints to working with wildlife, notably those listed under endangered species legislation.

Dealing with these challenges will require new concepts, tools, and approaches.

The evidence for immunotoxic effects resulting from EDC exposure in wildlife is primarily limited to vertebrates, in which mechanistic support has been substantiated through a variety of in vitro and in vivo studies and laboratory animal studies (e.g. Luebke et al., 1997). Here lies perhaps the major impediment to advancing the concept of wildlife immuntoxicology and of ecotoxicology in general: while field and semi-field studies provide the best evidence for a 'real world' context, they generally fail to provide mechanistic evidence for the reasons listed above. A combination of research strategies entailing both field- and laboratory-based research has generally provided the most robust 'weight of evidence' for wildlife (**Figure 2.26**).

2.11.3.1 Marine mammals

Effects of EDCs on immune function in free-ranging marine mammals have been inferred from a number of studies, the most comprehensive of which were on the immunotoxic effects of contaminants in captive harbour seals (De Swart et al., 1994; 1995a; 1995b; 1996a; 1996b; Ross et al., 1995; 1996), in which a range of immunological parameters were measured in animals fed fish from the contaminated Baltic Sea over the course of 2.5 years. Differences in adaptive and innate immunity (such as natural killer cell activity and delayed-type hypersensitivity responses) were observed between experimental and control groups, with these differences attributed primarily to the PCBs in their diet.

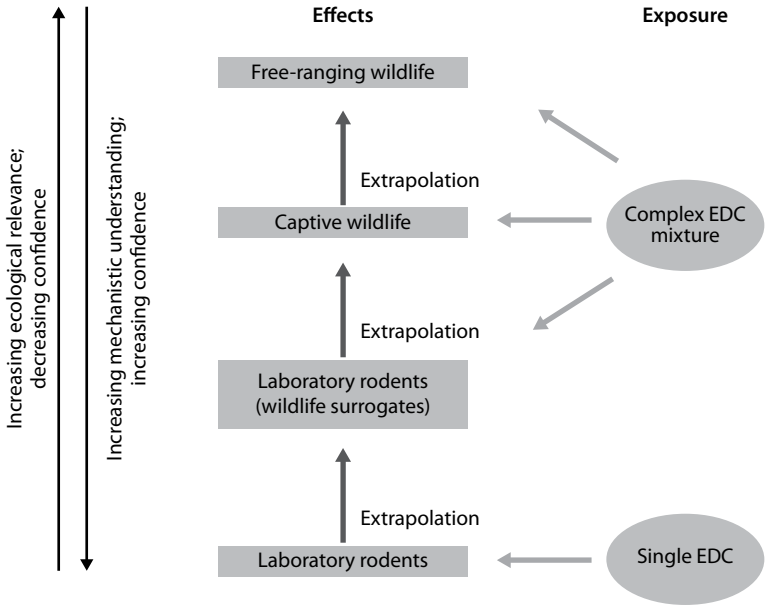

Figure 2.26. Since uncertainties exist for toxicological studies in free-ranging wildlife, assessment of EDC effects on the immune and other systems are best served by a "weight of evidence" from a combination of approaches. A wealth of toxicological information has been generated for many EDCs using laboratory rodent studies, but similar approaches are neither practical nor ethically acceptable in wildlife. In this manner, ecologically relevant information can be generated (i.e. free-ranging wildlife) that has a mechanistic basis in toxicology (i.e. laboratory rodent studies), and uncertainties can be minimized by appropriate and critical extrapolations among each of these lines of research (Modified from Ross, 2000).

Other studies also indicate associations between tissue concentrations of contaminants in marine mammals and changes in immune blood parameters, suspected of indicating immunotoxicity (Beckmen et al., 2003). Reduced mitogen-induced T-cell proliferation associated with elevated PCB and DDT blood levels was observed in free-ranging bottlenose dolphins off the coast of Florida, suggestive of a contaminant-induced inhibition of the cellular immune response (Lahvis et al., 1995). However, age may also have contributed to these results. Thymic atrophy and splenic depletion in 61 by-caught and stranded harbour porpoises were correlated with increased PCB and PBDE levels (Beineke et al., 2005). However, lymphoid depletion was also associated with emaciation and an impaired health status in these animals.

Mechanistic evidence for the effects of EDC exposure on immune function in marine mammals comes mainly from in vitro studies. For example, beluga whale splenocyte proliferative responses were reduced after exposure to a mixture of PCB and DDT congeners (De Guise et al., 1998). Organochlorines, individually and in mixtures, modulated in vitro respiratory burst (Levin, Brenda & De Guise, 2007), T-cell proliferation (Mori et al., 2006) and B-cell proliferation (Mori et al., 2008) in several cetacean species.

Despite the inherent challenges, evidence suggests that EDCs have affected immune function, thereby resulting in increased susceptibility to infectious diseases in vertebrates, notably marine mammals (Aguilar & Borrell, 1994; Hall et al., 1997; Jepson et al., 1999; Bennett et al., 2001; Jepson et al., 2005; Davison et al., 2011).

2.11.3.2 Birds

Immunological effects have been reported in captive male, but not female, American kestrels, exposed to PCBs, including an increase in total white blood cell counts amongst other responses (Smits et al., 2002). In an earlier study, Smits & Bortolotti (2001) also observed that PCB-exposed adult female American kestrels had a higher antibody response than did controls, whereas adult males exposed to PCBs had suppressed antibody production. These sex-specific responses in PCB-exposed birds provide further evidence of the endocrine disrupting behaviour of PCBs. These studies are further supported by those of Fernie et al. (2005) in which environmentally relevant concentrations of PBDEs modified cell-mediated and humoral immune functions in captive nestling American kestrels, and resulted in structural alterations in immune organs. In addition, there were alterations in the spleen (fewer germinal centers), bursa (reduced apoptosis) and thymus (increased macrophages). In wild glaucous gulls, the numbers of white blood cells were positively related to circulating concentrations of PCBs; Bustnes et al. (2004) and Sagerup et al. (2000) reported that glaucous gulls with high PCB and DDT levels had higher nematode burdens than individuals with low levels, suggesting an effect of these EDCs on host resistance. This evidence follows in the path of earlier pioneering work wherein ten-day-old mallard ducklings which had been fed PCBs exhibited higher mortality to duck hepatitis virus (Friend & Trainer, 1970).

2.11.3.3 Amphibians

Associations between EDCs and immunocompetence have been reported for amphibians, where a limited number of studies have shown that exposure to p,p'-DDT and PCBs correlated with poor immune responses (e.g. Gilbertson et al., 2003; Albert et al., 2007), parasitic infections and mortality events (Mann et al., 2009; for a review of wildlife exposure to POPs, see Chapter 3.2.1).

2.11.3.4 Fish

Immunological effects caused by exposure to environmental contaminants, including some EDCs, have been reported across a range of fish species, many of these related to aquaculture (reviewed by Wester, Vethaak & van Muiswinkel, 1994; Cuesta, Meseguer & Esteban, 2011). Planar PCBs are considered the most toxic as they bind and activate the Aryl hydrocarbon receptor (AhR) and cytochrome P4501A (CYP1A) expression, while non-planar congeners can interfere with AhR signalling but also affect cells via AhR-independent pathways (Duffy & Zelikoff, 2006). Interestingly, treatment of rainbow trout with 10-70% sewage treatment works effluent (containing PAHs among other contaminants) increased their in vitro lymphocyte proliferation but decreased the number of circulating lymphocytes (Hoeger et al., 2004). This effluent failed to alter other measured immune functions including respiratory burst, phagocytosis, lysozyme activity, leucocyte populations other than lymphocytes and *A.Salmonicida*-specific IgM production (Hoeger et al., 2004). In another study, however, exposure to a mixture of chemical contaminants (including some EDCs) caused impairment in the immune system that ultimately led to increased host susceptibility to infectious diseases (Arkoosh et al., 1998).

2.11.3.5 Invertebrates

Invertebrates represent about 95% of total species in the animal kingdom but only a few studies evaluating the effects of EDCs on the immune system have been done in these taxa (reviewed in Galloway & Depledge, 2001).

Molluscs

Different EDCs have been shown to affect immune function in marine bivalves (Canesi et al., 2003; Renault, 2011). Moreover, Porte et al. (2006) reviewed the effects and alternative mechanisms of action of natural and environmental estrogens in mussel (*Mytilus* sp.) immunocytes. Organotin compounds were found to be effective modulators of the immune system in molluscs (Fisher, Wishkovsky & Chu, 1990; Cooper et al., 1995; Cima et al., 1998; St.-Jean, Pelletier & Courtenay, 2002). Interestingly, contamination of the eastern oyster, by tributyltin increased the intensity of *Perkinsus marinus* infection, but no modulation of cellular or humoral parameters were detected

(Anderson, Unger & Burreson, 1996; Chu et al., 2002). An investigation on the effects of chronic exposure of eastern oyster to the insecticides DDT, toxaphene and parathion, noted the presence of an unidentified mycelia fungus in exposed oysters (Lowe et al., 1971).

Crustaceans

In marine crustaceans, there are a scarcity of data to support the notion that EDCs affect the immune system or alter their susceptibility to infectious disease agents. In one study, sub-acute concentrations of CB-15, but not CB-77, reduced haemocyte count but increased recoverable hemolymph volume (Smith & Johnston, 1992).

Echinoderms

The main immune responses of echinoderms are considered to be phagocytosis and the production of reactive oxygen species (ROS). These immune responses have been reported to be affected by EDCs. PCBs increased ROS production and delayed the timing of peak production in the echinoid *Paracentrotus lividus* (Coteur et al., 2001) but not in the sea star, *Asterias rubens* (Coteur et al., 2003). Butyltins affected the phagocytic activity of the amoebocytes of the polar seastar *Leptasterias polaris* (Békri & Pelletier, 2004).

Tunicates

Previous research has shown that in vitro exposures of the colonial ascidian, *Botryllus schlosseri*, to tributyltin and dibutyltin (1 μM) inhibited phagocytosis. Furthermore, exposure to monobutyltin (10 μM) resulted in a slight but significant decrease of this activity (Cima et al., 1995; 1997). The immunotoxicity of organotins, reflected by altered phagocytic activity, chemotaxis or ROS generation, have also been identified in the tunicates, *Styela plicata* (Raftos & Hutchinson, 1997) and *Ciona intestinalis* (Cooper et al., 1995)

Annelids

The commercial PCB mixture Aroclor 1254 suppressed natural cytotoxicity (Suzuki et al., 1995) and phagocytosis (Burch et al., 1999) in the coelomocytes of the earthworm *Lumbricus terrestris*. Exposure in vivo to Aroclor 1254 also decreased host resistance to subsequent challenge of *L. terrestris* with the bacterium *Aeromonas hydrophila* (Roch & Cooper, 1991).

2.11.4 Evidence for a common mechanism of endocrine-immune diseases and disorders in human and wildlife species

The basic similarity in the immune systems of humans and other vertebrates has been used in support of an extensive history of toxicological and pharmacological research. The main function of the immune system in all organisms is to provide protection against infectious agents. Two basic defence systems have evolved in biota: the innate immune system (natural immunity) and the adaptive immune system (acquired immunity). The innate system can be found in all multicellular organisms (Hoffmann et al., 1999), while the acquired system is only found in vertebrates (Du Pasquier, 2001).

The AhR is found in diverse species, including mammals, birds, fish and invertebrates and, in addition to its poorly understood role in xenobiotic processing, it serves to modulate immune modulation in humans and laboratory rodents. The AhR provides a defensible mechanistic basis for extrapolation of toxicological and immuntoxicological effects associated with dioxin-like compounds. Contaminants in the Baltic Sea herring (containing PCBs among other contaminants) fed to laboratory rats (Ross et al., 1997) and harbour seals (De Swart et al., 1994; 1995a; Ross et al., 1996) caused a similar pattern of immunotoxicological effects in the two species, supporting a common mediation of this toxicity, and lending support to the notion that extrapolation from surrogate species can be used. Freshwater amphibian and fish immune function is reduced by ecologically relevant concentrations of atrazine, and this is regularly accompanied by elevated infections (reviewed by Rohr & McCoy, 2010). Similarly, both direct in vitro and in vivo exposure to atrazine decreases immune function (reviewed by Rowe, Brundage & Barnett, 2008) and disease resistance (Karrow et al., 2005) in rodents.

Hotchkiss et al. (2008) noted the sometimes controversial nature of associations between EDC exposures and adverse effects in humans, but the effects of PCBs on immune function is one case where the data are robust enough to support a cause and effect relationship (reviewed by Brouwer et al., 1999; Selgrade, 2007). Furthermore, animal laboratory studies corroborate many of these adverse effects observed in the field and, in some cases, provide mechanisms to explain the effects. Selgrade (2007) concluded that "suppression of immune responses in rodents is predictive of suppression of immune responses in humans and that there is a relationship between immune suppression following developmental exposure to the toxicants and enhanced risk of infectious or neoplastic disease in humans" for PCBs.

It is now evident that a 'weight of evidence' approach provides the best avenue for establishing causation in the case of complex EDC mixtures and effects on the endocrine and immune systems of wildlife (Ross, 2000). Laboratory-based screening protocols certainly provide the most cost-effective and mechanistically-based approach to predicting the effects of EDCs in the real world. However, continued vigilance in the field is also needed.

2.11.5 Main messages

- It is increasingly clear from both data in the laboratory and from human and wildlife samples that EDCs play a role in the development of immune-related disorders and are at least partially responsible for the rise of many of these diseases in recent years.
- Allergies, asthma, and airway disorders, endometriosis, and autoimmune thyroid disease in humans may have roots in EDC exposure.

- Systemic inflammation, immune dysfunction and immune cancers such as lymphoma and leukemia in humans have been connected to EDCs. These chemicals may exert their effects through nuclear receptor signaling pathways that have well-established ties with the immune system through crosstalk with inflammatory pathways. They may also interact with non-nuclear membrane-bound steroid receptors, might affect levels of endogenous steroids, and could work through both the HPA and HPG axes.
- Despite multiple inherent challenges, great strides have been made in establishing cause-and-effect linkages between exposure to EDCs and adverse effects in wildlife.
- During the last decade, a number of wildlife studies have shown that, in addition to their well documented role in regulating reproductive function, estrogens and androgens also modulate the immune system.
- Wildlife immunotoxicology may provide an indication of contamination and shed light on the mechanisms of endocrine disruption for some chemical compounds.
- Studies of caged fish and molluscs, as well as laboratory-based exposures, confirm that sewage effluent modulates the function of the immune system in some freshwater species.

2.11.6 Scientific progress since 2002

- The molecular mechanisms connecting a variety of nuclear hormone receptors to NF-kB (one of the master regulators of inflammation and immunity) have been elucidated.
- Exposure to phthalates and dioxin-like compounds have been linked with the development of endometriosis.
- Developmental immunotoxicity (DIT) has been further explored, linking compounds such as diethylstilbestrol and genistein to postnatal immune disorders.
- Links between PVC products and airway disorders and asthma have been expanded and strengthened.
- Triphenyltin has been linked by several studies to neutrophil and natural killer cell abnormalities.
- Animal data indicate that embryonic exposure to estrogens leads to prostate inflammation in the resulting offspring.
- New data connect bisphenol A to inflammation, allergic sensitization, increased antibody production, and Th2 immune responses.
- Atrazine has been found to affect both natural killer cells and T cells.

2.11.7 Strength of evidence

There is sufficient evidence that EDCs play a role in the development of immune-related disorders and are at least partially responsible for the increase in these diseases in recent years. Further epidemiological and laboratory investigation is warranted to connect correlative data on exposure with underlying causative mechanisms responsible for disease etiology.

There are substantial data linking systemic inflammation, immune dysfunction and immune cancers such as lymphoma and leukemia with EDCs. Together, these new insights stress a critical need to acquire a better understanding of how EDCs affect normal immune function and immune disorders, how windows of exposure may affect disease incidence (particularly for childhood respiratory diseases), and how these effects may be passed on to generations to come.

There is good evidence that EDCs acting through nuclear hormone receptor pathways can directly affect the HPA axis, particularly through their actions on the adrenal glands (Marx et al., 1998).

There are good epidemiological data linking PAH, PCBs and other persistent POPs with autoimmune thyroid disease (Burek & Talor, 2009; Guarneri & Benvenga, 2007; Langer et al., 2008). The mechanistic basis of these associations requires further investigation and offers opportunities for new research directions.

There are sufficient data linking exposure to phthalates, PCBs and dioxins with endometriosis (Cobellis et al., 2003; Heilier et al., 2005; Porpora et al., 2006; Reddy et al., 2006). Further investigation into the mechanistic basis of these links will be important for the future.

There are strong links between EDC exposure, particularly phthalates, and the rising incidence of asthma throughout the developed world (Bornehag et al., 2004; 2005; Kolarik et al., 2008). These links are also supported by animal studies (Bornehag & Nanberg, 2010), suggesting that the links are causal and should be explored in detail in the future.

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2.12 Endocrine disruptors and population declines

2.12.1 Trends in wildlife populations

It is clear that many wildlife populations have declined or are declining in numbers. There is worldwide concern about the loss of species or reduced populations of amphibians, mammals, birds, reptiles, freshwater and marine fishes, and invertebrates (Myers & Worm, 2003; Butchart et al., 2004; Stuart et al., 2004; Clausnitzer et al., 2009; Cumberlidge et al., 2009; Hoffmann et al., 2010; 2011; Vié, Hilton-Taylor & Stuart, 2009; Vorosmarty et al., 2010) (**Figure 2.27**). These declines are more severe in some regions than others, but most have been linked to human activities such as direct harvesting (over-exploitation), and development resulting in habitat loss, environmental contamination, or global climate change (e.g. Myers & Worm, 2003; Hayes et al., 2010; Zhou, Cai & Zhu, 2010). These activities have directly or indirectly affected the ability of many species to survive and reproduce, both of which are critical for a healthy population.

While there is strong evidence that EDCs are affecting the survival and reproduction of individuals (Cheek, 2006; Milnes & Guillette, 2008; Hamlin & Guillette, 2010, 2011; Letcher et al., 2010), making the link between these effects and changes to the population numbers or biodiversity in a region is much more difficult. The natural abundance of fishes, mammals, reptiles, amphibians, birds and invertebrates is often not well understood, but can be affected by chemical exposure (through direct toxicity and endocrine mechanisms), resource extraction, the availability of resources (food, habitat), and competition from or predation by other species. Understanding the role of exposure to EDCs in the decline of species or biodiversity in a region is challenging because of the presence of other natural or human stressors, the mixtures of chemicals (both EDCs and non-EDCs) that many populations are exposed to and the difficulty in assessing exposures (see Chapter 3), and our limited understanding of the ecology of the population. Declines in the abundance of one species will in turn affect the health and balance of its ecosystem because of the interdependencies of organisms within the environment.

2.12.2 Evidence for EDCs causing population declines in wildlife

The best evidence of EDCs causing declines in wildlife species has been from associations between exposure to a chemical with known impacts on the endocrine system (mainly from lab studies) and changes in the numbers of wild animals. As chemical exposures increase, populations decline; conversely, as chemicals are removed from the market and wildlife exposure decreases, populations recovers. The two best examples of these types of relationships are for the pesticides DDT and tributyltin (TBT).

2.12.2.1 Tributyltin and invertebrate populations

TBT is an anti-fouling compound that was common in paints used on ships in the 1970s through to the 1990s. Exposure to TBT caused imposex (development of male sex organs) in female snails and led to reproductive failure and declines or extirpations of several species in harbours and other areas with high TBT use (Titley-O'Neal, Munkittrick &

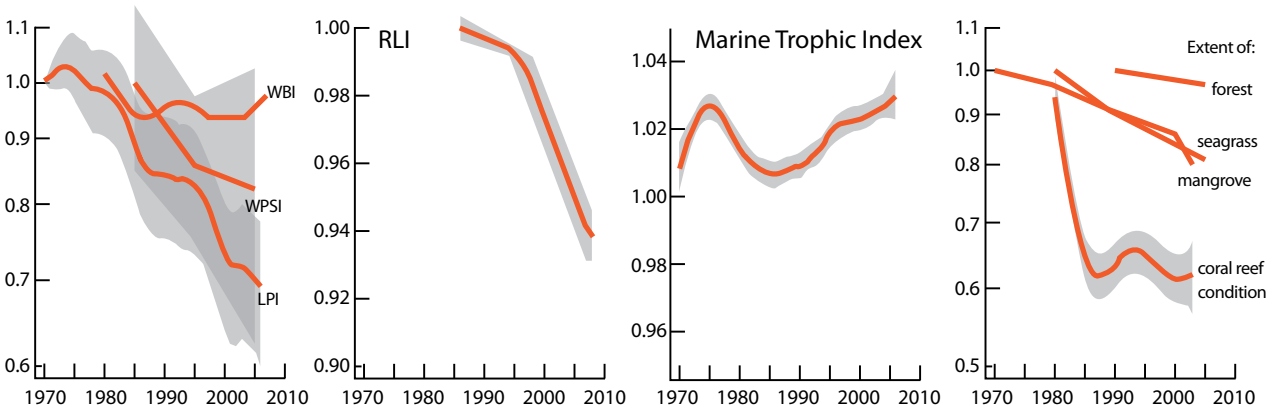

Figure 2.27. Indicator trends for the state of biodiversity. Data scaled to 1 in 1970 (or for first year of data if >1970), modeled, and plotted on a logarithmic ordinate axis. Shading shows 95% confidence intervals except where unavailable (i.e. mangrove, sea grass, and forest extent). WBI, Wild Bird Index (The Global Wild Bird Index aims to measure population trends of a representative suite of wild birds for which robust data are available, to act as a barometer of the general health of the environment and how it is changing), WPSI, Water Bird Population Status Index (similar to WBI but only for water birds and focused on direction of change in population rather than magnitude of change), LPI, Living Planet Index (state of biological diversity for vertebrates around the world. Derived from the ***Living Planet Database (LPD)*** which contains over 10,000 population trends for more than 2,500 species of fish, amphibians, reptiles, birds and mammals), RLI, Red List Index (The ***Red List Index (RLI)***, based on the IUCN Red List of Threatened Species, is an indicator of the changing state of global biodiversity. It defines the conservation status of major species groups, and measures trends in extinction risk over time). Figure based on Butchart et al. (2010) and reproduced with permission from the publisher.

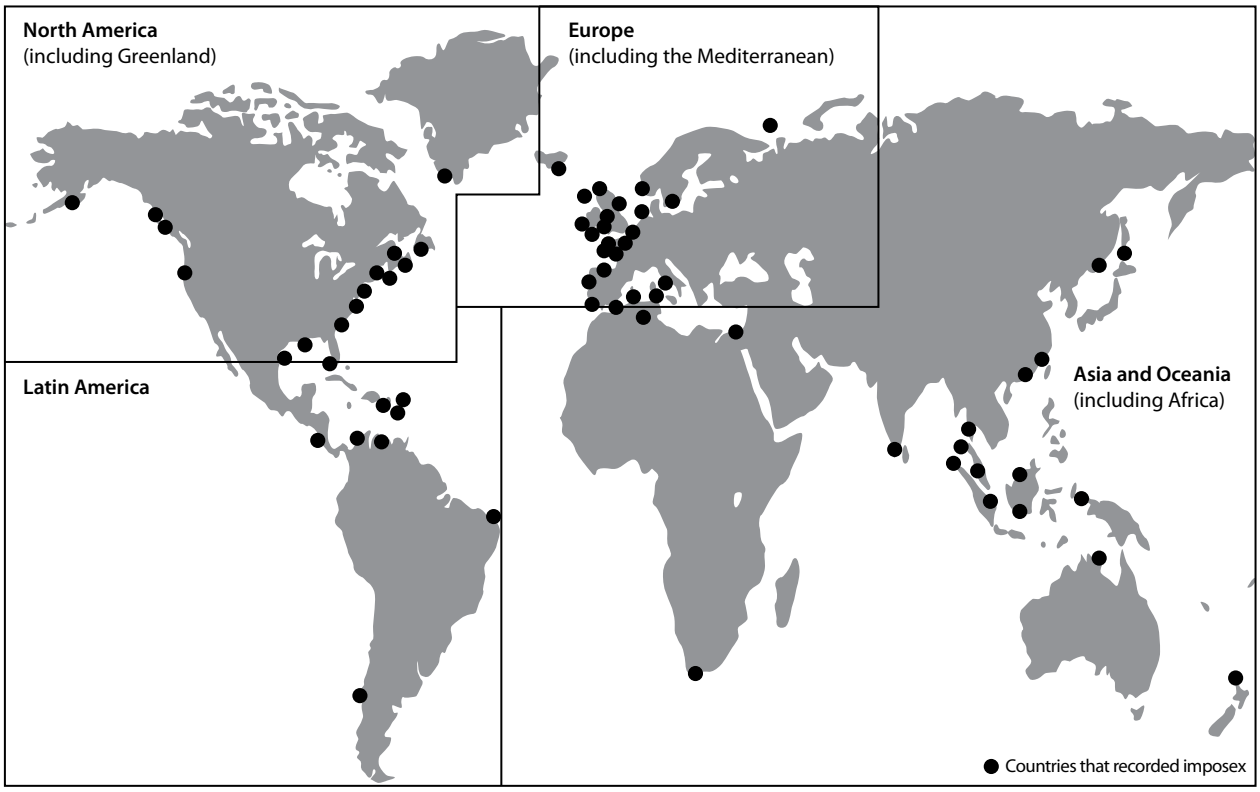

Figure 2.28. Geographic regions of the world where female gastropods were reported as affected by imposex, intersex and ovo-testis between 1990 and 2009 (Titley-O'Neal et al., 2011). Figure based on data from the reference given.

MacDonald, 2011; **Figure 2.28**). The use of TBT has been restricted (but not banned completely worldwide) since the 1990s and, as a result, snail populations have recovered in locations where environmental concentrations of TBT have declined (Jorundsdottir, Svavarsson & Leung, 2005; Morton, 2009; **Figure 2.29**). Similarly, other invertebrates also historically affected by TBT have shown recent signs of recovery in abundances, although an endocrine disrupting mechanism has not been identified. For example, the populations of North Sea brown shrimp declined steeply prior

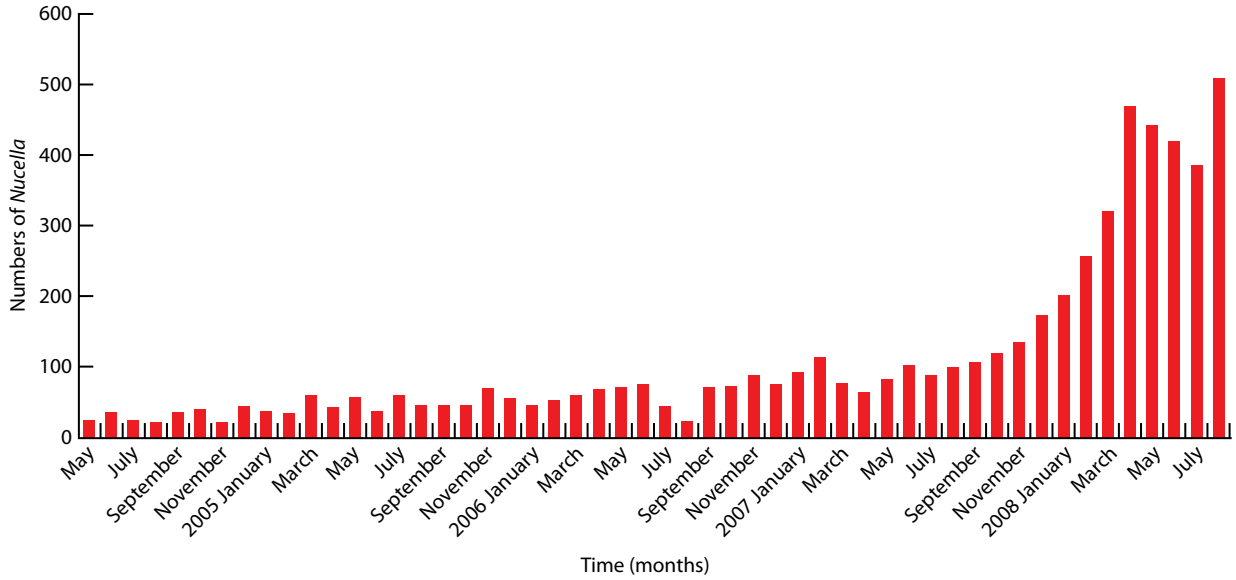

Figure 2.29. The numbers of dog whelks (*N. lapillus*) recorded from a single location (Mewsbrook Groyne at Littlehampton on the southeastern coast of England) every month from May 2004 to August 2008 coinciding with the period immediately after TBT was banned globally as a ship anti-foulant (Morton, 2009). During the study period, the size of the population of *N. lapillus* grew from ~25 individuals to >500, i.e., a 20-fold increase. (Figure redrawn from Morton, (2009); Used with publisher's permission)

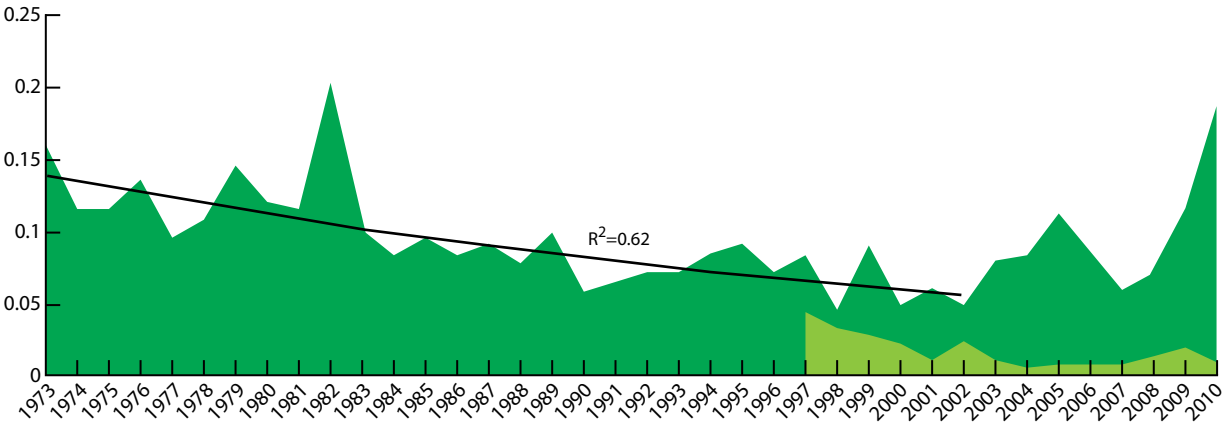

Figure 2.30. Effects of TBT on the North Sea brown shrimp (*Crangon crangon*) stock prior to and since the TBT ban in 2003. Long-term annual time series of landings per unit effort (LPUEs) for the Southern Bight (Y-axis, average monthly LPUEs in kg FW x horsepower-1 x fishing hours-1) for the North Sea brown shrimp (dark green) and cod and whiting (light green) during 1973–2010 and 1997–2010, respectively (X-axis). (Figure redrawn from Verhaegen et al. (2012); Used with publisher's permission)

to an EU ban in 2003, and corresponded with accumulation of unacceptably high levels of TBT in their tissues, such that they could not be consumed by people. Levels have decreased approximately 10-fold since the ban took effect, coinciding with a recovery of the shrimp stock after 30 years of gradual decline (**Figure 2.30**; Verhaegen et al., 2012).

2.12.2.2 Organochlorine pesticides and bird populations

Similar population declines and recoveries for top predator bird species have been observed with the heavy use and subsequent restriction of the insecticide DDT. In the environment, DDT is broken down into DDE, a form that concentrates up through the food web to elevated levels in upper-trophic-level birds such as osprey, falcons and eagles, and this chemical interferes with the hormones (prostaglandin signalling) controlling eggshell production. As DDE exposure increased, eggshell thickness decreased in birds worldwide (Hickey & Anderson, 1968; Grove, Henny & Kaiser, 2009; Blus, 2002), affecting both the survival of the chick and the reproductive success of the species. When DDT was heavily used in North America and Europe, numerous bird populations (e.g. brown pelicans, merlins, double-crested cormorants, great cormorants, peregrine falcons, bald eagles, osprey) with high DDE exposure declined because they were unable to successfully reproduce (Blus, 2002). Field monitoring programs were invaluable in linking the declines to DDE (Blus & Henny, 1997). Populations of some of these birds have recovered or are recovering due to bans on the use of DDT in many countries. As an example, after DDT was banned in North America in 1972, osprey populations in the US increased from 8000 in 1981 to 14 200 in 1994 to 16 000-19 000 in 2001 (Grove, Henny & Kaiser, 2009). There is a clear association between the DDT ban (as evidenced by declining levels of DDE in osprey eggs) and the recovery of this species (**Figure 2.31**; Henny et al., 2010). This raises the possibility that avian species where

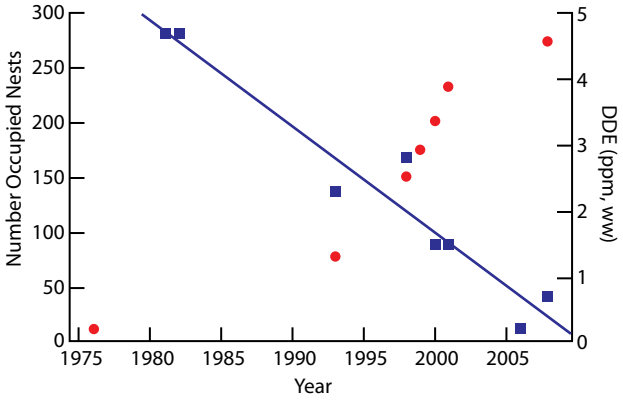

Figure 2.31. Number of osprey nests occupied (left Y axis; circles) versus DDE concentrations (squares; right Y axis; geometric means, ppm wet weight, and best fit line) in osprey eggs from Willamette River, Oregon, USA. Figure based on data from Henny et al. (2010).

DDT is still used for malaria control may be adversely affected. Bouwman et al. (2008) found several POPs (HCB, DDT, HCHs, chlordanes and PCBs) at detectable levels in eggs from 8 bird species in South Africa. Of those, the African darter had thinner eggshells that were associated with higher egg concentrations of DDE, PCBs and other POPs. Monitoring the impact of DDT on birds is essential in areas where ongoing spraying occurs.

2.12.2.3 Mercury and bird populations

There is ongoing concern about exposures of birds to methylmercury - the form of mercury that biomagnifies up through food webs to high concentrations in top predators - and how it affects populations. For example, concentrations of mercury in eggs of ivory gulls collected from Seymour Island, Canada, have steadily increased since 1976 to levels which are

Table 2.8. Nesting effort of white ibises and body feather mercury (Hg) concentrations in great egret chicks in the Florida Everglades. The number of white ibis nests are negatively correlated with great egret feather mercury concentrations (Spearman-rank rs = −0.77, p = 0.04). Data are from a seven-year standardized dataset of great egret chick-feather mercury concentrations (as a measure of temporal changes in mercury bioavailability) and annual numbers of white ibis nests (Heath & Frederick, 2005). (Table reprinted with permission of the publisher).

Year	Maximum number of White Ibis nests	Mean Great Egret feather Hg
1994	5182	17.40
1995	8177	11.48
1997	5989	19.10
1998	4971	8.67
1999	14014	7.36
2000	32204	5.96
2001	13144	8.35

now among the highest measured in seabirds, and these high concentrations appear to have had a long-term effect on breeding productivity (Braune, Mallory & Gilchrist, 2006; Gilchrist & Mallory, 2005). Similar concerns have also been reported in other bird species. The mechanisms of endocrine impairment and net effects on demography of bird populations are poorly understood. However, a recent long-term lab study mimicked the dietary methyl-mercury exposure (0.05–0.3 ppm wet weight) of wild ibises and found increases in male–male pairing behaviour (to 55% of males), and decreases in egg productivity (to 30%) and fledgling production (to 35%) in captive ibises (Frederick & Jayasena, 2011). Endocrine disruption caused by mercury exposure could, therefore, lead to altered demographic patterns in wild bird populations (e.g. Burgess & Meyer, 2008). Indeed, Heath & Frederick (2005) suggest that breeding population size of ibises in the Florida Everglades may be inversely correlated with annual methyl mercury exposure (**Table 2.8**).

2.12.2.4 PBDEs

PBDEs are found at elevated concentrations in birds, especially those that are high in the food web and those near urban centers (Chapter 3.2). Lab studies have shown that reproduction is compromised in American kestrels exposed to environmentally relevant concentrations of PBDEs in their diet. More specifically, these birds had delayed times to egg laying, and laid eggs that had thinner shells and smaller weights (Fernie et al., 2009). Although no field studies have linked raptor abundances with PBDE exposure, lab studies like this suggest that populations of wild birds accumulating high concentrations of PBDEs may also be compromised.

2.12.2.5 Fish populations and estrogens

Understanding the links between EDCs and fish abundances downstream of point source inputs of municipal or industrial effluents or in systems receiving non-point source contamination (e.g. runoff from agriculture) is challenging mainly because EDC exposure is one of several stressors in the aquatic environment. However, population modeling based on lab studies has predicted declines in the abundance of fishes exposed to low, environmentally relevant concentrations of estrogens and their mimics (e.g. Grist et al., 2003). A long-term, whole-lake experiment showed dramatic declines in the fathead minnow population after exposures to low ng/L concentrations of EE2 (Kidd et al., 2007). Male fish have high incidences of intersex in rivers receiving sewage treatment works effluents that contain estrogens and anti-androgens (e.g. roach in the UK; Jobling et al., 2006), and this condition decreases their reproductive success when in competition with normal males (Harris et al., 2011). EDCs are likely impacting fish abundance and genetic diversity (see below), but these impacts are difficult to detect and, hence, our ability to link EDCs directly to population declines for fishes remains an ongoing challenge (Mills & Chichester, 2005; Sumpter & Johnson, 2008).

2.12.2.7 Marine mammals

Several studies have suggested a role for chemical exposure in the decline of marine mammal populations through their effects on reproduction and survival. Although the evidence is speculative due to the difficulty of associating the chemicals directly to lower numbers of individuals, declines in the sea otter, northern fur seal, Steller sea lion and the Galapagos sea lion may be partially due to their exposure to diverse mixtures of PCBs, DDT, other POPs, mercury and other metals (Alava et al., 2011; Barron, Heintz & Krahn, 2003; Beckmen et al., 2003; Kuker & Barrett-Lennard, 2010; Towell, Ream & York, 2006). The numbers of Baltic grey seals declined in the late 1970s to less than 4 000 individuals from an estimated 88 000 to 100 000 animals 100 years earlier (Harding & Härkönen, 1999). More recent population estimates indicate around a 7% increase in the populations per year likely due to lower POPs exposures in

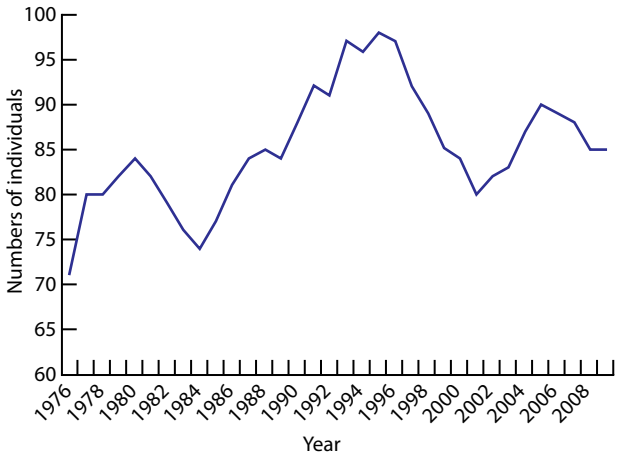

Figure 2.32. Abundance of southern resident killer whales from 1976-2009 (data from the Center for Whale Research) (Pudget Sound Partnership (2009); redrawn; Used with publisher's permission)

the last decade (Olsson, Karlsson & Ahnland, 1994; Karlsson et al., 2005). The southern resident killer whales (SRKW) also experienced an unexplained 20.4% decline in their population between 1995-2001 (see **Figure 2.32**) (Centre for Conservation Biology, 2012). This is an alarming rate of decline for an already small population; the SRKW represents the smallest of four resident communities (consisting of only 89 individuals in three pods) within the eastern North Pacific Ocean and is the only killer whale population to be listed as endangered.

There are three current hypotheses that are posed as reasons for the decline in the SRKW: 1) a decline in the whales' primary prey, Chinook salmon; 2) noise disturbance from private and commercial whale watching vessels; and 3) exposure to high levels of endocrine disrupting POPs (e.g. PCBs, PBDEs and DDT), which are stored in the whales' fat. Because they are long-lived top predators, killer whales accumulate high concentrations of POPs, including PCBs and PBDEs. Killer whales are known to be the most contaminated marine mammals in the world, due to the high levels of toxic anthropogenic chemicals that accumulate in their tissues (O'Neill & West, 2009; Hickie et al., 2007). PCB concentrations measured in biopsies collected from killer whales (Ross et al., 2000; Krahn et al., 2009) exceed the effects threshold established for harbour seals (17 mg/kg PCBs in blubber; see section 2.2) by several times (Kannan et al., 2000; Ross et al., 1996). As PCBs restrict the development of the reproductive system in cetaceans (see Chapter 2.2), high contamination levels lead to low pregnancy rates and endocrine and immune system disruption; both systems are critical to mammalian health and survival (Ross et al., 2000). These or other contaminants may be a factor in the decline of endangered populations of killer whales (Ross, 2006; Krahn et al., 2004), although the mechanism via which these effects may occur is unknown. In a very recent study (Buckman et al., 2011), biopsies from the SRKW tissues revealed that PCB tissue loads were strongly correlated with increases in the expression of aryl hydrocarbon receptor, thyroid hormone α receptor, estrogen α receptor, interleukin and metallothionein 1, thus providing the first evidence of endocrine disruption related to exposure to PCBs in these animals. Reduced exposure to these contaminants will be important to the recovery and long-term survival of the southern resident killer whales and of other killer whale populations.

2.12.3 Evidence that EDCs cause declines in genetic diversity in wildlife populations

Declines in the abundance or extirpation of a species is the most severe response to EDC exposure but there are other more subtle but potentially devastating impacts on wildlife populations over the long term. EDCs could also impair reproduction and an individual's ability to contribute genetically to the next generation (fitness). Chemical exposure is known to decrease the genetic diversity of populations either directly because of mutations to the DNA (genotoxic effects) or indirectly because only the tolerant individuals survive and reproduce, reducing the genetic information in the next generation (Medina, Correa & Barata, 2007; Bickham, 2011). It is likely that this also occurs in response to EDCs because of their impacts on reproductive success of the parents and survival of the offspring. For example, laboratory exposure of zebra fish to a potent pharmaceutical estrogen, EE2, changed the genetic composition of the offspring because it reduced the spawning success of some individuals and increased the success of others (Coe et al., 2008). Estrogen exposure in male fish has also been shown to cause chromosomal abnormalities (aneuploidy) in sperm and in the embryos they fertilize (Brown et al., 2008). Reduced genetic diversity in a population could threaten its ability to survive changes in the environment, increase its risk of extinction, and may in turn impact how communities and ecosystems function (Medina, Correa & Barata, 2007). At a higher level of biological organization, for example, it has been reported that marine communities have lower biodiversity in polluted than in non-polluted areas (Johnston & Roberts, 2009). Although a direct link between EDC exposure and reduced genetic diversity in populations or communities has not yet been demonstrated, it is possible that this may occur given the effects of EDCs on the reproductive success of wildlife.

2.12.4 Human populations

Increasing human populations in many places in the world have been of major concern due to their impact on food and energy resources. Therefore, since the 1960s major national and international bodies, including the Population Council and WHO, have been operating large family planning programs (Special Programme of Research, Development and Research Training in Human Reproduction, World Health Organization, 1211 Geneva 27, Switzerland). These programmes seem to have

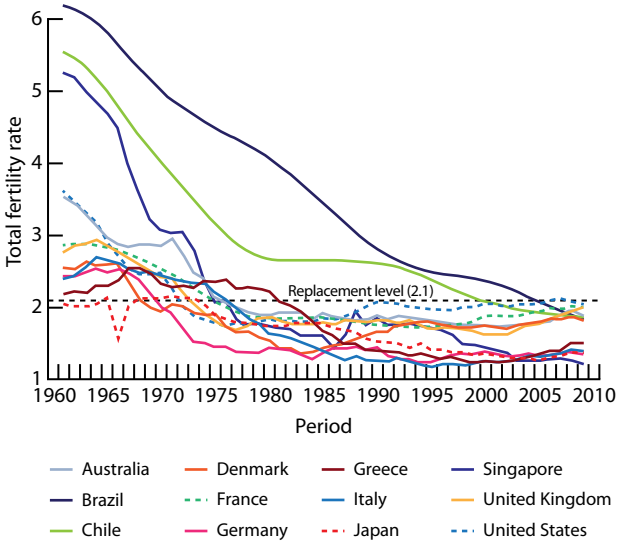

Figure 2.33. Fertility (1961-2009) in countries where current fertility rates are below replacement level. Diagram based on data presented by the World Bank (http://data.worldbank.org/indicator/SP.DYN.TFRT.IN).

been successful. Besides contributing to reductions in fertility rates (see **Figures 2.33 and 2.34**), they have also resulted in improved economic status of individuals and resulted in worldwide improvement in women's reproductive health and social status in general. China took part in this development by introducing the one child family programme in the 1970s.

In spite of the drastic reductions in fertility rates are still increasing. There are two main reasons for this. The first is that, although fertility rates seem to be declining all over the world, there are still areas, particularly in developing countries, where fertility is significantly above the replacement level (an average of 2.1 children per woman) (**Figure 2.33**). Somalia and Tanzania are examples of African countries with high fertility and it is noteworthy that all high fertility countries belong to less industrialized areas (**Figure 2.34**). The second main reason for the current increase in the world population is that changes in fertility rates are not completely reflected in population statistics until 30-60 years after they occur. The reason is that humans live much longer than before due to improved health conditions. Therefore today we see rather stable, but ageing, human populations in countries like Japan and Europe, where fertility rates have been below the replacement level for 20-40 years. However, we shall soon begin to see significant reductions in populations of these industrialized countries, although immigration may modify this development.

It has generally been believed that the low fertility rates have been due to contraception combined with political initiatives (China) or changes in social family structures (Japan, South Korea, Singapore, Chile, Europe, Australia) (Lutz, 2006; UN DESA, 2011). Reproductive health problems among men and women may also be important factors behind the low fertility rates (Jensen et al., 2008). In countries like Denmark where health statistics are well developed, there are accurate figures of the use of assisted reproduction techniques (ART); in Denmark, 8% of all children are now born after ART, including in vitro fertilisation, intra-cytoplasmatic sperm injection, intrauterine insemination by partners' sperm and intrauterine insemination by donor sperm (European Science Foundation, 2010). In spite of the common practice of ART, almost 25% of men are still childless (voluntary or involuntary). These numbers, taken together with recent data showing that poor semen quality is widespread (20%-40% of young men have suboptimal semen quality) (Jørgensen et al., 2012), suggest that reduced fecundity may also play a role in the current low fertility rates in Denmark. Trends in conditions causing low female fecundity have been less well examined (Crain et al., 2008). However, it is assumed that male and female factors contribute equally to human infertility, suggesting that female infertility may also play a role in these current fertility trends (see also section 2.2).

As human reproduction is a very slow process (30-40 years between generations) compared to many animal species, adverse trends in reproduction may not have a full population effect until after one or two generations. In other words, the low fertility rates we are witnessing today will not have full societal effect in our time. The European Science Foundation (2010) has recently highlighted the evidence that environmental factors play a role in adverse trends in male reproductive problems (testicular cancer, poor semen quality, low testosterone levels and other genital abnormalities; see also section 2.3) and urged both its European members and global research bodies to take part in the endeavor to identify the causes of the adverse environmental reproductive trends.

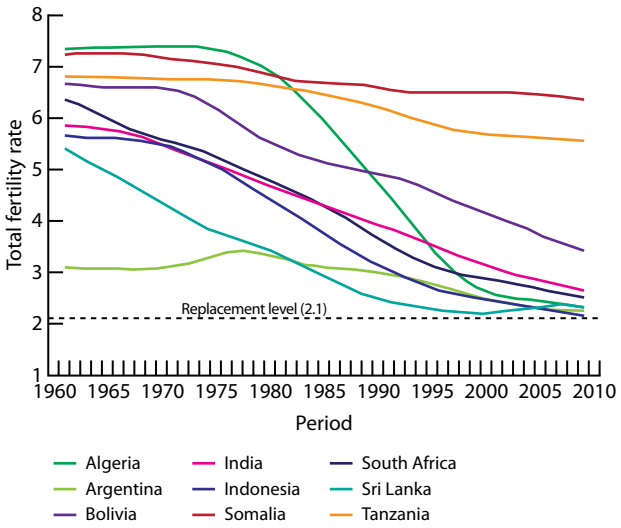

Figure 2.34. Fertility (1961-2009) in countries where current fertility rates are above replacement level. Diagram based on data presented by the World Bank (www.worldbank.org), from http://data.worldbank.org/indicator/SP.DYN.TFRT.IN.

2.12.5 Main messages

- Wildlife species and populations continue to decline worldwide and this is due to a number of factors including over-exploitation, loss of habitat, climate change, and chemical contamination.
- Although declines (and sometimes recoveries) in abundances of birds, marine mammals, fish, and snails have been related to changes in exposure to EDCs, making a clear link between endocrine effects in individuals and population declines is challenging.
- It is clear that historical declines in some wildlife populations (e.g. birds and snails) were because of the effects of chemicals (DDT and TBT, respectively) on the ability of these species to successfully reproduce. Bans on the use of these chemicals led to the recovery of some populations. For this reason, EDCs are strongly suspected to contribute to current declines in wildlife populations.
- In spite of concerns about rising human populations on a global scale, numerous industrialized countries have fertility rates well below the replacement level. It has generally been assumed that these changes are due to socioeconomic factors. However, widespread poor semen quality at subfertility levels may also contribute to this trend.

2.12.6 Scientific progress since 2002

In general, the evidence for endocrine disrupting POPs such as PCBs and OCs causing population declines has increased now, relative to 2002, due to the visible increases in populations of birds and seals, for example, following the restrictions on the use of these chemicals.

2.12.7 Strength of evidence

Wildlife Populations: While it is clear that the biodiversity and abundance of wildlife are threatened from a number of human activities, making direct links between declines in species and endocrine disruption from chemical exposure remains a major challenge. Relationships between exposure to EDCs and decreases in animal abundance or genetic diversity are correlative at best because of the difficulty in isolating effects of chemicals from other stressors (i.e. loss of habitat, overharvesting or climate change). To date the best evidence of a relationship between EDCs and wildlife populations is from a temporal relationship between a measure of exposure (e.g. DDT in bird eggs or TBT in snails) and population parameters (e.g. number of active bird nests or snails, respectively). When the species' exposure to the chemical declined through bans on its use, populations recovered. Current exposures to chemicals are compromising the endocrine system of many species and, as such, are believed to be playing a role in the lower abundances of wildlife.

To date, the quality and strength of the evidence linking EDC exposure to most wildlife population declines is insufficient. An endocrine mechanism for wildlife declines is probable but not conclusive.

Human Populations: Similarly, endocrine mechanisms linking EDCs to steep declines in fertility rates, such that in many countries they are below replacement levels, are plausible, but not explored.

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Chapter 3

Human and wildlife exposures to EDCs

Karen A. Kidd, Georg Becher, Åke Bergman, Derek C.G. Muir and Tracey J. Woodruff

3.0 Introduction

A decade ago, the first IPCS *Global Assessment on the State-of-the-Science of Endocrine Disruptors* (IPCS, 2002) focused mainly on a few environmentally persistent organic pollutants (POPs; primarily PCBs, DDTs, PCDFs/PCDDs), with only very brief descriptions of other chemicals believed to contribute to endocrine disruption (ED) such as phytoestrogens and the flame retardants, polybrominated diphenyl ethers (PBDEs). The limited scope of this first report was primarily because there was much less information about the extent to which other chemicals might affect the endocrine system and even less information about exposure to these chemicals.

Over the past decade, understanding of the types of chemicals that may be endocrine disruptors and on how humans and wildlife are exposed to them has increased dramatically. A larger number of chemicals belonging to diverse classes are now identified as EDCs, and they include additives in materials and consumer goods (pharmaceuticals, personal care products, electronics, food packaging, clothing, etc.), metals, and current-use pesticides (**Table 3.1**). These chemicals come from a variety of sources, enter the environment during production, use or disposal of chemicals or products, and have a range of behaviours in the environment.

Over the last decade, some important exposure-related issues have emerged for EDCs. Now there is a better understanding that exposure to even very low concentrations of EDCs can increase the risk of effects (Chapter 1). Further, it is also understood that there are additional, important sources of exposure to EDCs, including dust from indoor environments and direct contact with consumer products. Concerns for the fetus and for young children has increased because, for some sources of EDCs, exposure is greater for young children (i.e. dust and particulates) than for adults (Lunder et al., 2010; Wormuth et al., 2006), and there is a greater understanding of the enhanced susceptibility to EDCs during these early developmental periods (Woodruff et al., 2008; see also Chapter 2). It is also evident that both human and wildlife exposures to EDCs consist of complex mixtures of chemicals that are persistent (remain intact for many years) and bioaccumulative (concentrate in fat or protein) or less persistent and not bioaccumulative (rapidly excreted). However, there is limited understanding of the types of mixtures that humans and wildlife are exposed to and how they affect the endocrine system, even though combined exposures can result in a greater risk than exposure to any one chemical at a time (National Research Council, 2008). In addition, effects related to exposure to EDCs could be complicated by exposure to non-EDC chemicals and possibly to other environmental, biological or physical stressors. Indeed, these are the main reasons why the evidence linking exposure to chemicals with endocrine disruption seen in human and wildlife populations (reviewed in Chapter 2) are often not definitive; all disorders and diseases are probabilistic and multicausal.

This chapter describes the current knowledge on the types, sources, environmental fate, exposure routes, and levels of "known" and "potential" (or "possible") EDCs (see Chapter 1.3 for definitions) in humans and wildlife. Although some of the chemicals from the 2002 document (e.g. PCBs) are included both here and in Chapter 2, the focus in this document is on chemicals that have been more recently identified as or suspected to be EDCs and those that are still produced and used. The end of each sub-chapter contains the main conclusions and the chapter ends with the main messages.

3.1 The EDCs of concern

This chapter is not intended to be a comprehensive description of all EDCs, but it identifies and describes major classes of chemicals with known or potential ED properties, and example chemicals from each class (**Table 3.1**). The EDC classes are defined according to common chemical properties and structural features, and on their use and occurrence (**Table 3.1**). This does not mean that the chemicals described here have only endocrine disrupting properties, as several have other toxicological properties and/or health effects. In addition, some of the chemicals could be included with several different classes due to their broad uses but, for simplicity's sake, are only presented in one place. Known or potential EDCs have been identified based on several reviews or authoritative reports (US EPA 2009; 2010a; Kortenkamp et al., 2011; Ryu, Yoon & Oh, 2011; McKinlay et al., 2008; EEA, 2012; Pongratz & Vikström Bergander, 2011; ChemSec 2011; TEDX, 2011; Lintelmann et al., 2003). **Table 3.1** presents only a portion of the EDCs that have been identified in the scientific literature, and additional chemicals are listed in the above-mentioned reviews. It is of note that there are many chemicals described here to which we and wildlife are exposed, but for which there are very little or no epidemiological data or data from animal studies described in Chapter 2.

3.1.1 Types and sources of EDCs

Hundreds of individual anthropogenic and natural chemicals are known or suspected to interact with endocrine systems in

humans and wildlife, and their sources of exposure, chemical properties, and environmental fate vary widely. For the purposes of this report, the chemicals are grouped into eleven broad classes based on their physical-chemical characteristics or origin/application areas (**Table 3.1**). The classes include chemicals that are currently produced for commercial purposes, and those that are no longer manufactured or are being phased out of production. The previous assessment on EDCs was primarily on POPs (IPCS, 2002) but this has broadened more recently to include less persistent and less bioaccumulative organic chemicals (e.g. current-use pesticides, plasticizers, pharmaceuticals, natural hormones, phytoestrogens, product additives) and metals. Some EDCs are persistent in the environment, bioaccumulate through food webs to high concentrations in wildlife and humans, and can be transferred to the developing fetus and the newborn through the placenta or breast milk, respectively. Other EDCs are less persistent in the environment and do not remain in humans and wildlife for very long (short half-lives), as for bisphenol A with a half-life of 4-8 hours (Vokel et al., 2002); more specifically, they are not bioaccumulative yet they are a concern because exposure to them can be continuous. Of all of the chemicals on the market (slightly less than 145,000 chemicals are currently preregistered by REACH (ECHA, 2011)), large numbers are persistent enough to reach humans and wildlife but are not accumulated in the body. These chemicals have been classified as *pseudo persistent,* i.e. through continuous emission to the environment they essentially become "persistent" pollutants even if their half-lives are short (Daughton, 2003). Some chemicals affect the endocrine system in their original form, whereas others undergo metabolic transformations in the body or are abiotically transformed to forms that make them active in endocrine systems. Humans and wildlife are exposed to a diverse number of EDCs through a variety of routes and, as described below, the levels found in body tissues are affected by environmental and socioeconomic factors. Despite an improved understanding of the types of EDCs that are in the environment, there are still knowledge gaps.

In contrast to a decade ago, there is a better appreciation that industrial and consumer products can contain known or potential EDCs. For example, cosmetics and other personal care products (shampoos and other hair products, toothpaste, soaps, lotions) contain fragrances (e.g. galaxolide), solvents (e.g. cyclic methyl siloxanes), preservatives (e.g. parabens), plasticizers

Table 3.1. Endocrine disrupting chemicals (EDCs) can be grouped in multiple ways. In this table known or potential EDCs are grouped into 11 categories with examples of individual EDCs. Bolded chemicals were selected since they are regarded to be of specific interest as EDCs, and are described in more detail in the text.

Classification	Specific Examples of EDCs[1]
Persistent and bioaccumulative halogenated chemicals	
Persistent Organic Pollutants (POPs) (Stockholm Convention) (section 3.1.1.1)	PCDDs/PCDFs, **PCBs**, HCB, **PFOS**, **PBDEs**, PBBs, Chlordane, Mirex, Toxaphene, **DDT**/DDE, Lindane, Endosulfan
Other Persistent and Bioaccumulative Chemicals (section 3.1.1.2)	**HBCDD,** SCCP, PFCAs (e.g. **PFOA**), Octachlorostyrene, PCB methyl sulfones
Less persistent and less bioaccumulative chemicals	
Plasticizers and Other Additives in Materials and Goods (section 3.1.1.3)	Phthalate esters (**DEHP**, BBP, DBP, DiNP), Triphenyl phosphate, Bis(2-ethylhexyl)adipate, n-Butylbenzene, Triclocarban, Butylated hydroxyanisole
Polycyclic Aromatic Chemicals (PACs) including PAHs (section 3.1.1.4)	**Benzo(a)pyrene**, Benzo(a)anthracene, Pyrene, Anthracene
Halogenated Phenolic Chemicals (HPCs) (section 3.1.1.5)	2,4-Dichlorophenol , Pentachlorophenol, Hydroxy-PCBs, Hydroxy-PBDEs, Tetrabromobisphenol A, 2,4,6-Tribromophenol, **Triclosan**
Non-halogenated Phenolic Chemicals (Non-HPCs) (section 3.1.1.5)	**Bisphenol A**, Bisphenol F, Bisphenol S, Nonylphenol, Octylphenol, Resorcinol
Pesticides, pharmaceuticals and personal care product ingredients	
Current-use Pesticides (section 3.1.1.6)	2,4-D, **Atrazine**, Carbaryl, Malathion, Mancozeb, **Vinclozolin**, Procloraz, Procymidone, Chlorpyrifos, Fenitrothion, Linuron
Pharmaceuticals, Growth Promoters, and Personal Care Product Ingredients (section 3.1.1.7)	Endocrine active (e.g. Diethylstilbestrol, Ethinylestradiol, Tamoxifen, **Levonorgestrel),** Selective serotonin reuptake inhibitors (SSRIs; e.g. **Fluoxetine**), Flutamide, 4-Methylbenzylidene camphor, Octyl-methoxycinnamate, Parabens, **Cyclic methyl siloxanes** (D4, **D5,** D6), Galaxolide, 3-Benzylidene camphor
Other chemicals	
Metals and Organometallic Chemicals (section 3.1.1.8)	Arsenic, Cadmium, Lead, Mercury, **Methylmercury** Tributyltin, Triphenyltin
Natural Hormones (section 3.1.1.9)	17β-Estradiol, Estrone, Testosterone
Phytoestrogens (section 3.1.1.9)	Isoflavones (e.g. Genistein, Daidzein), Coumestans (e.g. Coumestrol), Mycotoxins (e.g. Zearalenone), Prenylflavonoids (e.g. 8-prenylnaringenin)

[1]See Appendix II for full names and abbreviations of the chemicals mentioned.

(e.g. phthalate esters), antimicrobials (e.g. triclosan), chemical stabilizing agents (e.g. phthalates), and metals (e.g. lead, arsenic, mercury). Most are added intentionally to these products but some may be contaminants with no added obvious benefit.

Pharmaceuticals for human or veterinary use contain EDCs and include contraception or other hormone therapies, lipid regulators, beta-blockers, anti-depressants, and antibiotics. Household, school and workplace products such as cleaners, toys, electronics, furniture, building materials, paints, paper, clothing, and lawn and garden supplies contain a range of chemicals including flame retardants (e.g. PBDEs), antimicrobial and chemical stabilizing agents, plasticizers, fragrances, solvents, preservatives, metals, and pesticides.

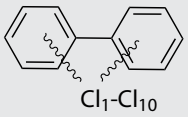
3.1.1.1 Persistent Organic Pollutants (POPs)

The majority of the POPs of the Stockholm Convention (http://chm.pops.int) are known as EDCs. They are listed as POPs as they have been shown to fulfill the criteria for persistency, bioaccumulation, toxicity and long-range transport as defined under the Stockholm Convention. In addition to these officially-acknowledged POPs, there are numerous chemicals that also fulfill some of the criteria of a POP. These are referred to in the table as "Other Persistent and Bioaccumulative Chemicals" (section 3.1.1.2, below).

These two groups of chemicals have high persistence and bioaccumulation potential, and are detected at elevated levels in wildlife and humans living close to where these chemicals were used or are still being applied, and in wildlife and humans far away from their sources. They also tend to be found at the highest concentrations in animals at the top of the food web (e.g. humans, seals, polar bears, birds of prey, crocodilians) and in tissues and body fluids that are high in fat (e.g. blubber, mothers' milk, egg yolk). Even though some of the POPs have been regulated or banned in most countries for several decades, such as the PCBs, many are still major global pollutants because of their persistence. This demonstrates the great challenge to eliminate highly persistent organohalogens from the environment and to prevent human and wildlife exposure. Furthermore, several types of POPs are composed of a number of congeners (homologues and isomers), making the total number of individual chemicals in commercial products and in the environment very high. For example, technical PCB products consist of about 130 congeners and PBDE-containing products can have up to 20-30 congeners. As a result, it is difficult to understand which specific PCBs or PBDEs are causing ED effects in wildlife and humans. Despite this, there is now a large body of evidence linking exposure to these chemical groups with endocrine diseases and disorders in both humans and wildlife (see Chapter 2, **Table 2.1** and sections 2.2 through 2.12).

Some POPs (e.g. PCBs, PBDEs) also undergo metabolism and have been shown to form ED active metabolites. These include PCB methyl sulfone and hydroxylated metabolites of PCBs (Letcher, Klasson-Wehler & Bergman, 2000) and PBDEs (Stapleton et al., 2009; Athanasiadou et al., 2008; Hakk & Letcher, 2003), even though some hydroxylated PBDEs are formed in processes other than internal metabolism (Ueno et al., 2008). Due to the persistent and bioaccumulative characteristics of the original (parent) molecules, there is a continuous internal source for formation of, e.g. hydroxylated PCBs and PBDEs. The PCB and DDE methyl sulfones, for example, are neutral compounds with high persistency and bioaccumulation potential (Letcher, Klasson-Wehler & Bergman, 2000). Intestinal microbial activity has been shown

Polychlorinated biphenyls (PCBs)



Characteristics: PCBs are technical mixtures of biphenyls with different numbers of chlorine atoms attached at different positions, making up a theoretical total number of 209 PCB congeners. PCBs exhibit high thermal and chemical stability and are very hydrophobic (log K_{OW} ranges from ~5.0 for Cl_2CBs to ≈ 8.9 for Cl_8CBs).

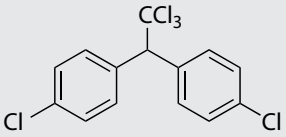
Origin and use: PCBs were produced from 1929 until the mid-1980s for primary use as insulating agents in transformer oils and capacitors, as heat transfer agents, and in sealants for construction (buildings).

Fate: PCBs are highly persistent in the environment, transported over long distances by air and water currents, and are globally distributed. As a result, wildlife and humans worldwide are exposed to PCBs. While some PCB congeners are easily metabolized, others are not. Some PCB congeners, particularly those with substitution at the 2,4 and 2,4,5 positions on the rings, accumulate through food webs to high concentrations in humans and wildlife.

Effects: Extensively studied: Possible endometriosis and fibroids in humans, fibroids, uterine tumours and adrenal problems in seals (Chapter 2.2 & 2.8). Strong experimental and molecular evidence for suppression of thyroid hormone in all vertebrate classes and epidemiological evidence of reduced cognitive function in children (Chapter 2.5 & 2.6). Limited evidence for increased prostate and breast cancer risk in humans and for genital carcinomas in sea lions (Chapter 2.7). Evidence for immune dysfunction in marine mammals and humans (Chapter 2.11). Limited evidence of increased diabetes risk (Chapter 2.10). Probable cause of population declines in fish-eating birds and mammals (Chapter 2.12).

Reviews: Hansen & Robertson, 2001; Ritter, Solomon & Forget, 1995; Waid; 1986; IPCS, 2003; 1992a; 1993

Dichlorodiphenyltrichloroethane (DDT)



Characteristics: Technical DDT is an organochlorine insecticide that consists mainly of 4,4'-DDT (structure shown) and 2,4'-DDT. DDT is very hydrophobic (log K_{OW} = 6.9). It is also semi-volatile and thus partitions into the atmosphere.

Origin and use: DDT was introduced during World War II, and has a broad range of agricultural and non-agricultural applications. Total global DDT production from the 1940s to present has been estimated at approx. 4.5 Mt (Li & Macdonald, 2005). Almost all uses of DDT were banned in the US, western Europe, Japan, and many other countries in the early 1970s, and in China and the former Soviet Union in the 1980s (Voldner & Li, 1993). The Stockholm Convention has given an exemption for the production and public health use of DDT for indoor application to control vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives. However, both WHO and the United Nations Environment Programme, share a common commitment to the global goal of reducing and eventually eliminating the use of DDT without compromising the burden of vector-borne diseases (WHO, 2007a). In 2009, 13 countries in sub-Saharan Africa and 3 in southeast Asia used DDT to control malaria through indoor spraying (WHO, 2010a).

Fate: DDT and its related compounds are very persistent in the environment; as much as 50% can remain in the soil 10-15 years after application. DDT undergoes dehydrochlorination to DDE, which is a very persistent and bioaccumulative degradation product. In anaerobic sediments, dechlorination is the major degradation route, yielding DDD. DDT compounds are found globally in all environmental media due to their long-range atmospheric transport, great persistence and high bioaccumulation.

Effects: Extensively studied: Chapter 2, **Table 2.1**. Possible cause of endometriosis and disruption of ovarian cyclicity in humans. Eggshell thinning, feminization, homosexual behaviour and population declines in birds (Chapter 2.2 & 2.12). Lowered testosterone and demasculinization in polar bears and alligators and intersex in fish and frogs. Methylsulfonyl-DDE and o,p'-DDD cause adrenal hyperplasia and "Cushing disease-like" problems in seals (Chapter 2.8). Some evidence of suppression of thyroid hormone in marine mammals, birds and amphibia (Chapter 2.6). Limited evidence for increased breast cancer risk in humans (Chapter 2.7) and of leukemia and lymphoma. Limited evidence of increased obesity risk with perinatal exposure (Chapter 2.10). Probable cause of population declines in fish-eating birds and mammals (Chapter 2.12)

Reviews: Ritter, Solomon & Forget, 1995; Stemmler & Lammel 2009; WHO, 2007a; IPCS, 2011.

Perfluorooctanesulfonate (PFOS)

Characteristics: PFOS is a synthetic surfactant consisting of a perfluorinated C_8 chain and a terminal sulfonate group. Commercial mixtures contain both linear and branched isomers. PFOS is hydrophobic, oleophobic and proteinophilic (associated with proteins). It is exceptionally stable to degradation under natural conditions.

Origin and use: PFOS and the perfluorooctane sulfonyl fluoride (PFOSF) based products are produced by electrochemical fluorination. PFOS is commonly used as a salt or incorporated into larger polymers via amide or acrylate substituents. Production in the USA and Europe was phased out in 2001/02 but increased in China at that same time. PFOS-based polymers were incorporated into stain repellents and other surface coating agents. PFOS salts continue to be used in fire-fighting foams and in the semiconductor and photolithographic industry.

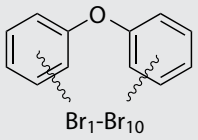
Fate: PFOS can be formed by environmental microbial degradation (or by metabolism in larger organisms) from PFOS-related precursors, i.e. molecules containing the PFOS-moiety. Due to the perfluorination, PFOS is highly resistant to any transformation. Volatile PFOS precursors, such as perfluorosulfonamides, are subject to atmospheric transport while long-range transport in oceans has been documented for PFOS. PFOS is bioaccumulative, but binds preferentially to proteins in liver and blood rather than accumulating in fats.

Effects: Little studied: Lowered female fecundity and altered menstrual cyclicity through occupational exposure. Reduced fetal growth (Chapter 2.2).

Reviews: Lindstrom, Strynar & Libelo, 2011; Stock, Muir & Mabury, 2010; UNEP, 2006

to be essential in formation of aryl methyl sulfone metabolites (Bakke, Bergman & Larsen, 1982; Bakke et al., 1983). A large number of PCB methyl sulfone metabolites are accumulated in humans and wildlife (Chu, 2003; Karasek et al., 2007; Letcher et al., 2009; Linderholm et al., 2007). The phenolic metabolites of POPs are covered in the group of halogenated phenolic compounds (section 3.1.1.5).

Polybrominated diphenyl ethers (PBDEs)



Characteristics: PBDEs are technical mixtures of diphenyl ethers with different numbers of bromine atoms attached at different positions, making up a theoretical total number of 209 PBDE congeners. PBDEs are brominated aromatic compounds of high chemical stability under natural conditions, but are broken down when heated. The PBDEs are hydrophobic (log K_{OW} of tetraBDEs = 6.77; heptaBDEs = 9.4).

Origin and use: PBDEs have been produced since the early 1970s for applications as flame retardants in textiles, electronics, electric articles, furniture and building materials. PBDEs have been subdivided into PentaBDE, OctaBDE and DecaBDE, representing the types of PBDEs produced commercially. DecaBDE (consisting predominantly of BDE-209) remains the major PBDE mixture in production worldwide with 85% of its global use occurring in North America and East Asia (BSEF, 2003).

Fate: PBDEs are very persistent in the environment, transported long distances by wind and air currents, and globally distributed. Debromination of DecaBDE - by sunlight in surface soils and on aerosols, and in the gastrointestinal tract of fish, mammals and birds - is a major transformation process that results in formation of less brominated BDEs (Schenker et al., 2008). Thus while "Penta" and "Octa" BDEs have been phased out, debromination of DecaBDE could be an additional source of emissions of the lower brominated congeners along with the large inventory of in-use PBDE products. While some PBDE congeners are easily metabolized, others are not. Some PBDE congeners bioaccumulate and biomagnify through food webs, and are present in wildlife and humans at high concentrations. Wildlife and humans worldwide are exposed to PBDEs.

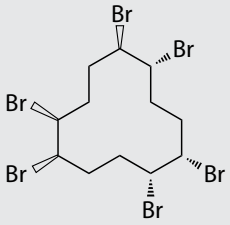
Effects: Limited evidence for earlier age at menarche and cryptorchidism in humans (Chapter 2.2 and 2.3), eggshell thinning, delayed hatching and reduced weight of hatchlings in birds (Chapter 2.2). Strong experimental evidence for suppression of thyroid hormone in humans and Arctic wildlife (Chapter 2.5 & 2.6). Limited evidence for cognitive disorders. Probable contributing cause of population declines in marine mammals (Chapter 2.12).

Reviews: Alcock, Mac Gillivray & Busby, 2011; Daso et al., 2010; EFSA, 2011a; Yogui, & Sericano, 2009; IPCS, 1994.

3.1.1.2 Other persistent and bioaccumulative chemicals

There are numerous other persistent and bioaccumulative organohalogens (chlorinated, fluorinated or brominated) besides those described above, including hexabromocyclododecane (HBCDD), short-chained chlorinated paraffins (SCCPs), hexachlorobutadiene, polychlorinated naphthalenes (PCNs) and pentachlorophenol which are under review for inclusion in the Stockholm Convention. Since HBCDD has been studied in quite some detail it is included as a case compound in the present chapter. However many other brominated flame retardants (BFRs) are not well or not at all studied with respect to their ED properties or environmental fate. For example, a number of BFRs, used as substitutes for PBDEs, have high persistence and bioaccumulation potential. Although there have been reviews of emerging BFRs (Covaci et al., 2011; Law et al., 2006; Eljarrat & Barceló, 2011; de Wit et al., 2011), very little exposure data have been published on any of these chemicals. Another large group of highly persistent chemicals are the perfluorocarboxylic acids (PFCAs). Perfluorooctanoic acid (PFOA) (see text box) is the most commonly assessed compound. Like PFOS (see section 3.1.1.1), the PFCAs all have a perfluorinated alkyl chain but with a carboxylate functional group.

Hexabromocyclododecane (HBCDD)



Characteristics: HBCDDs are cycloaliphatic compounds with six bromine atoms. Technically produced HBCDD is primarily a mixture of three stereoisomers, namely α-, β-, and γ-HBCDD, with enantiomeric pairs. γ-HBCDD is the dominant isomer in technical mixtures, with lower concentrations of α- and β-HBCDD. The HBCDDs have low water solubility and a high affinity for fats or organic carbon in soils and sediment. The γ-HBCDD isomer can be both abiotically and metabolically transformed to α-HBCDD. The dominant isomer in biota is α-HBCDD (see insert). Log K_{ow} (technical HBCDD): 6.6; (α-HBCDD): 7.9.

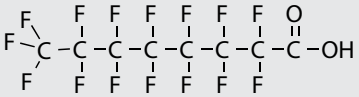
Origin and use: HBCDD has been produced from the early 1970s for use as a flame retardant in insulating materials in construction.

Fate: HBCDDs are transported long distances and generally present in wildlife and humans at concentrations in the low ng/g fat.

Effects: Little studied: Often found in association with PBDEs and PCBs in tissues and sometimes in association with the same effects.

Reviews: EFSA, 2011b; Law et al., 2005; 2008a; 2008b; Tanabe et al., 2008.

Perfluorooctanoic acid (PFOA)



Characteristics: PFOA is a fluorosurfactant consisting of a perfluorinated C_7 alkyl chain with a terminal carboxylate group.

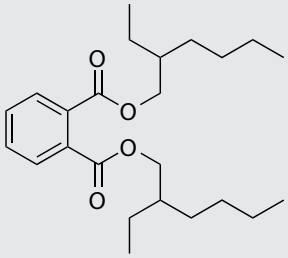
Origin and use: PFOA has been manufactured since the 1940s for industrial applications. The major application is as an emulsifier in the production of fluoropolymers (e.g. Teflon^{R}), but it is also used as an industrial surfactant in a variety of other processes. PFOA is also formed by the transformation of precursors such as polyfluorotelomers (including polymers incorporating the fluorotelomers) and by polyfluoroalkyl phosphates and phosphonates.

Fate: There are no indications of any transformation of PFOA in the environment. Neutral precursors of PFOA are subject to long-range atmospheric transport, and PFOA is transported long distances in ocean currents. The bioaccumulation potential of PFOA seems to be low in fish, but the presence of detectable concentrations in higher trophic levels (polar bear, caribou, walrus) has generated concerns regarding the biomagnification potential of PFOA in food webs. The voluntary PFOA Global Stewardship Program and the UNEP Strategic Approach to International Chemicals Management perfluoro initiative (both led by the US EPA) involve reductions of PFOA emissions and transitioning to alternatives.

Effects: Little studied to date but very limited evidence of in utero or perinatal exposure in association with adverse pregnancy outcomes in people (Chapter 2.2) and with obesity (Chapter 2.10). Adrenal glands are a potential target for these compounds (Chapter 2.8)

Reviews: Houde et al., 2011; Lindstrom, Strynar & Libelo, 2011; Stock, Muir & Mabury, 2010; Ahrens, 2011

Di(2-ethylhexyl) phthalate (DEHP)



Characteristics: DEHP is a diester, and an oily liquid at room temperature. It has low water solubility, and a log K_{OW} of around 7.5.

Origin and use: DEHP is widely used (>95%) as a plasticizer for polymers (mainly PVC but also other vinyl resins and cellulose ester plastics), and can be up to 30% of the product weight. The addition of DEHP improves the flexibility and workability of the plastics. Flexible PVC is used in medical devices, toys, cables, flooring, and other building materials. DEHP is also used as an additive in advanced ceramics for electronics and structural materials, and in printing inks, lacquers, paints, adhesives, sealants and rubber. In the EU DEHP is no longer permitted for use in toys and childcare articles and, in the USA, the Consumer Product Safety Improvement Act (2008) banned the use of six phthalates in toys and child care articles at concentrations greater than 0.1%.

Fate: DEHP has low reactivity under abiotic conditions except in the atmosphere. It is readily taken up by biota but undergoes metabolic transformations that are catalyzed by lipases and esterases in the gastrointestinal tract and primarily by lipases in other tissues. The monoester of DEHP is the primary metabolite of DEHP but several other metabolites are known.

Effects: Chapter 2, **Table 2.1** on DEHP and other phthalates. Limited epidemiological evidence of associations with fibroids and endometriosis in women. Extensive evidence for testis dysgenesis syndrome in experimental rodents; lowered testosterone, reduced anogenital distance, cryptorchidism, hypospadias and reduced semen quality. Limited evidence for associations between phthalate concentrations in mothers, urine and reduced anogenital distance, testosterone and sperm counts in their children. Very limited evidence for hyperactivity in girls exposed in utero (one study). Phthalate monoesters activate both rodent and human PPARα and PPARγ and also affect pancreatic beta cell function so may have strong negative impacts on human immune function (Chapter 2.11) and diabetes (Chapter 2.10). Limited studies show high plasma concentrations of DEHP are associated with endometriosis in women. Moreover, in accord with rodent studies showing effects on immunity, two case-control prevalence studies describe an association between the concentration of DEHP in indoor dust and asthma and wheezing in children.

Reviews: IARC, 2000; European Commission, 2008; Lyche et al., 2009.

There have been recent reviews of PFCs in the environment and biota (Houde et al., 2006; 2011). Other major classes of PFCs are perfluorinated alkylphosphonates and –phosphinates, and perfluorocyclohexane sulfonates (D'eon & Mabury, 2011; Lindstrom, Strynar & Libelo, 2011).

3.1.1.3 Plasticizers and other additives in materials and goods

The number of chemicals that can be included under this subheading is very large and they belong to very different classes of chemicals. Still, only a few classes of these chemicals have been investigated for their ED properties or toxicological profile in general. There is also an overlap in this section with several of the groups of chemicals described below such as halogenated and non-halogenated phenolic chemicals, pesticides, and additives in personal care products (sections

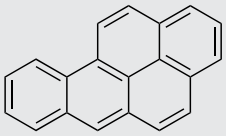
3.1.1.5, 3.1.1.6, 3.1.1.7), i.e. the individual chemicals can be listed in more than one group.

Plasticizers, e.g. phthalate esters (like DEHP, DBP, DiNP and BBP), adipic acid esters (e.g. DEHA) and non-pesticide organophosphate esters (e.g. triphenyl phosphate (TPP)), are commonly used additives in a number of materials and consumer products. Adipic acid esters and non-pesticide organophosphate esters are also used as additives for purposes other than as plasticizing materials. DEHP is used as an example of a phthalate ester plasticizer but it also has other applications (see text box). TPP is also used as a flame retardant additive in association with brominated flame retardants.

3.1.1.4 Polycyclic aromatic chemicals

Other ubiquitous persistent but non bioaccumulative compounds are polycyclic aromatic hydrocarbons (PAHs) that are generated during incomplete combustion of organic material (e.g. coal-fired power plants, residential heating, smoking) and they are also present in food (Boström et al., 2002; Fatoki, Ximba & Opeolu, 2011; Srogi, 2007). Several of the PAHs are referred to as EDCs, with the most well-known marker of PAHs being benzo[*a*] pyrene (BaP, see text box; e.g. Irigaray et al., 2007). PAHs are also commercial chemicals, some of which - e.g.-anthracene - are of very high concern (ECHA, 2012). Further, pyrene (four symmetrical fused rings) and anthracene (three rings) are both high production volume chemicals (US EPA, 2011b) that are used to make dyes and dye precursors.

Benzo[a]pyrene (BaP)



Characteristics: BaP is a polycyclic aromatic hydrocarbon. It is a pale yellow solid and has low water solubility (log K_{OW} = 6.1). BaP and other PAHs are classed as persistent organic pollutants under the United Nations Economic Commission for Europe POPs protocol.

Origin and use: The release of BaP to the environment is widespread as it is a product of incomplete combustion. BaP is mainly released from anthropogenic activities involving the combustion of fossil fuels and wood as well as from industries such as coke ovens and smelters. Major sources are transportation and domestic wood and coal combustion. Smoking of tobacco products or consumption of food, in particular barbecued food, constitutes an additional source of BaP.

Fate: Released BaP is largely associated with particulate matter, soils and sediment. In this form, BaP can undergo long-range atmospheric transport such that remote areas far from the source are exposed. In the gaseous phase, BaP can undergo rapid photooxidation.

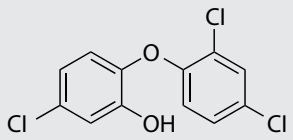
Effects: Exposure to PAH contaminated estuaries associated with neoplasia in wildlife but other contaminants also present (Chapter 2.7).

Reviews: IARC, 2010; Boström et al., 2002; Srogi, 2007

3.1.1.5 Halogenated and non-halogenated phenolic chemicals

Halogenated and non-halogenated phenolic chemicals (HPCs and non-HPCs) have emerged as EDCs of concern. The compounds can be of commercial origin, metabolites of POPs or other persistent organohalogens, or of natural origin. Halogenated phenols like PCP, 2,4,6-tribromophenol, triclosan and tetrabromobisphenol A are commercially produced for a variety of purposes, including use as pesticides (e.g. wood preservatives), antimicrobials and flame retardants. Many that persist are from the metabolism of POPs such as certain PCB (Letcher, Klasson-Wehler & Bergman, 2000) and PBDE (Athanasiadou et al., 2008; Hakk & Letcher, 2003) congeners and HCB (Koss et al., 1979). Phenols are also formed via demethylation of methoxyl groups in neutral compounds, such as pentachloroanisole and methoxychlor. HPCs are in many cases proteinophilic; as a result, they are found in blood and in proteineous tissues.

Triclosan



Characteristics: Triclosan is a trichlorinated phenoxyphenol with broad-spectrum antimicrobial action. It is relatively hydrophobic (log K_{OW} = 4.76). Methyl triclosan, a biotransformation product, is considered more persistent and bioaccumulative (log K_{OW} = 5.2).

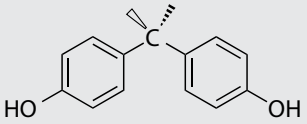
Origin and use: It is widely used in personal care products, but is also increasingly used in consumer products such as kitchen utensils, toys, bedding, socks, and trash bags.

Fate: Triclosan binds readily to soils and is not expected to evaporate from soil or water surfaces. In aquatic environments, triclosan attaches mainly to the surface of suspended solids and sediments and it also bioaccumulates in organisms. Effluents from sewage treatment works (STW) contribute to the widespread occurrence of triclosan in surface waters. Chlorination of STW effluents leads to formation of chlorinated triclosan products that are photochemically transformed to tri- and tetra-chlorinated dioxins when discharged into natural waters (Buth et al., 2011). Aerobic biodegradation is one of the major and most efficient biotransformation pathways for triclosan. Microbial methylation of triclosan has been reported, leading to the more lipophilic methyl triclosan with a higher bioaccumulation potential (Lindström et al., 2002).

Effects: Very little studied but disrupts steroidogogenic enzymes involved in the production of testosterone and estrogen. Could lead to reduced reproductive success in both males and females. Limited evidence of this in laboratory studies (Chapter 2.2 & 2.3). Limited epidemiological evidence for associations with hayfever and other allergies in humans (Chapter 2.11).

Reviews: Dann & Hontela, 2011

Bisphenol A (BPA)



Characteristics: BPA is an industrial chemical containing two 4-hydroxyphenyl rings. As a phenol it is moderately hydrophobic (log K_{ow} = 3.3) and water soluble (solubility = 120 mg/L).

Origin and use: BPA is a high-production volume industrial chemical used mainly in the production of polycarbonate plastic (~95%) and epoxy resins. Polycarbonate plastics are used in reusable food and drink containers including baby, milk and water bottles, in tableware, and in water pipes. Several countries have now banned the use of BPA based polycarbonates in baby bottles. The walls of cans and lids of glass jars and bottles for food and beverages are lined with epoxy resins as a protective coating. BPA is also found in some PVC plastic, and in a variety of paper products (e.g. thermal paper) and recycled paper. The European Food Safety Authority has set a tolerable daily intake (TDI) of 0.05 milligrams/kg body weight.

Fate: BPA is readily biodegradable and its potential to bioaccumulate is low. It can leach from food containers into solids and liquids and has been found in a variety of foodstuffs consumed by humans.

Effects: Environmental estrogen in all vertebrates. Limited evidence for prenatal exposure leading to disruption of estrus and premature cessation of cyclicity and fibroids in rodents (Chapter 2.2). Early exposure induces alterations in the mammary gland that render it more susceptible to neoplasia and to estradiol at puberty (Chapter 2.7). Acts via estrogen receptors in fat cells and cells of brain to regulate adipose tissue deposition and food intake in rodents. No human data. Affects beta cell function, increases insulin resistance and glucose intolerance and secretion in adult male rodents. Limited epidemiological evidence for link with type 2 diabetes and altered liver function in humans (Chapter 2.10). Reduction of thyroid hormone and metamorphosis in amphibians. Learning ability compromised in deer mice (one study), epidemiological evidence of increased maternal urine concentrations associated with increased defeminization of behaviour and hyperactivity (one study), consistent with rodent data, suggesting an effect of BPA on sexual dimorphism of these types of behaviours (several studies; Chapter 2.6).

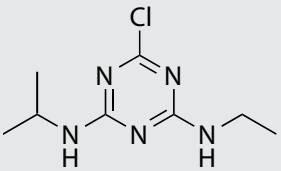
Reviews: WHO, 2011a; EFSA, 2010; WHO, 2011; 2010; Kang, Aasi & Katayama, 2007

Non-halogenated phenols include diethylstilbestrol (once an active pharmaceutical ingredient), bisphenol A, alkyl phenols and parabens. These chemicals have short half-lives but can, in the case of continuous elevated exposures, build up to concentrations of concern. Both HPCs and non-HPCs can also have a natural origin. For example, 2,4,6-tribromophenol and a hydroxylated PBDE (e.g. 6-OH-BDE47) are natural products, as are several phytoestrogens (see section 3.1.1.9).

3.1.1.6 Current-use pesticides

Increased public and regulatory attention during the 1970s and 1980s resulted in bans of pesticides, particularly those that are persistent and bioaccumulative, which led to the development and use of pesticides that are less bioaccumulative. Current-use pesticides are therefore generally characterized by shorter half-lives in the environment, with chemical properties that do not promote bioaccumulation in sediments or organisms. Still, some current-use pesticides are capable of building up to concentrations in soils and ground waters that may be of concern. These pesticides are used to control a wide range of pests and in a variety of applications. They can be used in building materials, during commercial or non-commercial crop production, by home owners for lawn and garden care, and for golf courses, and put into non-edible consumer products. The structural diversity among the almost 60 listed pesticides within the USA Endocrine Disrupting Screening Program (USA) is noteworthy (US EPA, 2009; 2010b), making predictions of relationships between chemical structure and ED activity very difficult. Many of these have been shown to be endocrine

Atrazine



Characteristics: Atrazine is a synthetic herbicide based on a triazine ring with chlorine and aminoalkyl substituents. Atrazine is relatively water soluble (33 mg/L) and chemically stable.

Origin and use: Atrazine has been widely used as a herbicide to control broadleaf and grassy weeds in major crops. The EU removed atrazine from its list of approved herbicides, but it is still used worldwide in many other countries. More than 35,000 tonnes were used annually in the USA alone in the mid-2000s.

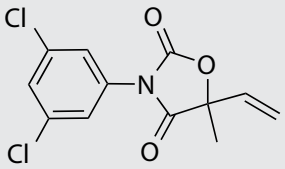
Fate: Atrazine is moderately to highly mobile in soils, especially where soils have low clay or organic matter content. It has a relatively long soil half-life, especially in cold climates. Because it is persistent and does not absorb strongly to soil particles, atrazine and its breakdown products have a high potential for surface and groundwater contamination. Atrazine is transformed to hydroxy compounds and via dealkylation in soils and surface waters.

Effects: Disruption of estrus in rodent studies (Chapter 2.2), intersex in frogs (Chapter 2.3). Exposure of rodents during prenatal period and during lactation results in depression of immune function in the the adult offspring. Amphibian and fish immune function is reduced by ecologically relevant concentrations, regularly accompanied by elevated infections (Chapter 2.11).

Reviews: Giddings et al., 2005; US EPA, 2006a

disruptors in vitro, albeit very few studies have been done in vivo, except for on a select few chemicals. Epidemiological evidence for effects of current-use pesticides and herbicides relates exposure to declines in male reproductive health (Chapter 2.3) and with breast cancer (Chapter 2.7). Several current-use pesticides have also been associated with prostate cancer (methyl bromide, chlorpyrifos, fonofos, coumaphos, phorate and permethrin) and with thyroid cancer (alachlor). In rodents, a number of pesticides induce thyroid follicular cell tumours, including amitrole, ethylene thiourea, mancozeb, acetochlor, clofentezine, fenbuconazole, fipronil, pendimethalin, pentachloronitrobenzene, prodiamine, pyrimethanil, and thiazopyr (Chapter 2.7). Finally, attention deficit disorder in children has been linked to elevated exposure to a variety of organophosphate pesticides (Chapter 2.6).

Vinclozolin



Characteristics: Vinclozolin is a dicarboximide fungicide. It is relatively hydrophobic (log K_{ow} = 3.1) and has a low vapour pressure (1.6x10^{-5} Pa).

Origin and use: Vinclozolin was used worldwide on oilseed crops, vines, fruits and vegetables. It was widely used in Europe until 2007 when a ban was implemented. The USA remains a major use area although uses on some crops were restricted in 2000 and current use is limited to canola and turf. These restrictions on use have led to reductions in quantities emitted to the environment.

Fate: Vinclozolin is transformed in plants and animals by cleavage of the oxazolidine ring to yield dichlorophenyl carbamate related metabolites. Vinclozolin is moderately persistent in agricultural soils with half-lives for total residues (vinclozolin plus its dichloroaniline-containing metabolites) of 179 to >1,000 days.

Effects: Concern about the toxicity of vinclozolin and/or its metabolites is related to its anti-androgenic activity and its ability to act as a competitive antagonist at the androgen receptor (EFSA 2008). Also its terminal metabolite, 3,5-dichloroaniline, is considered a potential carcinogen (US EPA 2000). See also Chapter 2.3, and **Table 2.1**. Anti-androgen that causes lowered testosterone, hypospadias, cryptorchidism, nipple development, and reduced penis size in male rodents. Feminization of male sexual behaviour and loss of sexual interest also in rabbits. Masculinization of females. Most sensitive period is during fetal development and transgenerational epigenetic effects have been reported (Chapter 2.3). Altered sex ratio in experimental studies with fish (Chapter 2.4). Reduced egg laying and fertility in birds (Chapter 2.2). Induces increased Leydig cell tumours in rats and so is a possible human carcinogen.

Reviews: EFSA, 2008; US EPA, 2000.

3.1.1.7 Pharmaceuticals, growth promoters, and personal care product additives

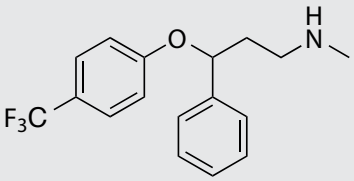
Endocrine-active pharmaceuticals have been developed over the years, and include chemicals to improve pregnancy outcomes (like diethylstilbestrol used to prevent miscarriages; see Chapter 2.1) and ones for contraceptives or hormone replacement therapies, such as synthetic estrogens (i.e. ethinylestradiol or EE2) and synthetic progestagen (e.g. levonorgestrel (Besse & Garric, 2009)) used in, for example, the birth control pill. With the exception of DES, which is no longer produced, many have been used for decades and are still on the market in a variety of formulations and products because of their effectiveness in birth control or hormone replacement therapies. The hormones present in pharmaceuticals, along with naturally-produced hormones, are excreted by women and men and not fully removed through the sewage treatment process. As a result, they are found in the effluents being discharged from sewage treatment works into receiving waters (Monteiro & Boxall, 2010). The feminizing and demasculinizing effects of estrogenic pharmaceuticals, in particular estradiol and ethinylestradiol, have been extensively studied in fish, both in the laboratory and in the field, where they cause intersex, and reduced fecundity and reproductive success in individuals (Chapter 2.3). Higher concentrations can cause population declines (Chapter 2.12).

Although there are many other pharmaceuticals on the market, the understanding of their potential effects on the endocrine system in wildlife, particularly in aquatic organisms, is currently very limited. However, some pharmaceuticals known to interfere with the endocrine system in wildlife include the selective serotonin reuptake inhibitor (SSRI) class of antidepressants. As for the oher endocrine-active pharmaceuticals, these chemicals are excreted by people using these pharmaceuticals and they enter the environment in complex mixtures mainly through the discharge of sewage or from the application of sewage sludge to soils (Monteiro & Boxall, 2010; WHO, 2011b).

Trenbolone acetate, a powerful anabolic steroid, is used in several countries as a growth promoter for beef cattle (Willingham, 2006). 17β-trenbolone is a relatively stable metabolic product of trenbolone acetate and enters the environment mainly in runoff from livestock feedlots. This metabolite has been shown to be strongly androgenic; for example, sex ratios of zebrafish exposed to trenbolone were skewed towards males (Morthorst, Holbech & Bjerregaard, 2010; Chapter 2.4).

Personal care products (shampoos, soaps, lotions, antiperspirants, cosmetics, toothpaste, etc.) contain a large number of chemicals, including some that are EDCs or suspected EDCs. Several of them have been mentioned above, such as parabens, triclosan, and phthalate esters. The cyclic methyl siloxanes are another class of chemicals used in personal care products. They are incorporated to reduce drying time in some rinse-off applications like shampoos and are used as carriers for aluminum salts in antiperspirants.

Fluoxetine



Characteristics: Fluoxetine is a secondary amine (making it ionizable), with two phenyl rings and limited water solubility (log K_{ow} = 4.05).

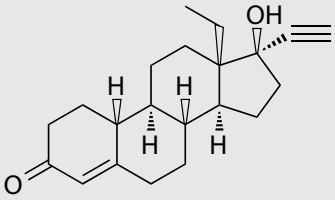
Origin and use: This is a selective serotonin reuptake inhibitor (SSRI) that is widely prescribed for depression, and for premenstrual dysphoric, panic, anxiety, obsessive-compulsive, and eating disorders.

Fate: This chemical is excreted mainly as metabolites by those taking the pharmaceuticals. Once in the sewage stream, fluoxetine and its metabolites may not be broken down during effluent treatment and can enter the environment with wastewater discharges.

Effects: Targets seratonergic modulation in the neuroendocrine brain and so has the potential to affect sex hormones, reproductive function and behaviour, feeding and energy metabolism in fish and other aquatic vertebrates (Chapter 2.6). Also influences the stress endocrine axis in fish (Chapter 2.8). Reduced growth and development, driven by reduced feeding rate in tadpoles (Chapter 2.6). Premature release of eggs and of non-viable larvae in freshwater molluscs (Chapter 2.6).

Review: Oakes et al., 2010

Levonorgestrel



Characteristics: Levonorgestrel is a steroid substituted with an ethinyl group. The compound is sparingly soluble in water, with a log K_{ow} of 3.

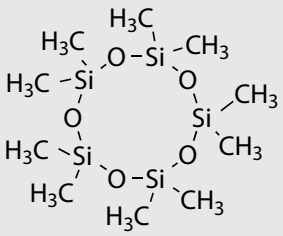
Origin and use: This is a progesterone-related chemical that is used in a number of oral contraceptives currently on the market.

Fate: This chemical is metabolized and excreted by those using levonorgestrel-containing pharmaceuticals. Once in the municipal wastewater stream, it can be found in the aquatic environment because it is not completely broken down during effluent treatment.

Effects: Little studied but detected at therapeutic levels in the blood of fish exposed to sewage effluents (higher than those in women on the contraceptive pill). Can cause reduced fecundity and cessation of egg laying in female fish and sub fertility and loss of sexual interest in male fish (Chapter 2.2 & 2.3).

Review: Besse & Garric, 2009

Cyclic methyl siloxanes



Characteristics: Cyclic methyl siloxanes, e.g. D4, D5 (the structure shown), and D6 are volatile, low-viscosity silicone fluids consisting of [-Si$(CH_3)_2$O-]× structural units in a cyclic configuration. They have an intermediate molecular weight (< 600 g/mol) and notable high vapour pressures (4.73-157 Pa). They are hydrophobic (log K_{ow} of 6.8 – 9.1).

Origin and use: D4, D5, and D6 are high production volume chemicals with a wide range of uses. They are used in hair and skin care products and antiperspirants and cosmetics (under the name cyclomethicone). The main use of D4 is for production of polydimethyl siloxane (PDMS, used for sealants and many other products) polymers and D4 is a residual (<0.1-3%) in these polymers. D5 and D6 are also used in production of PDMS. Volatile methyl siloxanes are used as an alternative to chlorofluorocarbons as a means of reducing the regulated volatile organic carbon content in products, and at parts per million levels as defoamers in pesticide formulations, pulp and paper, food, petrochemical, petroleum, chemical manufacturing, and water treatment. Thus there are direct releases to the atmosphere, to wastewaters and to indoor air.

Fate: D4, D5 and D6 are predicted to be persistent in air, with calculated atmospheric half-lives of more than 3 days. Thus they have the potential to be transported over longdistances in the atmosphere and have been detected in remote regions (Genauldi et al. 2011). Biodegradation studies indicate that D4, D5 and D6 undergo only limited transformation in most sediments or soils. However hydrolysis can be an important breakdown pathway in dry soils with high clay content (Xu, 1999; Xu & Chandra, 1999).

Effects: Little studied but some are estrogenic. Induce testicular atrophy and disturbances in spermatogenesis in several mammalian species (monkeys, dogs, rabbits and rats). Disruption of the estrous cycle and an increase in uterine weight in females (Chapter 2.2 & 2.3). No epidemiological studies.

Reviews: Environment Canada/Health Canada, 2008a; 2008b; 2008c. Genualdi et al., 2011.

3.1.1.8 Metals and organometallic chemicals

Many metals and metalloids are endocrine disruptors, disrupting a whole host of hormone pathways. They are found in rocks and soils and are common in ground and surface waters because of natural weathering processes. However, many metals are used in commercial products or are released into the environment during mining and metal smelting, the production of electricity using fossil fuels, and waste incineration. ED properties have so far been recognized for arsenic, cadmium, lead and mercury (mainly in its organic form methylmercury; see text box below).

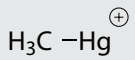
Arsenic is a metalloid found naturally in minerals worldwide and is present at trace levels in all soils, waters (ground and surface), and air (IARC, 2009). It is released to the atmosphere and to waters by weathering of rocks and volcanic eruptions. It is found in both organic and inorganic forms and its environmental fate and toxicity depend on the form of arsenic. High environmental levels of arsenic can be caused by combustion of fossil fuels, mining, and ore smelting. Similarly, arsenic has been used as a pesticide and in wood preservatives and these uses have led to localized contamination (IPCS, 2001).

Cadmium is a commonly occurring metal that is mainly found associated with ores of zinc, lead and copper. It is released from the earth's crust into water and air from natural weathering and from volcanic eruptions. Cadmium is also mined for a variety of uses including as protective plating on steel, stabilizers for PVC, pigments in plastics and glasses, and components of alloys and of electrode material in nickel-cadmium batteries. The mining, processing and disposal of cadmium-containing ores and products have resulted in high localized environmental contamination and this metal is also found as a contaminant in food (see reviews of cadmium by IPCS, 1992b; WHO, 2007b; IARC, 2009).

Lead is also a metal found commonly in rocks and soils worldwide, and is released through natural processes of geologic weathering and volcanic eruptions. Humans have used this metal in many different products including in batteries, ammunition, pigments for paints, ceramic glazes, crystal tableware and gasoline (as an additive), and it was put in solder used in food cans and water distribution pipes. A number of human activities can contaminate the air, water, and soils with lead including mining, smelting, burning of leaded gasoline or other fossil fuels, and recycling or disposal of lead-containing products (see also reviews of lead by WHO, 2007b; IPCS, 1995; 2012; IARC, 2006).

Metal exposure can target all five steroid receptor pathways (estrogen, progesterone, testosterone, corticosteroids and mineralocorticoids) and also receptors for retinoic acid, thyroid hormone and peroxisome proliferators (PPAR). It can affect embryonic, fetal and postnatal developmental processes in frog and fish models and in mice. Arsenic exposure, in particular, is strongly linked with increased prostate cancer risk (Chapter 2.7) and is also a powerful immunosuppressant (Chapter 2.11). Cadmium exposure has also been linked to prostate cancer in some, but not all epidemiological studies (Chapter 2.7). Some

Methylmercury



Characteristics: Methylmercury is an organic form of mercury that occurs naturally in the environment. It is proteinophilic, binding to the sulfhydryl groups in proteins.

Origin and use: Methylmercury is produced from inorganic mercury mainly by certain types of bacteria found aquatic systems. Inorganic mercury is present in rocks and soils and is released by weathering and by a number of human activities. The inorganic form of mercury has been used in a number of products (thermometers, fluorescent lights, batteries, switches, dental amalgams) and is released through natural (geological weathering, volcanic eruptions) and human (mining, chlor-alkali plants, coal and waste combustion) activities into the atmosphere or to terrestrial and aquatic systems. Methylmercury itself is not used in any consumer products or industrial processes but inorganic mercury is still used for mining and by industries in the production of various materials and goods.

Fate: The environmental cycling of methylmercury is complex and affected by many different physical, chemical or biological processes (**Figure 3.5**). Methylmercury has a high affinity for particles or dissolved organic carbon in waters. It also concentrates into bacteria, plants and algae at the bottom of aquatic food webs and then is transferred and biomagnified as those organisms are eaten by progressively higher levels in the food web. The biomagnification of methylmercury in aquatic food webs can lead to high concentrations in fish-eating fish, birds, and mammals (including humans). Many countries have fish consumption advisories to reduce the risk to human consumers, and there is a current global review of mercury led by UNEP to be finalized in 2013 (UNEP's Global Mercury Partnership (UNEP 2011a)). Human activities have increased the levels of mercury in the freshwater and terrestrial environment by 2-3 times.

Effects: Well studied element. Mercury crosses the blood-brain barrier and reduces the levels of key enzymes critical for reproduction, cognition, growth and development in vertebrate wildlife. Elevated exposure of fish and amphibians to methylmercury also impairs behaviours that are critical for successful reproduction, avoidance of predators and feeding; younger animals are more sensitive. In aquatic birds methylmercury exposure at environmentally relevant levels can interfere with reproductive success due to neuroendocrine disruptive effects on courtship behaviour and mate choice (Chapter 2.6). These effects may be relevant to the sustainability of bird populations (Chapter 2.12).

Reviews: Pacyna et al., 2010; WHO, 2010b; Dietz, Outridge & Hobson, 2009; Tan, Meiller & Mahaffey, 2009; Mergler et al., 2007; WHO, 2007b; 2007c; IPCS, 1990.

metals, such as cadmium and arsenic, also have diabetogenic effects in rodents and for arsenic, similar effects are indicated in epidemiological studies (Chapter 2.10). Lead has been particularly associated with delayed puberty (Chapter 2.2) and with cognitive problems in children, and incuding attention deficit disorder (Chapter 2.6).

Organotin compounds [e.g. tributyltin (TBT)] are also of concern due to their ED activity. TBT was heavily used in anti-fouling paints for boats and ships, but is now mainly banned from use because of its persistence, and its accumulation in and effects on aquatic wildlife, particularly snails, living in areas with heavy boat traffic (see Chapter 2.2, 2.4, 2.10-2.12). Tributyltin compounds (oxides, chlorides, hydrides) continue to be used in wood preservatives and as chemical intermediates (ECHA, 2011; US EPA, 2011b). Triphenyltin hydroxide/ acetate, once widely used as a fungicide (IPCS, 1999), has been restricted or banned for most agricultural uses in Europe and the USA However, other triphenyltins remain in commerce (ECHA, 2011; US EPA, 1999b; 2011b).

3.1.1.9 Natural hormones and phytoestrogens

Humans and mammals excrete the steroid hormones, e.g. estradiol, estrone, testosterone and estriol, used for controlling sexual development and reproduction. Considerable amounts of natural steroidal hormones are also produced by livestock and found in animal wastes. Some of these natural hormones are released directly to the environment in untreated sewage, or are not completely broken down during wastewater treatment and discharged in sewage treatment works effluents. In addition, animal wastes from agriculture can be a source of hormones to the terrestrial and aquatic environments when manure is applied to fields and the hormones are washed with rainfall into nearby streams. These endogenous steroidal EDCs are characterized by extremely high potency as compared to other non-hormone EDCs (for effects on fish see Chapter 2.3).

Phytoestrogens are plant-derived natural non-steroidal compounds that show estrogenic activity due to their structural similarity with the female sex hormones. Some typical phytoestrogens are the isoflavonoids, coumestans and prenylated flavonoids. A Canadian study on the levels of nine common phytoestrogens in a Western diet showed that foods with the highest relative phytoestrogen content were nuts and oilseeds, followed by soy products, cereals and breads, and legumes (Thompson et al., 2006; Pongratz & Vikström Bergander, 2011). The natural steroidal hormones and phytoestrogens are not included further in this chapter since the focus is on anthropogenic EDCs. However, it is important to recognize that these chemicals are also contributing to the overall issue of EDCs (see Chapter 2.2).

3.1.2 Structural features of EDCs

In general it is not possible to determine whether a chemical is an endocrine disruptor based on its structure, as there are a multitude of mechanisms and pathways by which a chemical can have an effect. In some cases, endocrine activity may not be attributed to the parent compound, but instead to one or several of its metabolites. Still there are some structural features that are indicative of estrogenic, thyroidogenic and glucocorticoid activities. Estrogenic compounds often include a phenolic ring, similar to endogenous (naturally-produced) estrogens, and no halogens, as exemplified in **Figure 3.1**. The figure also shows a few estrogenic compounds with very different structures. Typical thyroidogenic EDCs are substituted with chlorine or bromine atoms next to the OH group in a phenol and are similar in structure to natural thyroxin (T4), as exemplified in **Figure 3.2**. The aryl methyl sulfones shown in **Figure 3.3** indicate the importance of the sulfone functional group for interference with glucocorticoid hormone activity.

3.1.3 Environmental distribution and fate of EDCs

EDCs enter the environment during their production, use and disposal (**Figure 3.4**). When raw chemicals are manufactured, EDCs are released in industrial discharges. However, larger emissions occur when chemicals are incorporated into materials and products - such as plastics, furniture, carpets, and electrical equipment - by downstream manufacturers and when these goods are used. Emissions during manufacture or use can be to water, soils, and the atmosphere, due to the release of EDCs from materials and products, and from the disposal, incineration or recycling of wastes (e.g. e-wastes). The chemicals in products used in homes for cleaning, bathing, and health are washed down the drain or flushed down the toilet into the sewage system. Some EDC-containing sewage effluents enter the environment untreated, while others receive some form of treatment before they are released to aquatic systems. Other sources of EDCs to the environment include runoff from agricultural fields, emissions from urban areas, and long-range transport via air and ocean currents to remote environments. EDCs enter the environment from both point (discrete) and non-point (diffuse) sources as described below. There are no environmental distribution and fate characteristics that are specific to EDCs because of their broad range of physical and chemical properties.

3.1.3.1 Point sources

Sewage effluents have been identified as a source of a diverse mixture of EDCs to the aquatic environment. These waters from homes and industries include natural and synthetic hormones (estrogens, androgens), active ingredients in pharmaceuticals, metals, pesticides, personal care product additives, and industrial chemicals. Over one hundred pharmaceuticals (not including their metabolites) used by humans have been detected in effluents and surface waters at concentrations ranging from low parts per trillion (ng/L) to parts per billion (µg/L) and include analgesics, anti-inflammatories, anti-depressants, anti-epileptics, lipid regulators, several classes of antibiotics, β-blockers, antineoplastics, and hormones (Monteiro & Boxall, 2010).

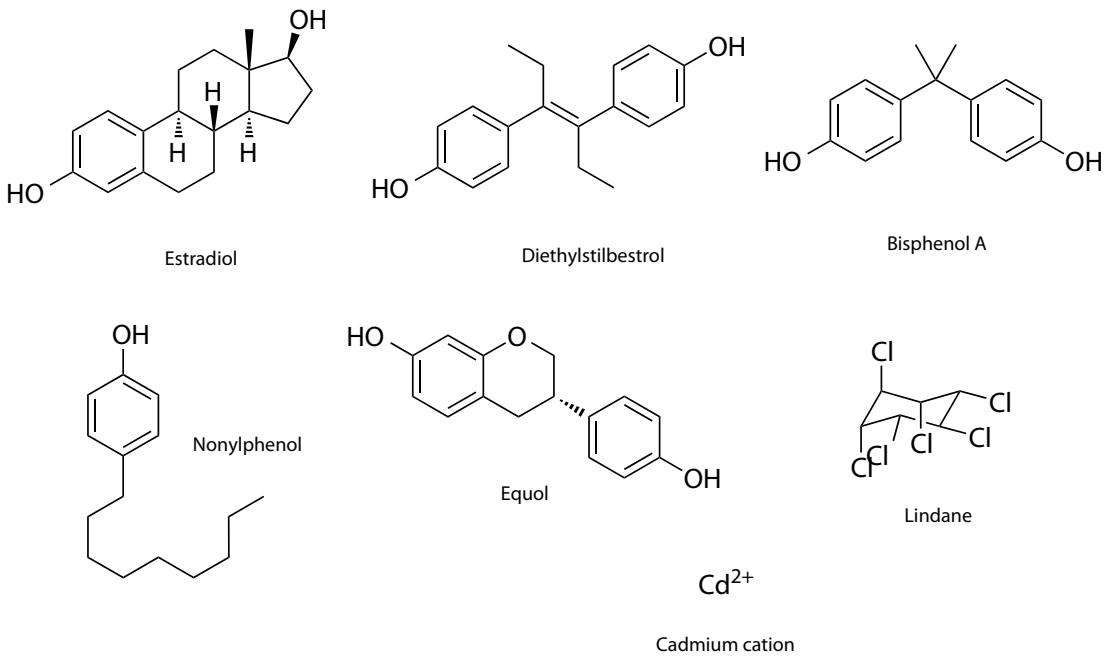


Figure 3.1. Chemical structures of a few estrogenic compounds, including natural 17β-estradiol. The examples show the great variability in the structures of chemicals that have estrogenic effects.

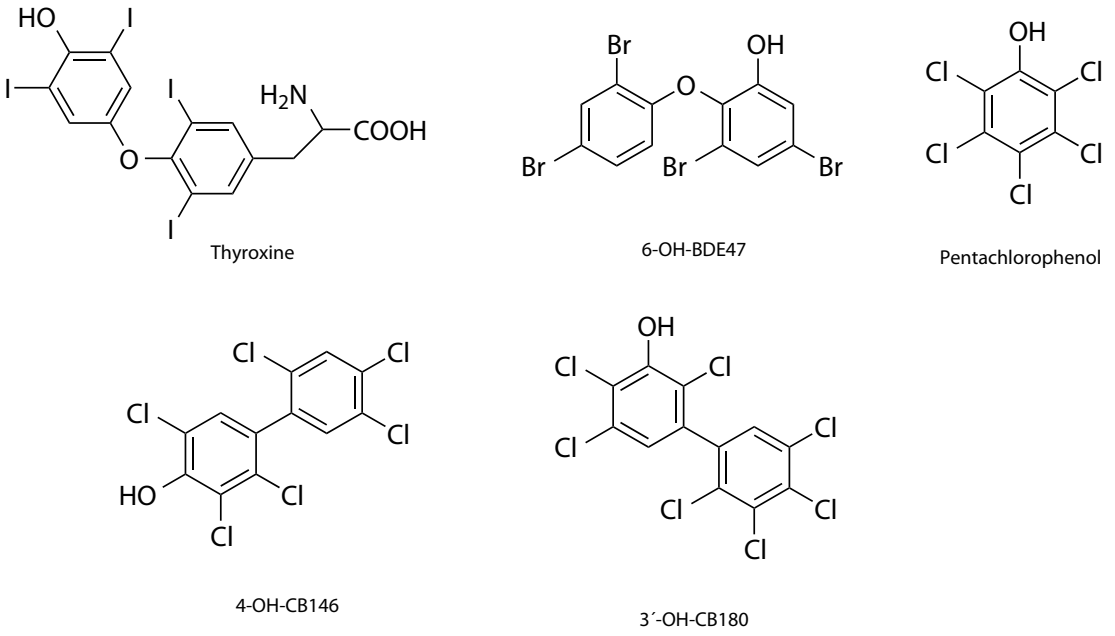


Figure 3.2. Chemical structures of a few thyroidogenic compounds, including natural thyroxin (T4). There is, at least in part, a general structural resemblance between the chemicals with effects on the thyroid system. Abbreviated names are given in full in Appendix II (List of chemicals).

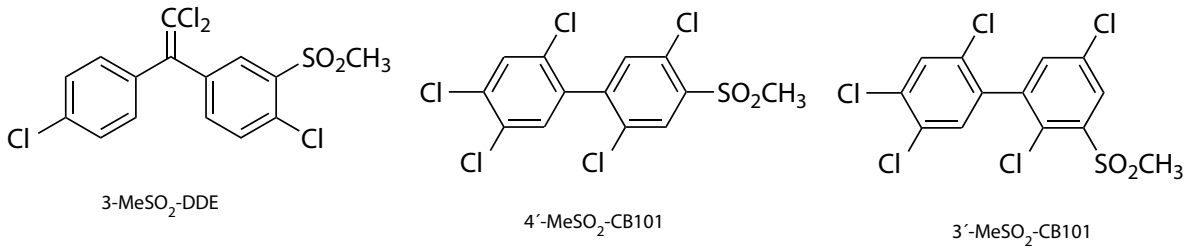


Figure 3.3. Chemical structures of aryl methyl sulfone compounds, all known to exhibit glucocorticoid activity. Abbreviated names are given in full in Apppendix II (List of chemicals).

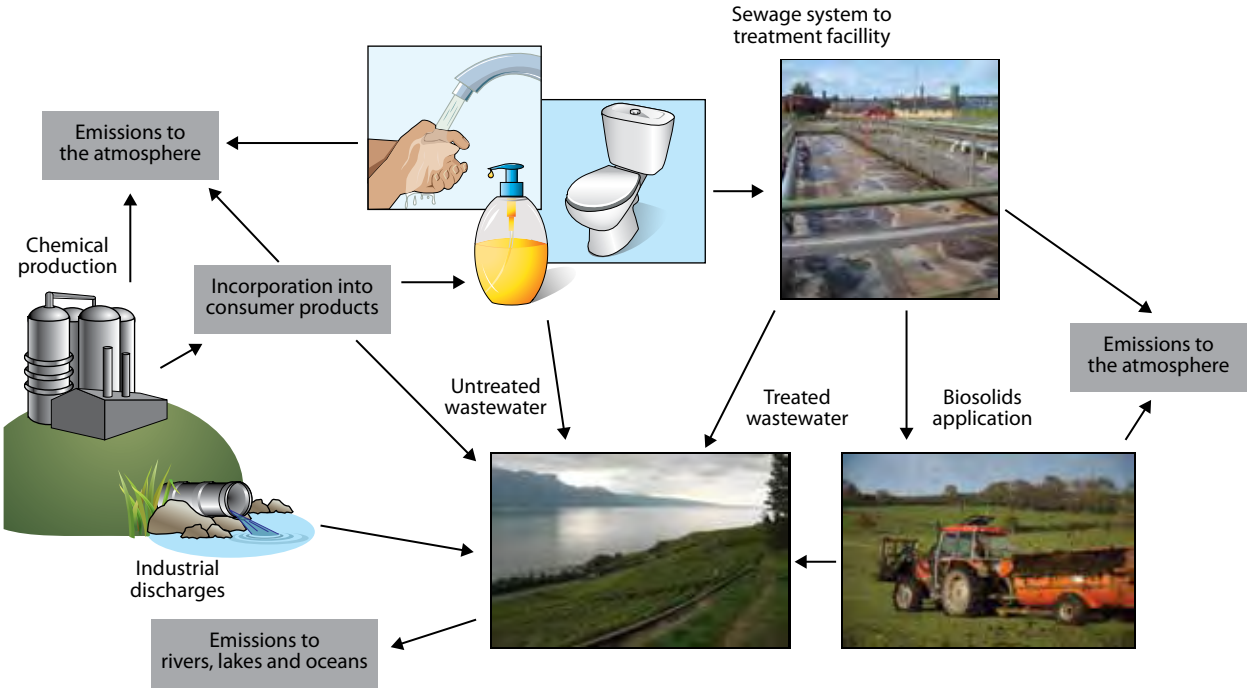


Figure 3.4. Environmental releases of EDCs from the manufacturing stage through use and disposal of personal care products and pharmaceuticals.

Some human pharmaceuticals are excreted in urine and faeces as metabolites (e.g. carbamazepine, an antiepileptic drug, has 5 different metabolites) and these compounds can be found in wastewaters at much higher levels than the parent chemical (la Farré et al., 2008). The kinds and concentrations of chemicals found in the effluents and receiving waters depend on the source of the wastewaters (industrial, municipal (this also depends on population demographics)) and the type of treatment the wastes received. Other point sources of concern include industries producing EDCs for commercial use; for example, effluents from pharmaceutical companies have been found to contain high concentrations of ciprofloxacin (Fick et al., 2009; Larsson, de Pedro & Paxeus, 2007). Globally, however, municipal wastewaters are believed to be a much greater source of pharmaceuticals to the environment (Monteiro & Boxall, 2010; WHO, 2011). The following are some examples of EDCs present in sewage and other point source discharges.

Triclosan: Sewage treatment works vary widely in their ability to degrade the antimicrobial triclosan. As a result, it is commonly found in municipal effluents and biosolids up to 3 µg/L and 33 µg/g, respectively, and is also detected in receiving waters (< 2.3 µg/L) and sediments (< 800 ng/g) (see review by Dann & Hontela, 2011). During the wastewater treatment process, triclosan is also converted via biomethylation into methyl triclosan, a chemical that is more persistent in the environment and more likely to accumulate in fish.

PFOS: Another EDC commonly found in sewage effluents and waters downstream of the discharges is PFOS. This chemical has been detected in effluents (up to 993 µg/L) and river waters (up to 193 µg/L) (see review by la Farré et al., 2008).

Bisphenol A: This chemical is present in municipal and industrial (producing chemicals and chemical products, printing paper, paper recycling, and packing-board paper plants) wastewaters. It is not completely broken down during wastewater treatment (37-94%) and is present in the effluent and sludge (Kang, Aasi & Katayama, 2007; Lee & Peart, 2000). As a result, it has been detected in ground and surface waters (up to 20 µg/L), in river sediments (up to 1.63 mg/kg dry weight), and in the leachate from landfills (Focazio et al., 2008; Kang, Aasi & Katayama, 2007).

Fluoxetine: Due to the variable effectiveness of sewage treatment in removing fluoxetine (ranges from <40 to >90%; Monteiro & Boxall, 2010), this antidepressant has been measured in municipal wastewater effluents and downstream waters at a number of locations worldwide. Concentrations up to 99 ng/L, 46 ng/L and 0.37 mg/kg dry weight have been reported in effluents, river waters, and treated sewage sludge, respectively (Monteiro & Boxall, 2010).

Levonorgestrel: Few environmental measures of this progestagen exist. However, some studies have measured it at low ng/L concentrations in municipal effluents (e.g. Fick et al., 2010).

The EDCs present in sewage occur as either the parent compound or as metabolites. Microbial transformations during sewage treatment can convert metabolized chemicals back to the parent compounds, resulting in the release of EDC-active chemicals; for example, conjugated ethinylestradiol excreted by women taking the birth control pill is converted back to the original parent form in sewage treatment works. Demethylation is known to play an important role in the transformation of phytoestrogens and methoxychlor to ED-active species (Cravedi & Zalko, 2012).

3.1.3.2 Non-point sources

In areas of food production, storm runoff from agricultural fields is an important non-point source of EDCs (pesticides, hormones, pharmaceuticals) to aquatic systems. The pesticides used on crops and for other agricultural purposes are found in ground water and in streams in these regions, especially after a heavy rainfall. Animal wastes are also washed into surface waters by rainfall and contain endogenous hormones, growth promoters and pharmaceuticals. Other EDCs are found in the sludge (biosolids) that remains after sewage is treated (Citulski & Farahbakhsh, 2010). If these solids are applied to fields, EDCs like triclosan are found in nearby surface waters (Edwards et al., 2009).

Urban areas have high rates of material, energy and chemical use, leading to emissions of a diversity of chemicals into the environment (Hodge & Diamond, 2010). Typical EDCs emitted in urban areas are: PAHs from fossil fuel combustion by vehicles; PCBs from older paints and sealants; BFRs from consumer goods with flame retardants; and PCDD/Fs from medical or municipal waste incinerators. Emissions are difficult to quantify as they originate from a myriad of sources, but clear, decreasing chemical concentration gradients have been found from urban to rural locations (Harrad & Hunter, 2006).

Some EDCs are transported long distances to remote environments via air and water currents. The atmospheric pathways are particularly important for highly persistent, semi-volatile EDCs such as PCBs, DDTs, pentaBDEs, current-use pesticides (e.g. endosulfan), as well as PFOS precursors (perfluorosulfonamido alcohols) and PFCA precursors (polyfluorotelomers). Highest concentrations of HCH isomers in the world's oceans are in the Arctic, great distances from where they were originally released (deWit et al., 2004). Highly persistent water soluble chemicals (e.g. PFOS and PFOA, see below) are thought to be transported to remote environments mainly via ocean currents (Armitage, MacLeod & Cousins, 2009; Armitage et al., 2009; Prevedouros et al., 2006) although atmospheric transformation of their volatile precursors also contributes to the burden in remote environments (Schenker et al., 2008; Wallington et al., 2006). The β-HCH isomer is also thought to be transported mainly via ocean currents. β-HCH is the predominant chemical form of HCH of concern for top aquatic predators because it biomagnifies and apparently resists transformation (Willett, Ulrich & Hites, 1998).

Non-point sources are also important for contamination of remote environments with PFCs. Transport by ocean currents is thought to be the main pathway for the global distribution of PFOS and PFOA (Armitage, Mac Leod & Cousins, 2009; Armitage et al., 2009; Stemmler & Lammel, 2010; Wania, 2007; Yamashita et al., 2008). Redistribution of these contaminants from lower latitudes to the Arctic Ocean

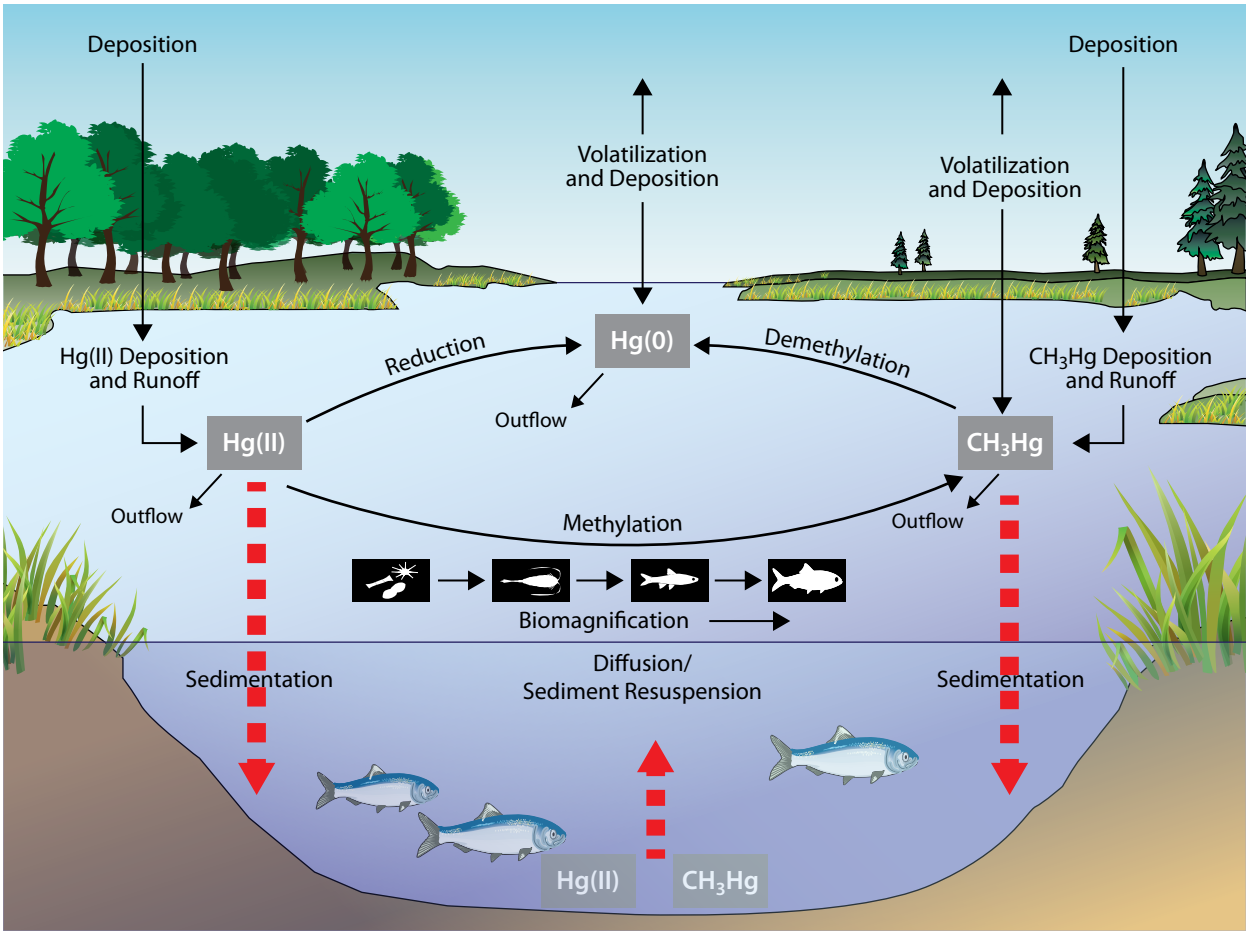


Figure 3.5. The complex cycling of mercury and methylmercury in the aquatic environment (modified from Watras & Huckabee, 1994).

is ongoing and the total mass (and average concentration) of PFOA and PFOS in the marine environment is expected to increase for the next 10 to 20 years based on modeled predictions (Armitage, Mac Leod & Cousins, 2009; Armitage et al., 2009; Wania, 2007). However, the withdrawal of PFOS from commerce in North America and Europe, as well as its inclusion in the Stockholm Convention, should lead to declining concentrations in the environment. On the other hand, there are many chemical precursors of PFOA that continue to be produced, resulting in continued emissions to the atmosphere as well as to surface waters (Armitage, Mac Leod & Cousins, 2009). Loss through settling and mixing to the deep waters of the ocean was estimated to remove approximately 25% of the total global emissions of PFOA over the period 1950 to 2004 (Armitage et al., 2009), which implies that these undegradable PFCs will be circulating in the world's oceans for many centuries after emissions to surface waters cease.

Mercury is an EDC that has very important non-point source emissions due to its release to the atmosphere from both natural sources (e.g. geological weathering) and human activities (e.g. burning of fossil fuels and wastes) (Pacyna & Pacyna, 2001; Pacyna et al., 2010). The mercury cycle is very complex and affected by a number of abiotic and biotic processes (**Figure 3.5**). Gaseous elemental mercury, one of the forms released to the air, has an atmospheric half-life estimated at between 6 months and 2 years (Lin et al., 2006; Strode et al., 2008) and can therefore undergo long-range transport and deposition (AMAP/UNEP, 2008). Rainfall removes some forms of mercury from the atmosphere. Once deposited, these mercury species can be chemically reduced to gaseous elemental mercury and then reemitted to the atmosphere (Steen et al., 2009). The repetition of the deposition and reemission cycle constitutes mercury's so-called "grasshopper" motion (Almeida et al., 2005). Thus, mercury that is emitted in one part of the world can eventually be transported to any other location. Mercury deposition in remote freshwater environments has increased two, to three-fold since the advent of the Industrial Revolution (Fitzgerald et al., 2005; Muir et al., 2009). For fish and wildlife the most important transformation is the conversion to methylmercury (**Figure 3.5**), because this is the form that biomagnifies through aquatic food webs and has ED effects in wildlife and humans (WHO, 2010b; 2007b; IPCS 1990; Chapter 2.6).

3.1.4 External exposure of wildlife to EDCs

Understanding the exposure of wildlife to EDCs is crucial but very challenging because some EDCs are not persistent in the environment or organism, and there are a large number of chemicals present in their food and habitat. Wildlife are not exposed to individual chemicals in isolation but to mixtures of chemicals that can affect the endocrine system in similar or opposite ways. It is clear that the timing and levels of exposure are critical to understand because some developmental stages for wildlife, e.g. during the fetal period (Hamlin & Guillette, 2011), are much more sensitive to EDCs than others. Although EDCs can be present in the environment, exposure must occur for the chemical to have an effect. While there is a better understanding of what is present in the abiotic environment, information on what is transferred into organisms – especially to target organs – is often incomplete or missing. In the absence of measured data, environmental fate and bioaccumulation models are generally used to predict exposures of wildlife to EDCs in regulatory risk assessments. Attributing endocrine effects to specific chemicals or groups of chemicals and understanding critical exposures remains a challenge and a research priority.

The exposure of wildlife to EDCs in the environment will come from air, water, food, soil or sediment and depends on the properties and persistence of the EDC. While some are rapidly transformed by sunlight, bacteria and chemical processes, others resist breakdown and can remain in the environment for months to years. As a result, exposures will vary considerably from one type of EDC to the next and it is not possible to define a "typical" route, level, or duration of exposure for the diverse group of substances that are known to be EDCs. Organisms can take up these chemicals from their diet, by inhalation or by absorbing them through the skin, where they travel in the blood to specific tissues and affect the endocrine system.

Wildlife exposures to EDCs are assessed through measures of their external environment (air, water, soil, sediments, food) and also by analysing the chemicals present in their tissues (see section 3.2.1.2). **Figure 3.6** shows the routes of exposure for fish (a representative aquatic organism) and polar bears (a representative marine top predator) to EDCs. Polar bears are typically not exposed to local sources; they have been shown to do little feeding while on land or near dumpsites in communities in the Arctic (Ramsay & Hobson, 1991). Yet as top predators they have some of the highest levels of PFOS, PCBs and organochlorine pesticides of any species due to the long-range transport, deposition and food web biomagnification of these chemicals.

While exposures of species living in remote locations, like the polar bear, are important to understand, aquatic and terrestrial species living in or near urban areas are often continuously exposed to EDCs via sewage treatment works outfalls, urban and agricultural runoff, and industrial effluents (**Figure 3.7**). Once effluents are discharged to aquatic environments, EDCs will be diluted in stream or river waters so that organisms living very close to the discharge will have the highest exposure. Although some EDCs are not persistent in waters (e.g. estrogens persist days to weeks), there is a constant discharge and fish and other organisms are continuously exposed to a mix of persistent and "pseudo persistent" chemicals. Additional exposure sources, especially in marine environments, are accidental or intentional discharges from oil tankers, ships and fuel extraction activities and oil spills.

Water exposure - The importance of water as a source of EDC exposure for wildlife depends on the type of chemical. Some EDCs are more soluble in water (e.g. some pharmaceuticals, current-use pesticides, natural hormones such as estrogens), are found at parts per trillion (ppt, ng/L) to parts per billion (µg/L) levels downstream of their sources, and water

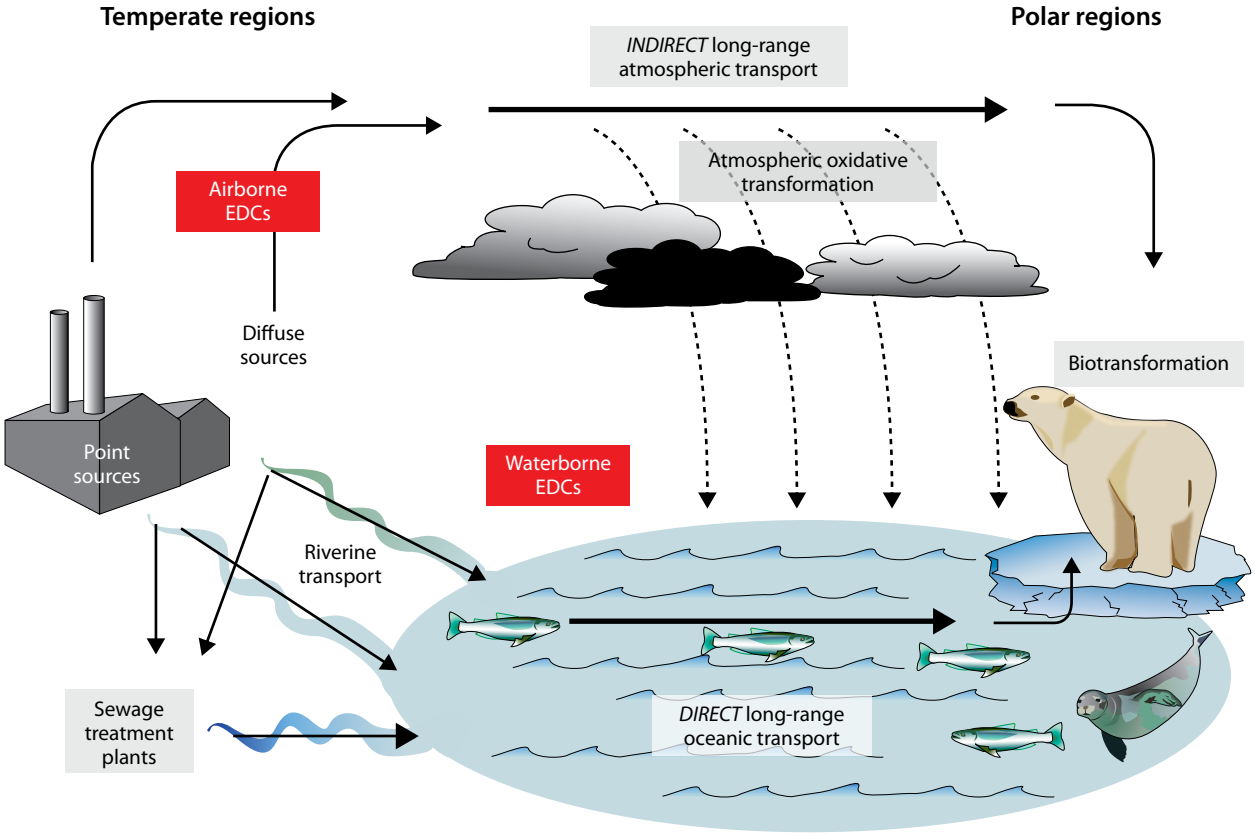


Figure 3.6. Routes of EDC exposure for biota in remote environments, illustrating the importance of long-range transport pathways for wildlife. Based on AMAP (2009).

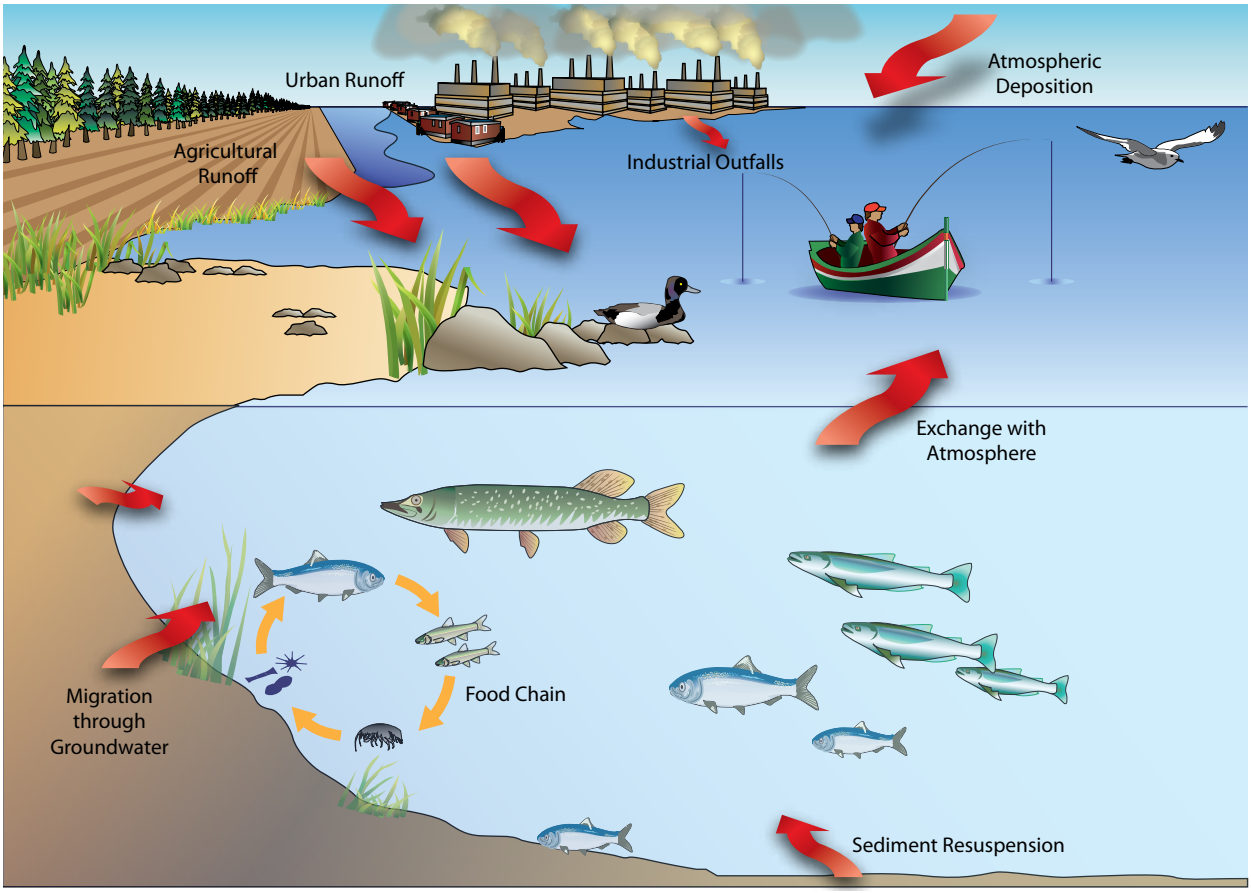


Figure 3.7. Exposure of fish and wildlife in urban regions due to continuous release of EDCs in effluents and to the atmosphere (Redrawn based on a figure from Chapter 4 of The Great Lakes: An Environmental Atlas and Resource Book, www.epa.gov/greatlakes/atlas/).

is the main route of exposure for wildlife to these chemicals. Fish will take these EDCs up through their gills, whereas birds and mammals will be exposed primarily through their drinking water. A diverse mixture of chemicals is present in surface waters and concentrations vary from one site to another and over time at the same site (e.g. Focazio et al., 2008).

Sediment or Soil Exposure- When EDCs are released into the environment, some will bind to soils or to particles and sediments in rivers or other waterways. Organisms living in or on the soils or sediments (worms, snails, some insects) are exposed to these particle-bound EDCs and the EDCs can concentrate into these organisms and up the terrestrial or aquatic food web. While there is a good understanding of how some of the POPs (e.g. PCBs) can, and do, bind to sediments and soils, much less is known about the environmental fate of chemicals of more recent concern. Very few measurements of sediment-bound pharmaceuticals, for example, have been made downstream of sewage treatment works discharges, and it is not well known whether there is much or any exposure of organisms to EDCs through this route.

Diet exposure- Diet is an important source of EDC exposure for wildlife. A number of EDCs are POPs with high affinities for fats and low solubility in water. Because of these properties, these chemicals are well known to concentrate in organisms and through food webs, with higher levels in fish-eating species than those that feed lower on the food web. This concentration – biomagnification – has been well described for many of the chlorinated pesticides (DDT, chlordane, toxaphene) and other POPs (PCBs, PBDEs, HBCDD), as well as for some metals (methylmercury) of concern to the endocrine system. The concentration of EDCs an organism is exposed to depends on the type of diet it consumes, as diets high in fats will have the highest levels of fat-soluble EDCs like POPs. In addition, because these chemicals are taken up into the body faster than they are lost, there is an accumulation of many of them as an organism grows and ages such that higher levels of POPs and methylmercury are found in the older, bigger animals. These processes are well understood for the POPs, but are still poorly understood for the EDCs that are only more recently being measured in the environment (e.g. triclosan, active ingredients of pharmaceuticals).

3.1.5 External exposure of humans to EDCs

The major exposure pathways for humans to many EDCs are via food and drinking water. However, over the past decade it has become clear that humans, in particular small children, are also exposed to EDCs via dust and particles in indoor environments like homes, schools, childcare centres, and offices. These EDCs are released from materials and goods in homes and at work; they are additives in electronics and electric products, textiles and furniture. Also handling of waste (e.g. e-waste) and recycling have been identified as sources of external exposure to EDCs for humans (see section 3.1.5.1). Humans can take up EDCs, or precursors of EDCs, through inhalation of air and particles, consumption of contaminated food and drinking water, and through direct dermal contact, e.g. with cosmetic products (**Figure 3.8**). Human exposure varies considerably and depends on individual habits (e.g. food choices), and the locations where people work and live.

For most adults, exposure to POPs and other persistent organohalogens is primarily through the consumption of fatty foods. However, persistent and bioaccumulative compounds such as BFRs and PFCs are still in use in a great variety of consumer products, and recent studies have demonstrated that concentrations are much higher in indoor than in outdoor environments (e.g. Harrad et al., 2010). It has become clear that infants and small children are at particular risk for exposure to these chemicals through their high hand-to-mouth activity, i.e. ingestion of contaminated dust/particulate matter (Haug et al., 2011; Goosey & Harrad, 2011; Lunder et al., 2010; Trudel et al., 2011). Adults can be exposed to POPs via inhalation but this is most commonly related to occupational exposure. For example, median concentrations of DDT, in blood of spray operators were 11 times higher than those of people living in homes that were sprayed (Table 1 of IPCS, 2011). Dermal uptake of POPs and other persistent and bioaccumulative compounds is regarded as a minor pathway for non-occupationally exposed people.

Humans can be exposed in several ways to other EDCs in materials and goods (e.g. triphenyl phosphate, phthalates, bisphenol A); for example, bisphenol A is in the lining of some food containers and in some paper products such as cash register receipts (Liao & Kannan, 2011; Muncke, 2009). A large number of chemicals are used as additives in indoor materials, food packaging, and other consumer products, and these compounds can leak from the packaging, materials and goods into food or onto dust that is ingested primarily by toddlers and infants. Skin uptake can be particularly relevant for chemicals used in, e.g. cosmetics and other personal care products, but can also occur when receipts are handled (Geens, Goeyens & Covaci, 2011).

Exposure of humans to PAHs occurs through both air (cigarette smoke, fossil fuel and wood combustion) and food. For non-smokers, exposure to airborne PAHs is highest in densely-populated urban areas and in rural areas where wood and coal are frequently used. Coal and biomass burning for cooking and heating in developing countries has been shown to lead to high indoor concentrations of PAHs. However, food appears to be the major source of PAH intake in industrialized countries, with grilled or charred meats, smoked food, contaminated cereals and vegetables as major sources (Srogi, 2007).

For metals, food and drinking water are the major exposure routes in the general adult population. For small children, however, normal hand-to-mouth activity can lead to considerable intake of metal-contaminated dust or soil. In addition, children eat, breathe and drink more per body weight than adults, and often have unique dietary patterns (e.g. consume more of a particular food group) that can increase

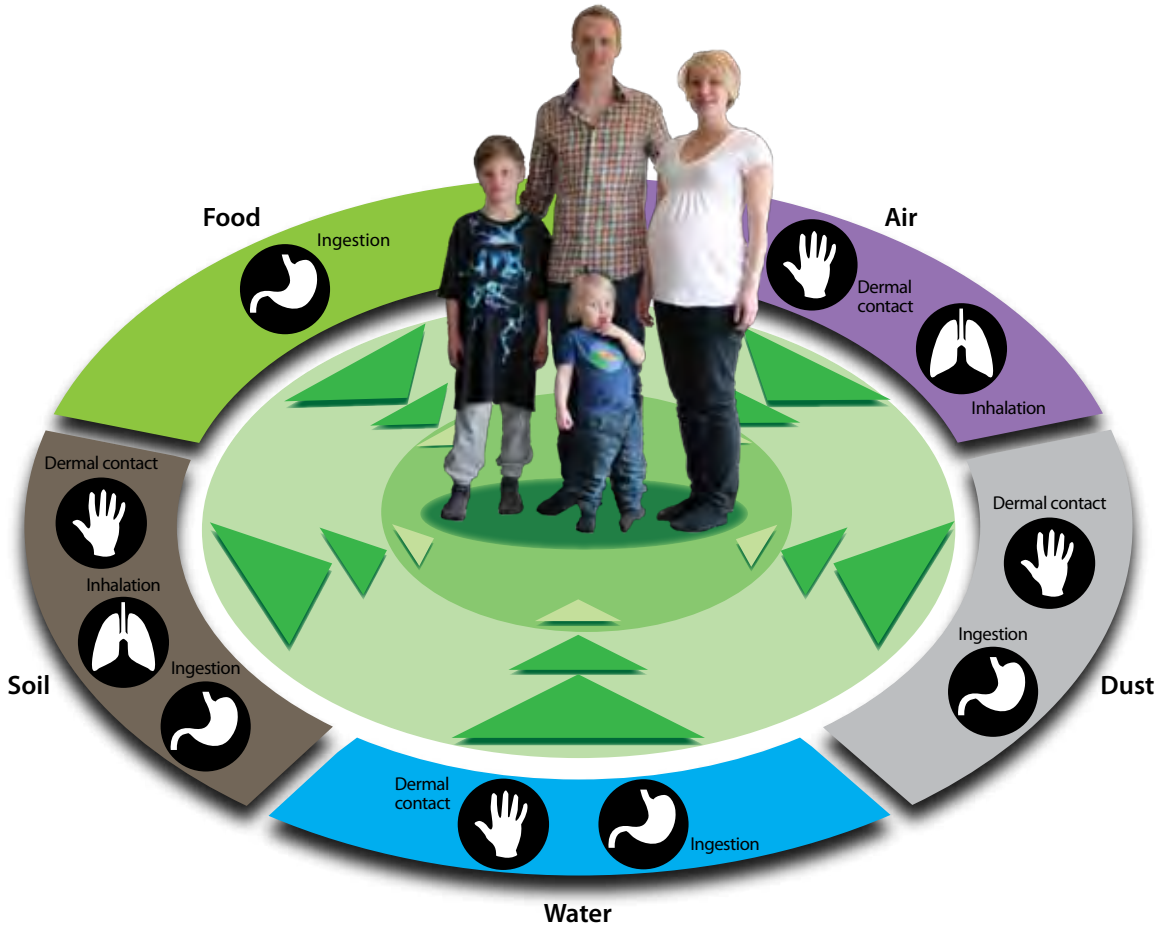


Figure 3.8. Routes of human exposure to EDCs, with each source showing the pathways of EDC uptake.

their exposure to chemicals in those products (WHO, 2007b; National Research Council, 1993). The exposure routes and toxic effects of metals are often dependent on the chemical form (speciation). For example, in contrast to inorganic arsenic species the organic arsenic compounds abundant in seafood are not toxic and are rapidly excreted. Consumption of contaminated ground water is a major source of human exposure to arsenic, with concentrations exceeding the WHO drinking water guideline value of 10 µg/L (IPCS, 2001) in several regions, notably Bangladesh and India (Bhattacharya et al., 2007; Smith, Linga & Rahman, 2000). For mercury, the organic species methylmercury is of major concern as it is accumulated up through food webs, crosses the blood-brain barrier, and is a potent neurotoxic agent (WHO 2010b; 2007c; IPCS; 1990; Chapter 2.6). For this metal, fish consumption is the main exposure route for humans, and many countries have national fish consumption advisories to reduce human exposure.

Exposure to halogenated and non-halogenated phenolic EDCs can occur via intake of food with such chemicals added as antioxidants, e.g. butylated hydroxyanisole (BHA), or through intake of food contaminated with phenolics (bisphenol A, PCP, TBBPA). However, food is possibly not a major pathway of anthropogenic phenolic compounds since their physical-chemical characteristics prohibit accumulation in consumed muscle tissues. Uptake of phenolic EDCs can occur via the skin when they are present in personal care products. Some of the phenolic chemicals are metabolites of POPs that are bound to, and accumulated in, blood and in other tissues (liver, lungs) of animals, i.e. proteinophilic compounds, that are ingested via food. The major proportions of these chemicals in vivo are, however, formed internally.

Food is regarded as the main source for current-use pesticide exposures in the general public. However, pesticides can also be inhaled and absorbed through the skin, particularly by people handling them directly during pesticide application or indirectly when the crop is harvested or processed. The former, in particular, could lead to high exposures.

Human exposure to pharmaceuticals can occur via drinking water, and may be most relevant in areas with extensive pharmaceutical industries and poor drinking water management. The presence of pharmaceuticals in drinking water depends on the source of the water (surface or ground) and its level of treatment (WHO, 2011b).

3.1.5.1 Case study: Exposure from E-waste

Waste electrical and electronic equipment, also commonly referred to as e-waste, describes end-of-life electrically-powered devices, including electronic products such as computers, television sets and cell phones as well as non-

electronics such as refrigerators and ovens. E-waste contains a large number of different chemicals in the plastic and metal components, and several are known or suspected to interfere with the endocrine system of humans and wildlife. More specifically, most e-waste contains a wide range of inorganic contaminants including lead, cadmium, and mercury, and organic pollutants such as PCBs, BFRs and the plastic additives phthalates (Brigden et al., 2005; Puckett et al., 2002; Robinson, 2009). The annual global volume of e-waste is estimated at 20–50 million tonnes and growing at a rate of 3%–5% (UNEP, 2005). The major producers of e-waste are the USA, European Union, China and Japan (Robinson, 2009), but the contribution by developing regions is forecast to increase dramatically in the next decade (Yu et al., 2010).

Despite the existence of the Basel Convention on the Control of Transboundary Movements of Hazardous Wastes and their Disposal since 1992 (UNEP, 2009b), it is estimated that 50%–80% of the e-waste collected for recycling has been exported to developing countries (Puckett et al., 2002). China is the largest recipient of exported e-waste (Liu, Tanaka & Matsui, 2006); others include India, Pakistan (Puckett et al., 2002), Vietnam, the Philippines (Terazono et al., 2006), Nigeria and Ghana (Puckett et al., 2005). Uncontrolled e-waste disposal and crude recycling techniques such as open burning of circuit boards and wires, acid-stripping of metals, and plastic chipping and melting (Wong et al., 2007) result in environmental release of not only contaminants contained in e-waste but also of by-products of the recycling and/or incomplete burning processes such as PCDD/Fs (Leung et al., 2007), their brominated and mixed halogenated homologues (Tue et al., 2010), PAHs and halogenated PAHs (Ma et al., 2009), as well as other EDCs (Owens et al., 2007).

Large quantities of e-waste end up in developing countries, where no infrastructure or protocols to safely recycle and dispose of hazardous e-waste exist, nor legislation dealing specifically with e-waste flow (Caravanos et al., 2011; Frazzoli et al., 2010). In Nigeria about 200 tons of e-waste materials were abandoned at riverbanks in dumping sites, where they are manually disassembled, working pieces are repaired and marketed, and useless junk ends up in open fires or in dumpsites (Frazzoli et al., 2010). The e-waste is recycled in a crude way, primarily involving manual disassembly and open burning to isolate copper from plastics. Some of the work is carried out by children, commonly using only rudimentary tools and with no protective equipment. Overall, there is high local contamination of the environment and people in areas with uncontrolled e-waste disposal and recycling.

Soils near e-waste dumpsites in developing countries are known to have high contamination. For example, soils at informal e-waste recycling sites in Asia have been extensively monitored for the occurrence of chemicals including several EDCs. Extremely high levels of lead, chromium, cadmium, PCBs, PBDEs, PCDD/Fs and PAHs were, e.g. reported in soils at an open e-waste burning area in Guiyu (China) (Wong et al., 2007). Elevated levels of antimony and mercury were also found in soil at an Indian e-waste recycling site (Ha et al., 2009). Other studies investigated dust samples and found high abundances of polybrominated and mixed halogenated dibenzofurans (PBDFs and PXDFs) at a Vietnamese e-waste recycling site (Tue et al., 2010b) and elevated chlorinated PAHs in Taizhou (China) (Ma et al., 2009).

Water and foodstuffs in the areas surrounding e-waste recycling sites in developing countries also become contaminated. In villages situated along the rivers where piles of e-waste are disposed of and burned, people use the river water directly for drinking, cooking and washing (Frazzoli et al., 2010). Fish collected near a recycling site in Qingyuan, China accumulated PCBs and PBDEs up to 16,500 and 1100 ng/g wet weight, respectively (Wu et al., 2008), whereas water birds in the same area accumulated these chemicals up to 120,000 and 2200 ng/g fat, respectively (Luo et al., 2009). In Taizhou, rice was found to be contaminated with lead and cadmium, at 2–4 times higher levels than the limit allowed in foodstuffs (0.2 mg/kg) (Fu et al., 2008), and chicken contained elevated PBDEs (up to 18 µg/g fat)(Liang et al., 2008).

High exposure levels of contaminants related to e-waste have been reported for workers and residents in e-waste handling areas and they are exposed from food, water and air. In Ghana, personal air samples collected from e-waste site workers and the environment revealed elevated levels of aluminium, copper, iron, lead and zinc (Caravanos et al., 2011). The blood levels of lead and cadmium in Guiyu children (mean 130 and 16 µg/L, respectively) (Zheng et al., 2008b) are higher than the levels known to cause neurodevelopmental deficiencies (10 and 0.6 µg/dL fat, respectively) (Chen et al., 2011). In the same region, a high exposure level of neonates to chromium (median 94 µg/L in umbilical cord blood) was associated with DNA damage (Li et al., 2008). Regarding BFRs, the PBDE exposure levels in Guiyu are among the highest ever reported (median 600 ng/g fat in human serum) (Bi et al., 2007). High PBDEs levels in women, independent of exposure source, will result in exposure of the fetus via the placenta and of the baby through nursing (Frederiksen et al., 2009; 2010a; Thomsen et al., 2010a; 2010b). The exposure to e-waste generated contaminants, such as dioxins and related compounds, is also significant. Relatively high PCDDs/Fs are found in mothers' milk from Taizhou (21 pg WHO-TEQ/g fat) (Chan et al., 2007). The daily intake of PCDD/Fs by Guiyu residents from inhalation alone was estimated at 1.8−5.8 pg WHO-TEQ/kg/day (Li et al., 2007), comparable to the WHO tolerable daily intake dose. Adverse health effects related to PBDEs, PCBs and other chemicals from e-waste recycling have not been well studied, but DNA damage (Wen et al., 2008) and hypothyroidism (Zhang et al., 2010) have been suggested.

The Partnership for Action on Computing Equipment (PACE) launched in 2008 is a multi-stakeholder group that provides a forum for governments, industry leaders, non-governmental organizations and academia to tackle the environmentally-sound management, refurbishment, recycling and disposal of used and end-of-life computing equipment (PACE, 2012).

3.1.6 Conclusions

EDCs

- The number of identified chemicals with ED properties has increased dramatically between 2000 and 2012.
- EDCs have diverse chemical and physical properties. Many are produced by humans while others are naturally-occurring in the environment. Some are POPs, a dominant class of EDCs known ten years ago, but the more recently-identified ones tend to be less persistent and less bioaccumulative, such as current use pesticides and plasticizers.
- Numerous EDCs have structures that resemble naturally-produced hormones. In addition, some chemicals can affect the endocrine system in their original form while others are more endocrine active after they are transformed in the body or environment.

Sources and environmental fate of EDCs

- EDCs are present in materials (i.e. packaging), goods (i.e. electronics, furniture, household cleaners), personal care products (i.e. cosmetics, lotions, soaps, shampoos), and pharmaceuticals (typically the active pharmaceutical ingredient).
- EDCs are released to water, soils, and the atmosphere during the production, use and disposal of materials and goods, during food production and processing, and through natural processes.
- The sources of EDCs to wildlife and humans are very diverse and include both point (e.g. effluent discharges) and non-point (e.g. agricultural runoff, urban emissions, long-range transport via wind and ocean currents) sources.
- EDCs have a range of fates in the environment. Some are persistent and will concentrate in soils, sediments or fatty tissues while others are more soluble in water and rapidly broken down.

Exposures

- Wildlife are exposed to EDCs in their diet, and through inhalation and dermal absorption.
- Wildlife living downstream of sewage treatment works discharges are exposed to many different EDCs including active ingredients in pharmaceuticals and additives in personal care and cleaning products.
- Wildlife higher on the food chain are particularly at risk for exposure to POPs and other similar chemicals with ED properties due to their biomagnification through the food web.
- Humans are exposed to EDCs from multiple sources including ingestion of food, dust and water, inhalation of volatile and particle bound contaminants, and dermal uptake.
- Disposal and recycling of e-wastes in developing countries has been identified as a source of EDC exposure for humans and wildlife.
- EDCs found in food include POPs, pesticides, additives in food packaging, metals, and PAHs.
- EDCs are present in personal care products, and their uptake through skin has been recently recognized as a significant route of human exposure.
- Some EDCs, like flame retardants used in furniture and other consumer products, are found at high concentrations in household dust. Young children can have higher exposures to EDCs than adults because of their hand-to-mouth activities and because they play close to the ground.
- Wildlife and humans are exposed to very complex mixtures of EDCs.

3.2 The EDCs found in wildlife and humans

3.2.1 Wildlife

3.2.1.1 Internal exposure

Distribution in the body - Once an EDC is taken up into the body of an invertebrate, fish, bird or mammal from the water or its diet, the EDC can be transported to different tissues, where it can be metabolized, excreted, or stored. Storage of EDCs occurs in the fatty tissues (liver, brain, adipose), in proteins (liver, muscle), and in the bones. Most POPs are lipophilic and stored in fatty tissues of wildlife. However, when fats are mobilized and used by a fish or bird to produce eggs, by mammals to produce milk to nurse offspring, or as a source of energy during periods of low food or starvation, this leads to higher concentrations of POPs in circulating media (blood) which may have adverse effects on the organism. Other EDCs are proteinophilic (e.g. perfluorinated chemicals, methylmercury) and are found at the highest concentrations in protein-rich tissues like liver.

There is also placental transfer of EDCs (e.g. POPs) to the fetus of wild mammals and this can cause effects in the young. Maternal transfer to the fetus is known to occur for PBDEs, mercury, organochlorine pesticides, and plasticizers and can result in a number of effects on the reproductive system of the offspring (see review by Hamlin & Guillette, 2011; see below under 3.2.2 and Chapter 2.2, 2.3 & 2.6).

Metabolism and excretion - There are several enzymes in wildlife that transform EDCs to metabolites. For vertebrates the liver is the main site of metabolism of many of the EDCs and this process typically makes it easier for an organism to remove the EDC from its body in faeces and urine. However, metabolism can also make an EDC more harmful (e.g. hydroxylated PCBs interfering with the transport of thyroid hormones in blood; Bergman, Klasson-Wehler & Kuroki, 1994; Letcher et al., 2000; see Chapter 2.5).

EDCs that have an affinity for fats are deposited into the high-fat eggs of birds and fish, where they can have an effect on the hatching and survival of the offspring (Hamlin & Guillette, 2011). Similarly, milk is high in fats and is a source of EDC exposure for nursing young due to the transfer of lipophilic EDCs like PCBs from the mother into her milk. For example, for High Arctic, Canadian subpopulations, polar bear cubs have higher levels of POPs than their mothers (Polischuk, Nordstrom & Ramsay, 2002), likely due to high exposure of the cubs during nursing.

3.2.1.2 What has been measured in wildlife

Over the last decade the understanding of the types and levels of EDCs found in wildlife has improved. While studies continue to focus on POPs, for example, in Arctic wildlife (e.g. Letcher et al., 2010), a broader range of chemicals are now known to occur in wildlife in remote and urban environments. In these environments, EDCs in some species, especially the long-lived top predators, can exceed levels known to cause effects.

Blood, milk, bile, fat, brain, and muscle tissues (or whole bodies of small organisms like mussels) are used to measure the internal dose of an organism to EDCs. These analyses provide information on what is getting into the body and can be more easily linked to effects on the endocrine system. However, some EDCs are very short-lived in the body and difficult to measure, or they will cause effects that appear later in life, making it impossible to link exposure to adverse responses.

The most well studied EDCs in wildlife tissues are the POPs, including DDT (and its main transformation product DDE), chlordanes, dieldrin, PCBs, "dioxins" (i.e. PCDDs/PCDFs and some dioxin-like PCBs), PBDEs, PFOS and PFCAs. In the previous document much of the discussion focused on PCBs and DDTs, PBDEs were only briefly mentioned, while PFOS and PFCAs were not mentioned at all (IPCS, 2002). The main POPs selected for discussion in this chapter are PCBs, PFOS, and PBDEs (select data are shown in sections 3.2.1.3 and 3.2.1.4). Other POPs with large tissue residue datasets include HCB, chlordane-related compounds, dieldrin/endrin, toxaphene-related compounds, mirex, and HCH isomers (in particular β-HCH). Some data exist for other POPs such as pentachlorobenzene, endosulfan, hexabromobiphenyl, and chlordecone in invertebrates, fish and other wildlife but they are not as comprehensive. Similarly, more limited datasets exist for other persistent organohalogen compounds, which are not on the Stockholm Convention POPs list such as chlorinated paraffins, polychlorinated naphthalenes, octachlorostyrene, pentachlorophenol/anisole, and methoxychlor.

The levels of POPs in wildlife vary from one location to another and are highly dependent on the extent of local contamination. For example, DDT (as 4,4'-DDT and 4,4'-DDE) levels were measured in fish and domestic animals in an area of South Africa where DDT spraying is on-going for malaria mosquito vector control. Average concentrations of DDT (on a fat weight basis) in tilapia were 4 to 6 times higher downstream of a sprayed area than at an upstream site (Barnhoom et al., 2010). Similarly, levels of DDTs in the fat of chickens were much higher (up to 700 times) from the sprayed than non-sprayed villages (Van Dyk et al., 2010).

Some of the chemicals used in pharmaceuticals and personal care products are being found in the tissues of wildlife living near sewage treatment works outfalls. The chemicals from personal care products include benzotriazole UV stabilizers, parabens, triclosan, and organophosphorous compounds (Dann & Hontela, 2011; Kim et al., 2011). Triclosan has been found in a range of aquatic organisms including algae, invertebrates, fish and dolphins (Dann & Hontela, 2011). A number of pharmaceuticals have also been found in wildlife in highly populated areas including the antiepileptic carbamazepine and the active ingredients of several antidepressants (fluoxetine, sertraline, venlafaxine, citalopram, norfluoxetine, diphenhydramine, diltiazem) in muscle or liver of wild fish or fish caged downstream of wastewater outfalls (Brooks et al., 2005; Metcalfe et al., 2010; Ramirez et al., 2009; Schultz et al., 2010, Bringolf et al., 2010). In addition, human contraceptives have been found in fish muscle at low parts per billion levels ((EE2; Al-Ansari et al., 2010) and in the plasma of fish exposed to municipal wastewaters (levonorgestrel up to 12 ng/mL; Fick et al., 2010). Less is known about whether pharmaceuticals and personal care products accumulate in terrestrial organisms. Some studies have shown that earthworms take up triclosan from soils that were treated with solids from municipal wastewater treatment plants or biosolids (Kinney et al., 2008).

There have been measurements of mercury in wildlife for over 40 years and, thus, there is extensive literature on this EDC (Das et al., 2003; Law 1996; Reijnders, Aguilor & Borrell, 2009; Thompson, 1996). Large databases for mercury in fish muscle are available (UNEP, 2002; US EPA, 1999a). Fish accumulate mercury primarily as methylmercury rather than other chemical forms of mercury (National Research Council, 2000; Scheuhamer et al., 2007). Fish-eating marine mammals and birds are therefore mainly exposed to methylmercury, because almost all of the mercury present in their diet is methylated. In tissues such as skeletal muscle, fur, feathers, and eggs, methylmercury is usually the predominant form (Endo et al., 2005; Scheuhammer, Wong & Bond, 1998).

PAHs have been widely measured in wildlife, often as part of monitoring responses to oil pollution events (Hellou, 1996). In marine mammals, levels of these compounds in blubber are generally lower than POPs (Hellou, 1996, Reijnders, Aguilor & Borrell, 2009). Lower molecular weight (2–4 ring) PAHs predominate in most marine mammal blubber samples (Fair et al., 2010; Taniguchi et al., 2009). Relatively high ΣPAH concentrations (on a fat weight basis) have been reported in blood. For example, sea otters from Alaska and the California coast had average Σ_{26}PAH in blood serum ranging from 3.1 to 9.8 μg/g fat (Jessup et al., 2010). Sea otter livers from the same regions had similar Σ_{16}PAH (16 unsubstituted "priority" PAH) concentrations when expressed

on a fat weight basis (Kannan et al., 2008). Highest Σ_{16}PAH were from sea otters collected in Prince William Sound, the site of the Exxon Valdez oil spill. There has been limited study of PAHs in seabird tissues. Measurements of Σ_{16}PAH in seabird livers from the Mediterranean and Eastern Atlantic found at low ng/g wet weight concentrations (Roscales et al., 2011). Seabird eggs have also been shown to generally have low PAH concentrations, with few distinctive differences between geographic areas (Pereira et al., 2009; Shore et al., 1999). Seabird fat from King George Island (Antarctica) had ΣPAHs (20 unsubstituted 2-6 rings + methyl naphthalenes) ranging from 1.5-5.7 µg/g fat, with naphthalene and methyl naphthalenes predominating. Phenanthrene was the most abundant unsubstituted PAH in seabird eggs from the UK coast, while methylnaphthalenes predominated in most other locations (Pereira et al., 2009). Seabird blood has been used as a bioindicator of PAH exposure from oil spills (Pérez et al., 2008). Higher plasma concentrations of Σ_{16}PAH were found in oil-exposed seabird colonies (Pérez et al., 2008; Troisi et al., 2007).

It is clear that wildlife contain a diverse mixture of EDCs. For example, the broad array of known or potential EDCs that have been detected in fish is illustrated in **Figure 3.9** from the Baltic Sea monitoring programme (HELCOM, 2010). Organotins, bis(2-ethylhexyl)adipate, and phenolics including triclosan and chloro- and bromophenols are the predominant compounds. HCB, PFOS and pentaBDEs are the only POPs measured among the 23 chemicals in these fish.

3.2.1.3 Spatial trends for wildlife

The previous global assessment on the State-of-the-Science of EDCs reviewed the spatial trends of PCBs and DDT-related compounds in wildlife with a focus on the Baltic, the Great Lakes, and the Arctic (see Chapter 6 Annex in IPCS, 2002). Here selected studies published since 2002 that have examined spatial trends of major EDCs in invertebrates, fish and other wildlife at global or large regional scales are discussed. Tanabe & Subramanian (2006) reviewed the regional and global spatial trends of POPs in invertebrates, fish and wildlife, with an emphasis on species that could be used for global biomonitoring. They discussed the essential characteristics of animal bioindicators and the important criteria were: (a) the broad geographical range of the species; (b) known feeding habits; and, (c) ease of sampling and sample processing. Thus, marine mammals such as dolphins (blubber), seabirds (especially their eggs), globally-distributed fishes such as tuna, and mussels have frequently been used to study large scale spatial trends of EDCs. Select studies that have examined broad regional or global trends in concentrations of EDCs in wildlife are discussed here. The vast majority of the studies on fish, mammals and birds are on POPs and not on the wider range of known or potential EDCs (see **Table 3.1**). This reflects the fact that vertebrate wildlife are generally not suitable for biomonitoring of more rapidly metabolized chemicals. Therefore results for selected contaminants in mussels were also included.

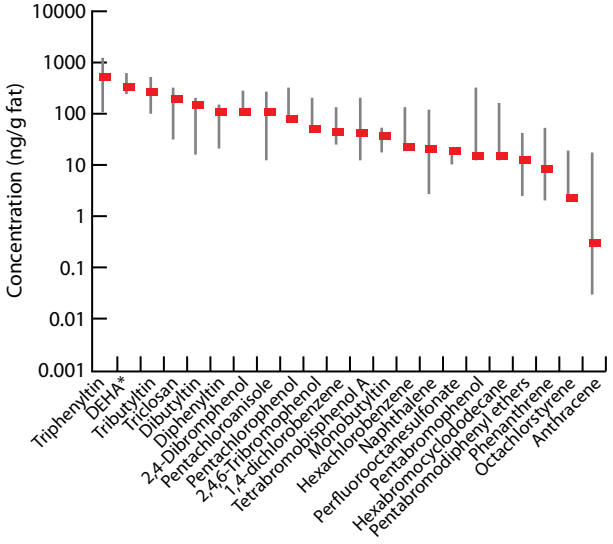


Figure 3.9. Concentrations (ng/g fat) of commonly observed chemicals in muscle of fishes from background areas in the Baltic Sea (HELCOM 2010, original data from Bignert et al., 2006). *DEHA is bis(2-ethylhexyl) adipate.

EDCs in mussels

Over the past 20 years, mussels and other bivalves (e.g. oysters) have been monitored in "mussel watch" programmes in many regions including; the USA, Caribbean, Central, and South America (Barra et al., 2006; Farrington & Tripp, 1995; Kimbrough et al., 2008); Japan, Korea, China, Vietnam and India (Ramu et al., 2007; Tanabe & Minh, 2010); Australia (Prest et al., 1995); the Persian Gulf (ROPME, 2011); the Baltic (HELCOM, 2010); the North Sea and northeast Atlantic (OSPAR, 2009); and the Mediterranean including north Africa (Scarpato et al., 2010). Only sub-Saharan Africa, and parts of the Russian Arctic, Alaskan and Canadian Arctic have not been included in various studies and this may reflect an absence of suitable species. While most of the monitoring has been on metals and POPs, PAHs have been included in many of these programmes (Farrington & Tripp, 1995; HELCOM, 2010; OSPAR, 2009) so that spatial trends in exposure to PAHs of coastal marine environments can be assessed over a broad area. Similarly, spatial trends of TBT have been assessed in the Baltic (HELCOM, 2010), the Northeastern Atlantic (OSPAR, 2009), and in East Asia (Sudaryanto et al., 2002; Choi et al., 2009). Recently the use of mussels has been extended to alkyl phenols, phthalates, bisphenol A, and triclosan (Gatidou, Vassalou & Thomaidis, 2010; Sánchez-Avila et al., 2011). Collectively these studies have shown that EDC concentrations in mussels vary considerably from one location to another and are typically highest in areas where their use and release is greatest. A large study of mussels from East Asian sites surveyed in Malaysia, Singapore, the Philippines, and Indonesia found concentrations of nonylphenol, octylphenol and bisphenol A that were

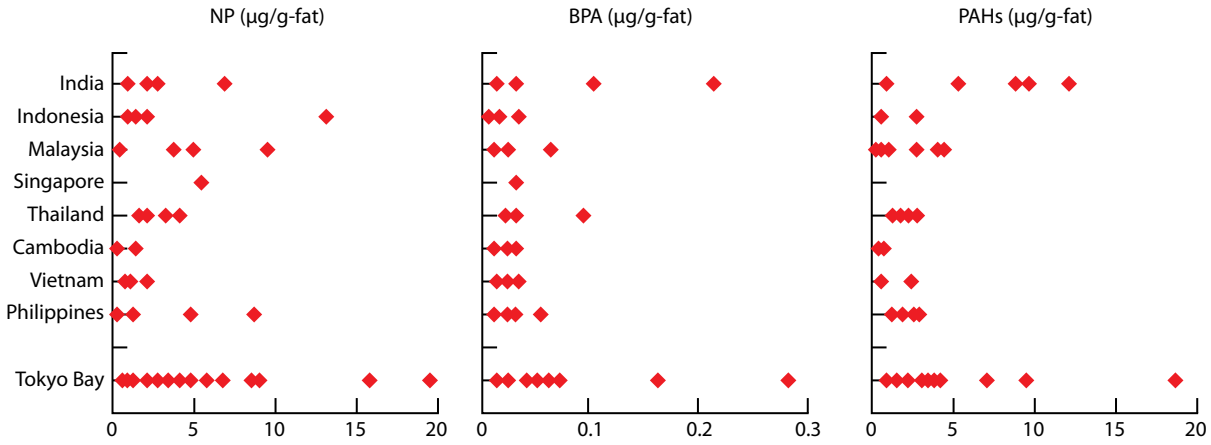


Figure 3.10. Concentrations (µ/g fat) of nonylphenol (NP), bisphenol A (BPA) and PAHs for mussels from South and Southeast Asia and in Tokyo Bay. (Figure based on Isobe et al. (2007)).

comparable to those observed in Tokyo Bay, while much lower concentrations were found in coastal marine areas of Vietnam and Cambodia (Isobe et al., 2007) (**Figure 3.10**).

PCBs and DDTs

The global distribution of PCBs and chlorinated pesticides in skipjack tuna *(Katsuwonus pelamis*) has been reported by Ueno et al. (2003). The geographic coverage of these studies included the Pacific Ocean, the Indian Ocean and the southwestern Atlantic (**Figure 3.11**). Unfortunately the North Atlantic was not included. Nevertheless these studies provide insights into the spatial trends of contamination by POPs in an important food source for humans and in regions with rapidly growing chemical manufacturing and product recycling industries. Elevated concentrations of ΣPCBs and ΣDDTs were found in tuna samples from the western Pacific, more specifically in the south China Sea and Sea of Japan. Relatively high levels of ΣPCBs were also found in tuna collected off the coast of Brazil. In contrast, ΣPCBs were relatively low in samples from the Indian Ocean while ΣDDTs was elevated in the Bay of Bengal region, likely contaminated from use of DDT for agriculture and for malarial vector control.

Many species of marine mammals are highly contaminated by POPs due to their top trophic positions and, in some animals, these chemicals have been linked with population declines (Chapter 2.12). The family *Delphinidae* (dolphins) is globally distributed and there are measurements, mainly in dead/stranded animals, from around the world (Houde et al., 2005). In general, the highest levels of ΣPCBs and ΣDDTs (sum of DDT-related compounds) were found in species inhabiting the midlatitudes of industrialized Asia, North America and Southern Europe (**Figure 3.12**), reflecting the areas where these chemical compounds have been intensively used. Very high concentrations in dolphin blubber are observed, sometimes exceeding 1000 µg/g fat. Possible declining POPs concentrations in dolphins are discussed in section 3.2.1.4.

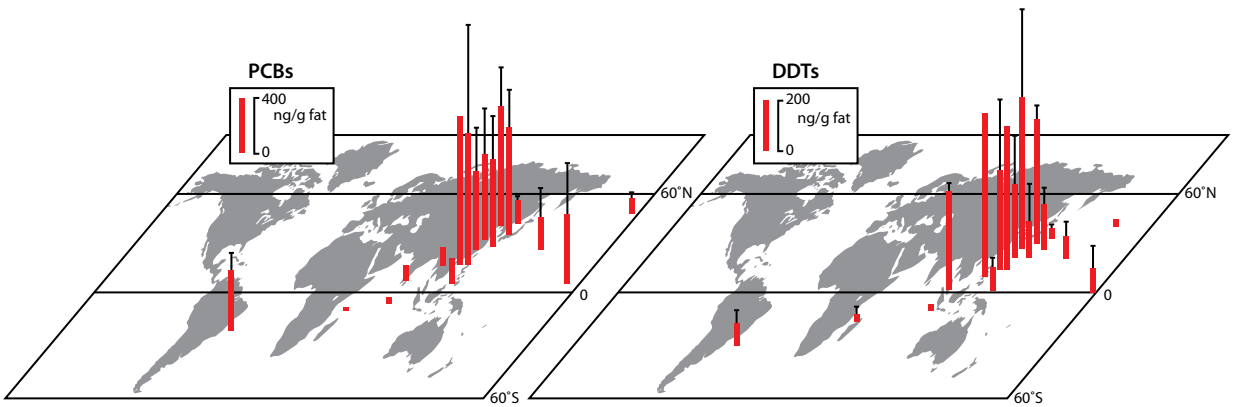


Figure 3.11. Geographical distribution of the sum of 117 PCB congeners and the sum of 4,4'-DDT, -DDE and –DDD concentrations (ng/g fat) in liver of skipjack tuna from east Asian waters, the central North Pacific, Indian and South Atlantic oceans. (based on Ueno et al., 2003).

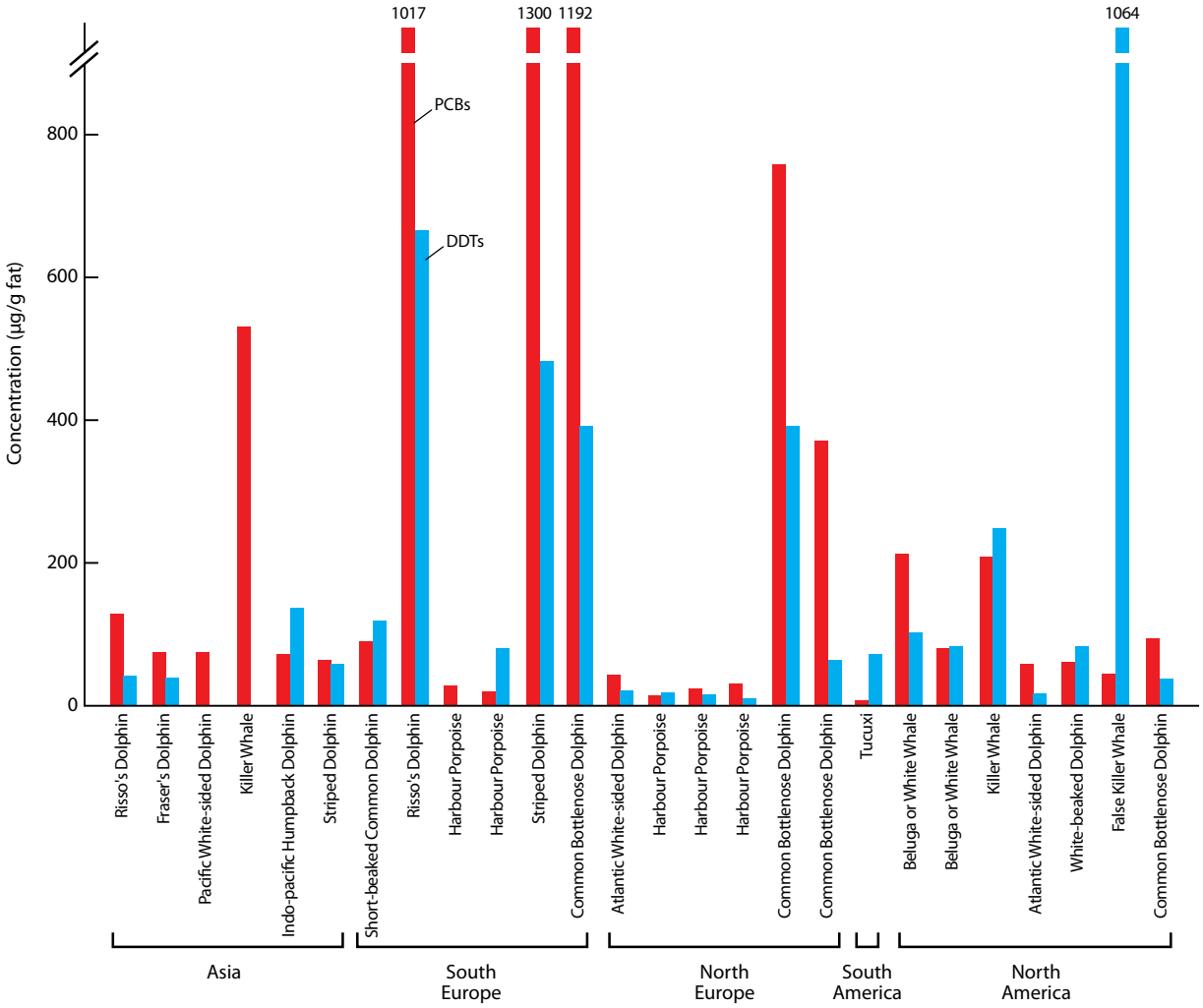


Figure 3.12. Mean concentrations (µg/g fat) of PCBs and DDTs in blubber of stranded male delphinoids from various regions. (based on figure from Houde et al., 2005).

PBDEs

Global contamination by PBDEs has been well documented over the past 10 years and reviews of spatial and temporal trends in wildlife are available (Chen & Hale, 2010; de Wit, Alaec & Muir, 2006, de Wit, Herzke & Vorkamp, 2010; Hites, 2004; Law et al., 2006; 2008b). The major congeners detected are BDE-47, -99, -100, -153 and -154, resulting from the use of PentaBDE and OctaBDE products. These congeners all exhibit significant trophic biomagnification, especially in aquatic food webs with fish and piscivorous birds as top predators.

Skipjack tuna were also used to assess the global distribution of PBDEs and HBCDD (Ueno et al., 2004; 2006) (**Figure 3.13**). Relatively high concentrations of ΣPBDEs and HBCDD were found in samples from the South China Sea and Sea of Japan, areas which are near large urban and industrial areas with known use, recycling and manufacturing sites for BFRs. Relatively high levels of ΣPBDEs were also found in tuna collected off of the coast of Brazil. In contrast ΣPBDEs and ΣHBCDDs were relatively low in samples from the Indian Ocean.

Chen & Hale (2010) examined the global spatial trends of PBDEs in birds (tissues or eggs) and found that North American birds exhibited much higher concentrations of ΣPBDEs than those from Europe and Asia (**Figure 3.14**). Differences in species and also in the number of congeners used to calculate ΣPBDEs did not account for the large regional differences. This geographical difference is consistent with the fact that the North American market has encompassed the bulk of the world's PentaBDE production (BSEF, 2003). Major differences were also apparent between remote and urban regions; the Canadian and European Arctic (Svalbard) and Antarctica were much lower in concentrations when compared to samples collected near urban areas. Chen & Hale (2010) also reviewed the available data on BDE-209 in birds around the world. Highest concentrations were observed in Chinese kestrels and USA peregrine falcons (**Figure 3.15**). The distinctive regional patterns observed with ΣPBDEs are not as apparent for BDE-209, with both North American and East Asian birds having high exposures. This may be

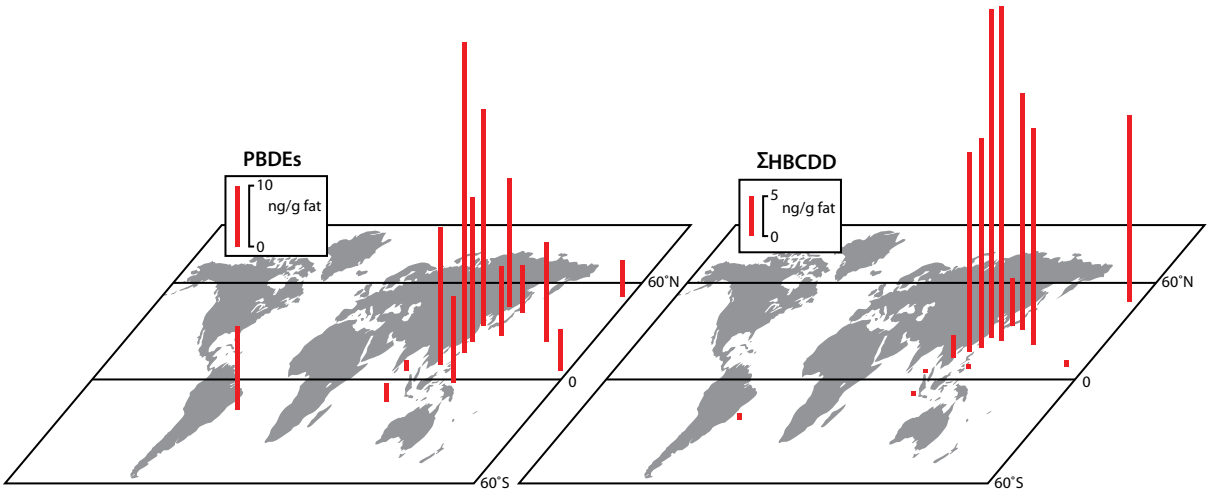


Figure 3.13. Sum of 11 PBDE congeners and sum of α–, β–, γ–HBCDD concentrations (ng/g fat) in muscle of skipjack tuna from east Asian waters, the central North Pacific, Indian and South Atlantic oceans (Ueno et al., 2004; 2006).

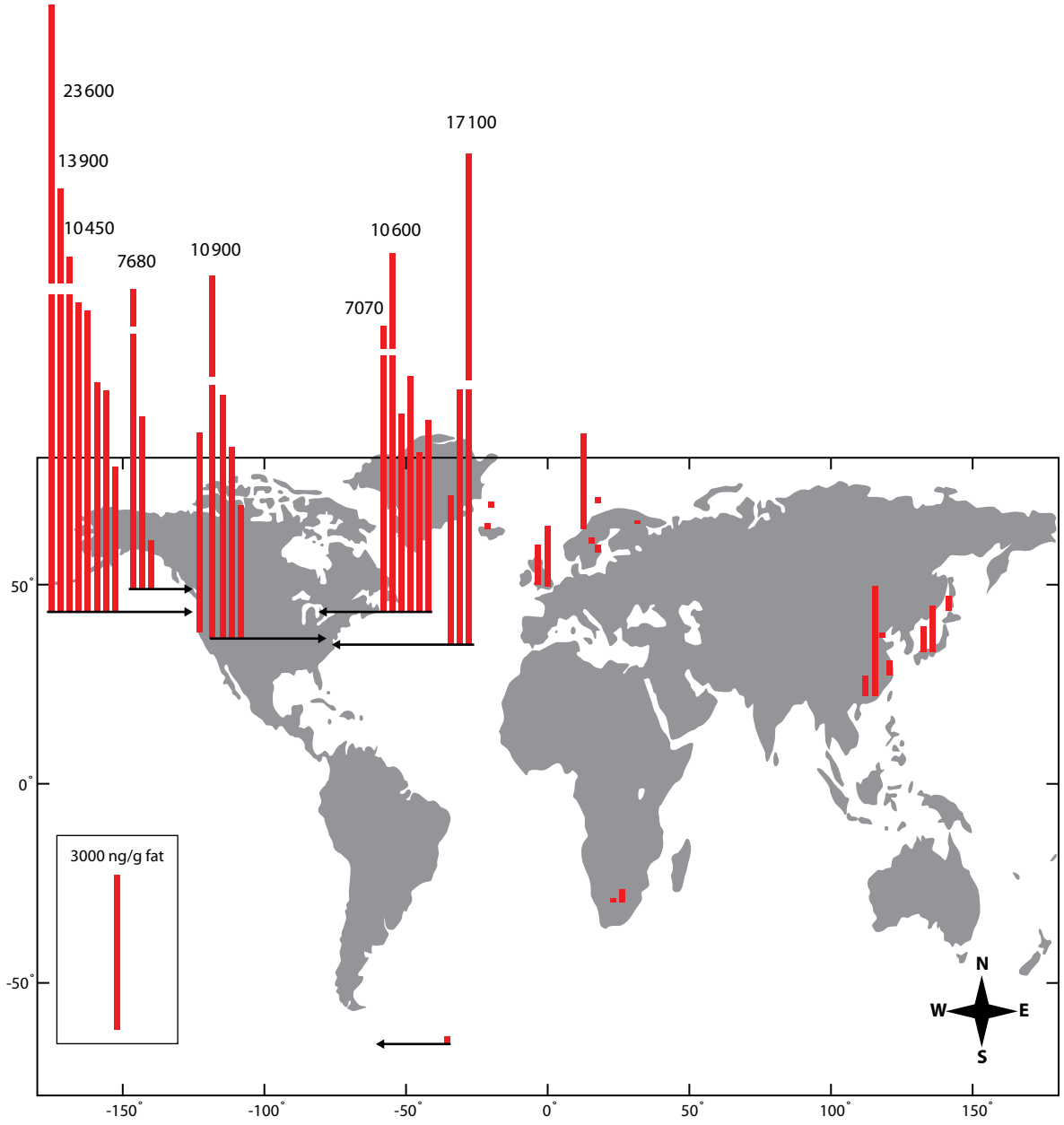


Figure 3.14. PBDE concentrations (ng/g fat) in bird tissues or eggs from North America, northern Europe, East Asia and South Africa (Chen & Hale 2010). (Figure redrawn; Used with publisher's permission)

explained by DecaBDE being the major PBDE product used in Asia, at least in the early 2000s (BSEF, 2003).

PFCs

Knowledge of PFCs in wildlife has also increased over the past decade (reviews by Beach et al., 2006; Houde et al., 2006, 2011); they were not included in the previous global assessment of EDCs (IPCS, 2002). PFOS in wildlife was first reported by Giesy & Kannan (2001). PFOS and related PFCs had been missed in previous surveys of halogenated contaminants in wildlife due to a lack of suitable analytical methodology; the sulfonates could not be readily analysed by gas chromatography, a method used for POPs like PCBs. Also the prevailing view that a halogenated compound with an ionisable carboxylate or sulfonate group would not biomagnify may have led regulators to give these PFCs low priority for exposure assessments. Subsequent reports identified many related compounds such as perfluorohexanesulfonate (PFHxS) and perfluorodecanesulfonate (PFDS), as well as the PFCAs with 8 to 14 carbon chains. Recently a new class of perfluoroalkyl sulfonates, the perfluoroethylcyclohexane sulfonates, was identified in Great Lakes fish and other aquatic biota (de Silva et al., 2011). Houde et al. (2006; 2011) have comprehensively reviewed the reports of PFCs in biota.

An initial large-scale study by Giesy & Kannan (2001) demonstrated the global distribution of PFOS in wildlife. Concentrations of PFOS in animals from relatively more populated and industrialized regions, such as the North American Great Lakes, Baltic Sea, and Mediterranean Sea, were greater than those in animals from remote marine

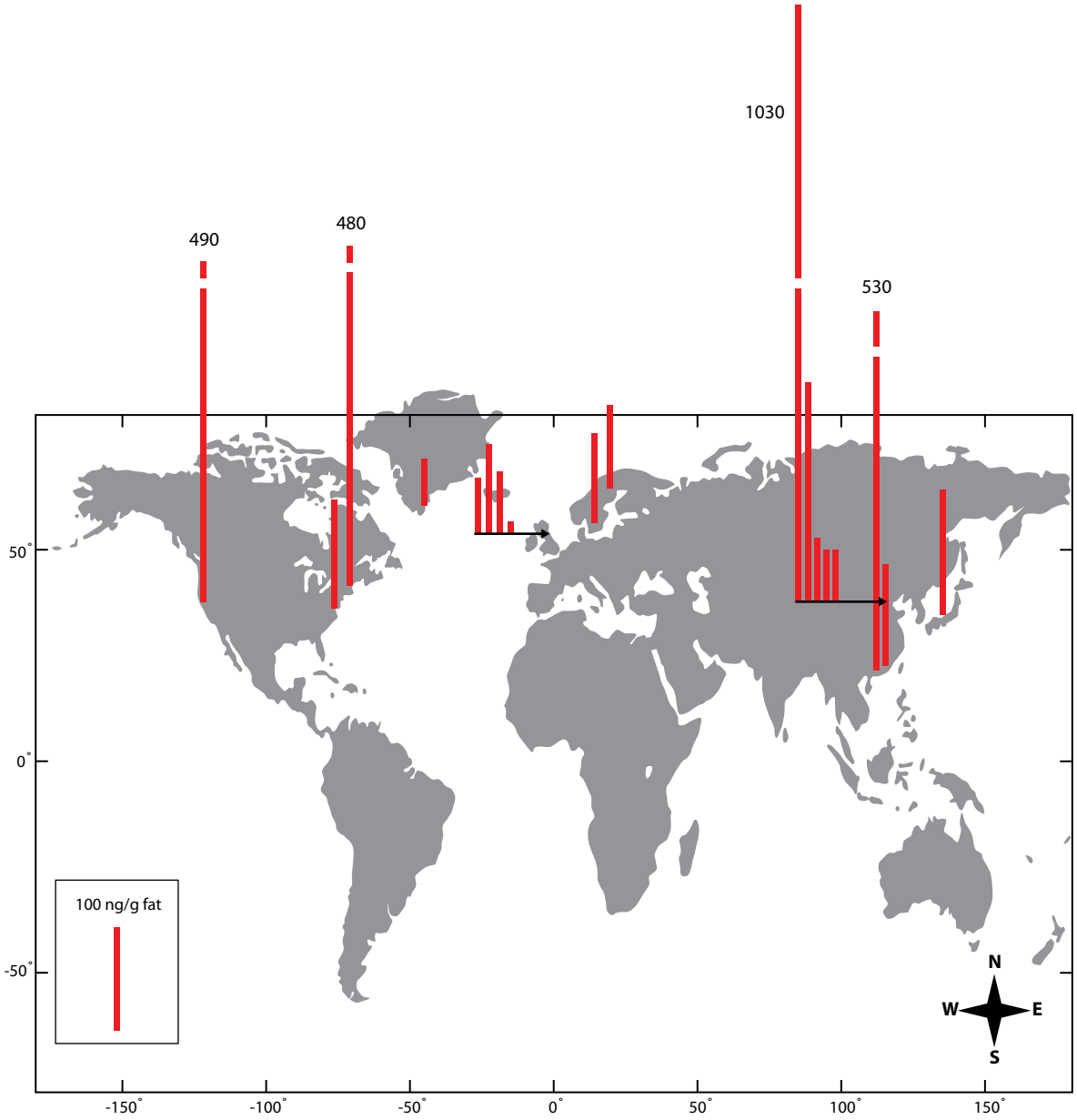


Figure 3.15. BDE-209 concentrations (ng/g fat) in bird tissues or eggs from North America, northern Europe, East Asia and South Africa (Chen & Hale 2010). (Figure redrawn; Used with publisher's permission)

locations, with the exception of polar bears from Alaska which had relatively high concentrations. PFOS remains the predominant PFC found in all species, tissues, and locations analyzed around the world (Houde et al., 2006; 2011). The geographical distribution of PFOS in marine mammals based on studies published in the period 2006 to 2010 is shown in **Figure 3.16**. Consistent with previous results, highest PFOS concentrations were reported for marine mammals in the Baltic and North Seas and in coastal Japanese waters, indicating the importance of urban and industrial sources.

Mercury/methylmercury

Although there is great interest in mercury in the global environment under initiatives such as UNEP's Global Mercury Partnership (UNEP, 2011a), the focus for large spatial comparisons has mainly been on atmospheric emissions, sources and air concentrations (AMAP/ UNEP, 2008). Earlier reviews have summarized the extensive measurements of mercury in marine mammals (Law, 1996), seabirds and terrestrial animals (Thompson, 1996). Das et al. (2003) have summarized mercury and methylmercury concentrations in livers of marine mammals published to about 2001. They noted that levels of mercury in liver varied several orders of magnitude among species and locations. Particularly important are the ages of marine mammals since mercury is usually correlated with age; other factors include diet, trophic level (determined with nitrogen isotope ratios), sex, location and metabolic rate. This applies equally to bird tissues (except eggs) and fish. Thus broad geographic comparisons of mercury concentrations are of little value without the supporting age, size, sex and diet information.

Total mercury in marine mammal and seabird tissues from the Arctic have been reviewed as part of the Arctic

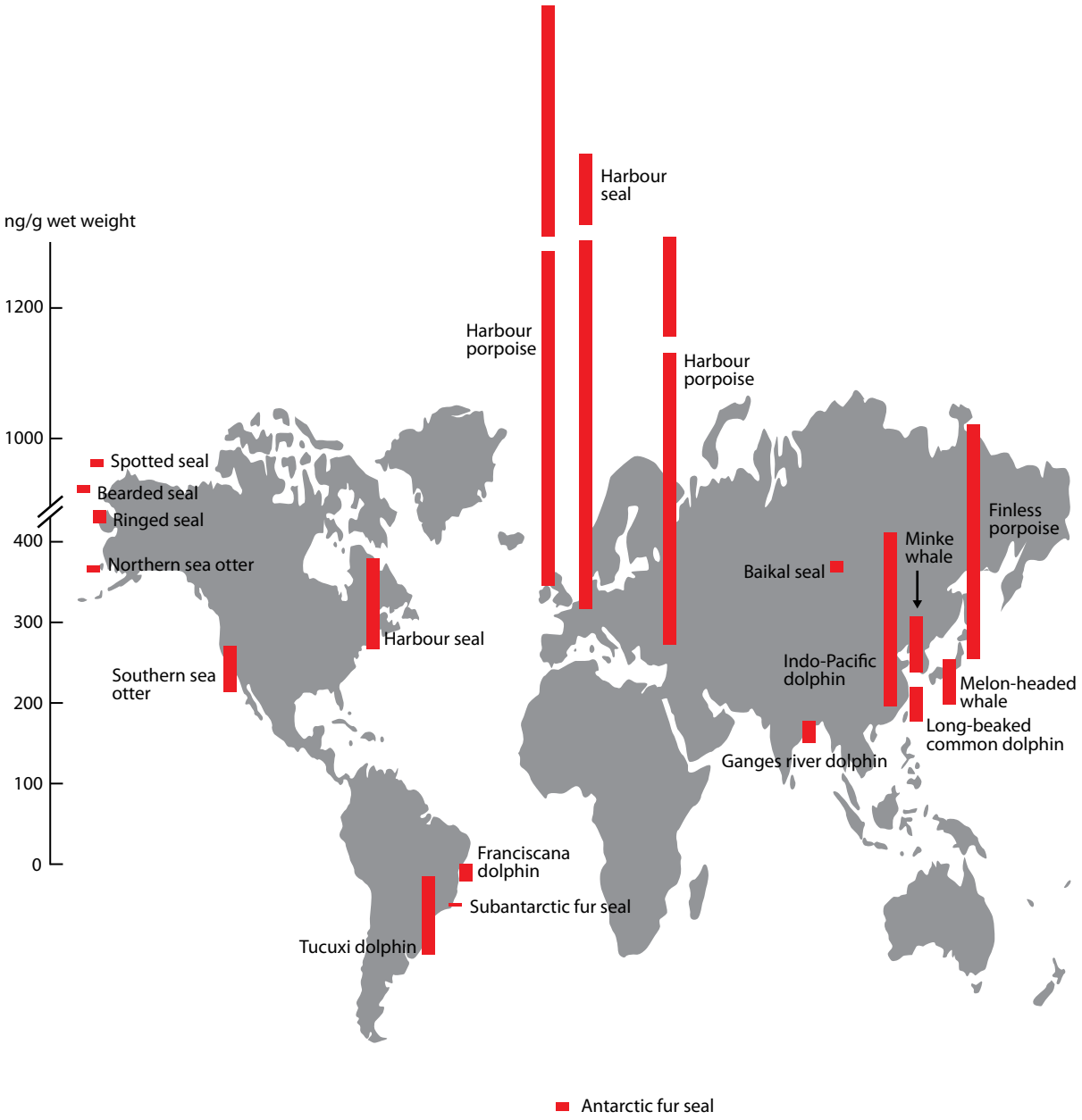


Figure 3.16. PFOS concentrations (ng/g wet weight) in liver of marine mammals worldwide (based on figure from Houde et al., 2011).

Monitoring and Assessment Programme (AMAP) (Dietz, Pacyna & Thomas, 1998; Ford et al., 2005; Riget et al. 2011). The use of common sampling and analysis protocols within the program allowed for geographic comparisons after adjustment for factors such as age. For example, spatial trends of mercury in ringed seal liver were compared using means adjusted to five year old animals (**Figure 3.17A**). Riget et al. (2005) used the same dataset, as well as samples from the White Sea in northwestern Russia, to study longitudinal trends of mercury in ringed seals (**Figure 3.17B**). Very high concentrations of total mercury were present in seals in the western Canadian Arctic but not further west at Barrow, Alaska, USA Natural geological differences in mercury may be important, although differences in diets and trophic levels of ringed seals in Hudson Bay and the Beaufort Sea could also explain some of the differences. Recent comparisons of mercury in polar bears from these two regions suggest that longer food chains (i.e. more trophic levels) and higher water concentrations of methylmercury best explain site differences (St Louis et al., 2011).

3.2.1.4 Temporal trends for wildlife

While information on spatial trends of EDCs in biota is available from a large number of global and regional monitoring programmes and literature reviews, there is much less information on temporal trends. The long term temporal trend programmes in the Baltic, the Great Lakes and the Arctic, which were highlighted in the 2002 IPCS assessment of EDCs (IPCS, 2002), continue to be the longest datasets for many POPs and mercury. Here some previous time trends cited in the 2002 EDC assessment are revisited and, where possible, expanded to include other regions and more chemicals.

PCBs and OC pesticides

Long term trends of POPs (PCBs and DDTs) in guillemot eggs have been studied at St. Karlsö (Gotland Island, Baltic Sea) since 1969, and archived samples from the late 1970s to present have been analyzed for many other halogenated organics and metals (Bignert et al., 2010). The common guillemot (*Uria aalge*) is a fish-eating bird that nests in a few remote colonies. These colonies stay in the Baltic region all year and thus guillemot eggs are representative of marine contamination in the Baltic. Time trends for PCBs (sum of 9 congeners), 4,4'-DDE, β-HCH and HCB in guillemot eggs are shown in **Figure 3.18**. All 4 legacy POPs continue to decline at about 5-10% per year. These declines parallel global declines in estimated emissions of these POPs (see inset graphics) and recovery of bird and mammal populations that were affected by their exposure (Chapter 2.12).

Many temporal trend datasets exist for POPs in Arctic wildlife (Riget et al., 2010). POPs in polar bears from East

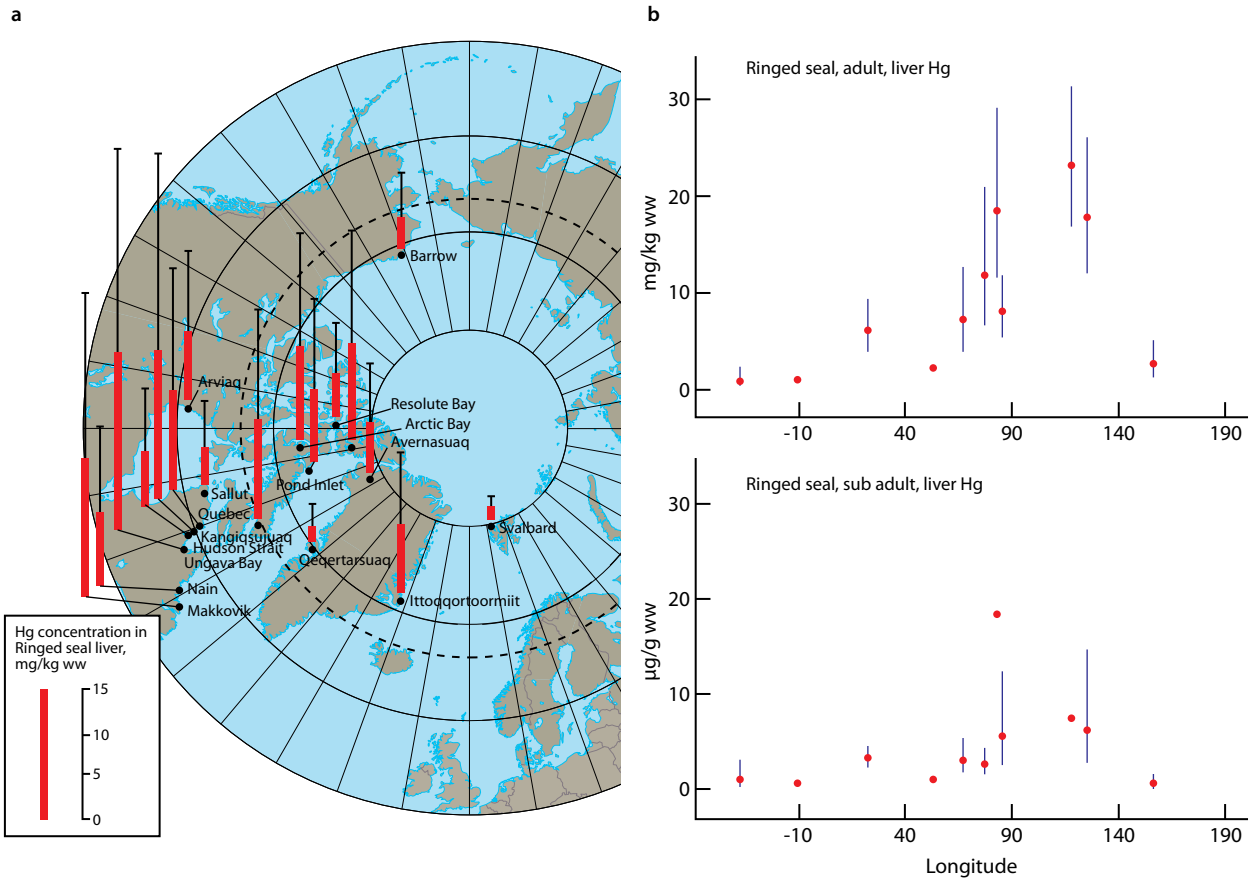


Figure 3.17. a. Mean concentrations (mg/kg wet weight) of total mercury in adult ringed seal liver from Greenland, northern Canada and Alaska based on sampling over the period 1995-2000. Data are mean-adjusted to five-year old animals (Ford et al., 2005). b. Trends of liver mercury in age-adjusted adult (top panel) and subadult seals (bottom panel) with longitude (Riget et al., 2005). Composite figure based on the references given.

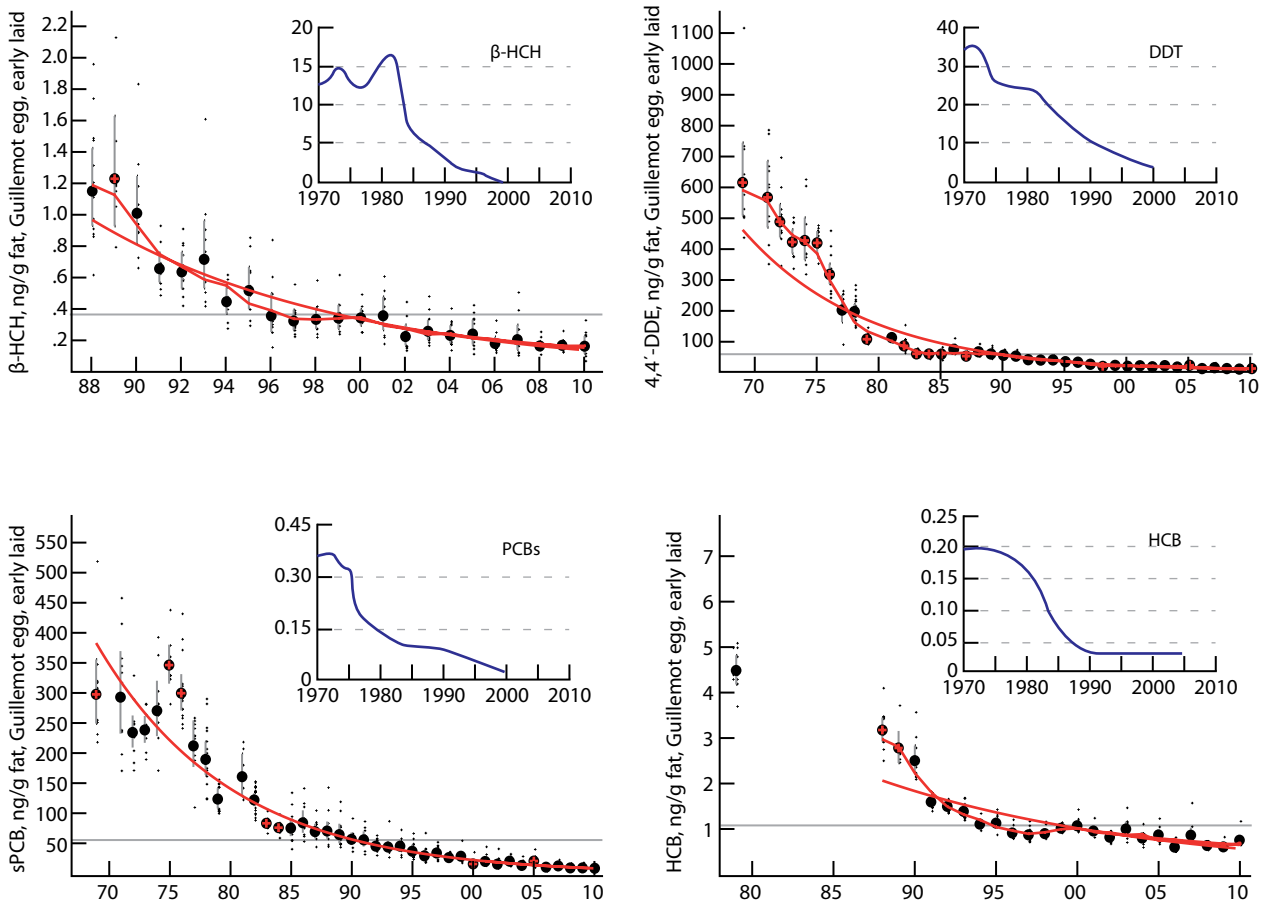


Figure 3.18. Temporal trends in concentrations (ng/g fat) of β-HCH, 4,4'-DDE, ΣPCB, and HCB in guillemot eggs (first laid) from Gotland (Baltic Sea), a long term monitoring site for the Swedish National Marine Monitoring Programme (Bignert et al., 2010). Estimated global emissions (kT) of PCBs (Breivik et al., 2002), 4,4'-DDT and β-HCH (Li & Macdonald, 2005) and European emissions of HCB (Pacyna et al., 2003) are shown in smaller inset graphics.

Greenland are among the longest time series in the Arctic. As top predators in the marine food web, polar bears are excellent biomonitoring species for chemicals that biomagnify. The East Greenland population has a well-defined and large home range away from local sources (Dietz et al., 2012). Samples are available from an annual hunt by the Inuit. PCBs have declined at an average rate of 3.8%/year in juvenile bears over the period 1983 to 2010 (**Figure 3.19**). A decline averaging -4.3 % for 4,4'-DDE, -3.5% for β–HCH and -2.0% for HCB per year was observed in juvenile bears. In contrast, no consistent decline was observed in adult female or male bears, reflecting the long half-lives of chlorinated POPs in these long-lived mammals.

Tanabe & Minh (2010) have reviewed the long term trends of PCBs and chlorinated pesticides in marine biota in the Asia-Pacific region. They noted that concentrations of POPs in marine mammals from inland and coastal areas in Japan and China, including Hong Kong, have decreased in comparison with their severely polluted status in the 1970s. On the other hand, species such as minke whale in Antarctica showed little change over time, reflecting the long residence time of these contaminants in open ocean food webs. Time trends of POPs in blubber of stranded striped dolphins from Gogo-shima and Taiji in southwestern Japan from a study by Isobe et al. (2009) are shown in **Figure 3.20**. The striped dolphin is an ocean-going species and may reflect contamination from coastal China as well as Japan. Thus, it is interesting that the temporal trends of POPs are quite different from those of top predators in the Baltic or the Arctic, with relatively steady levels of PCBs and no significant decreasing trends in ΣDDTs and ΣHCH concentrations, suggesting continuing discharge of these contaminants. Chlordane, which had been used as a termiticide for mostly wooden houses in Japan and possibly other locations along the east Asia coast, showed an increasing trend (statistically significant) even after the ban on these chemicals in 1986 (Isobe et al., 2009), possibly due to its ongoing release from older sites of use.

From reviews of information for Asia, it is also apparent that the ability to detect change (statistical power) in current temporal trend datasets is low, due to the small number of sampling years and a lack of annual sampling (Bignert et al., 2004). However it is bound to improve with additional sampling years given the availability of specimen banks (Tanabe, 2006). Although there are substantial numbers of studies on POPs in South America

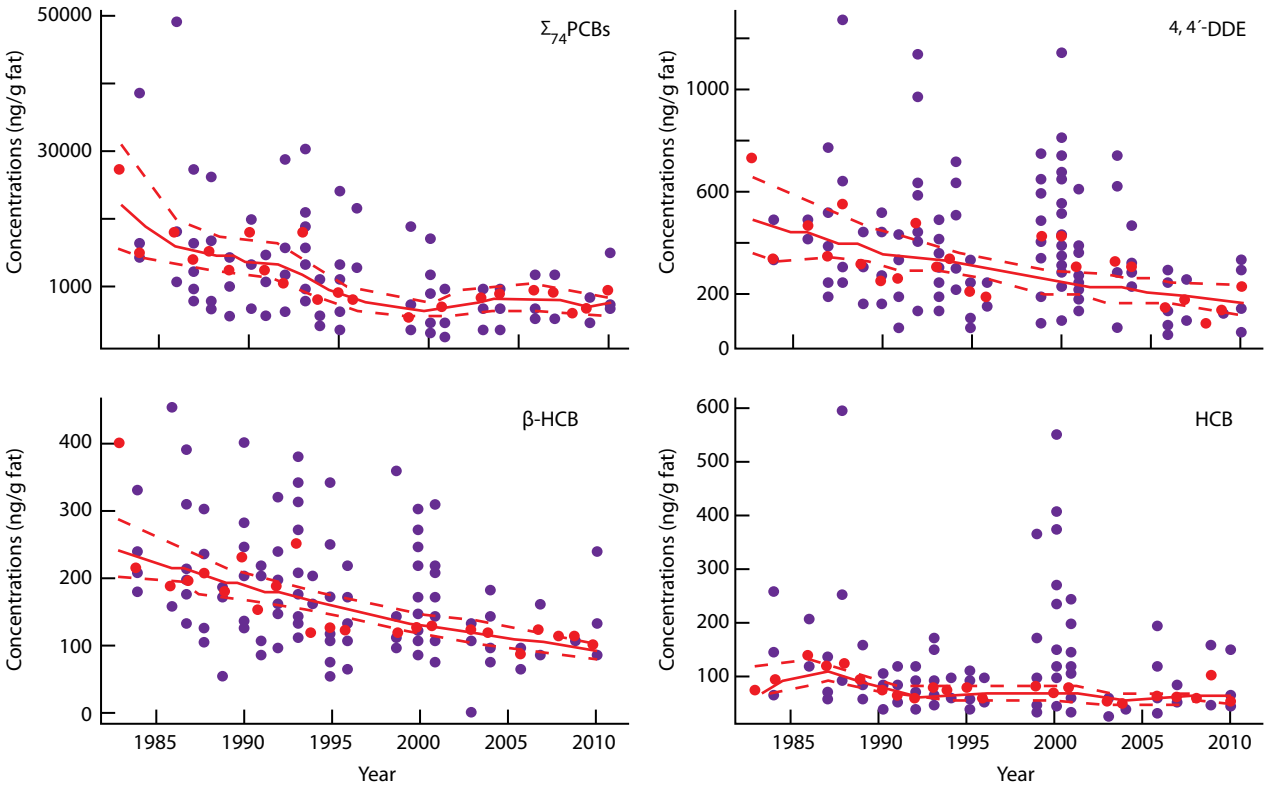


Figure 3.19. Long-term temporal trends in concentrations (ng/g fat) of PCBs (sum of 74 congeners), 4,4'-DDE, β-HCH and HCB in fat from juvenile polar bears from East Greenland. The filled red dots are median values. Red lines indicate significant trends and dotted lines the 95% confidence intervals. (based on figure from Dietz et al., 2012).

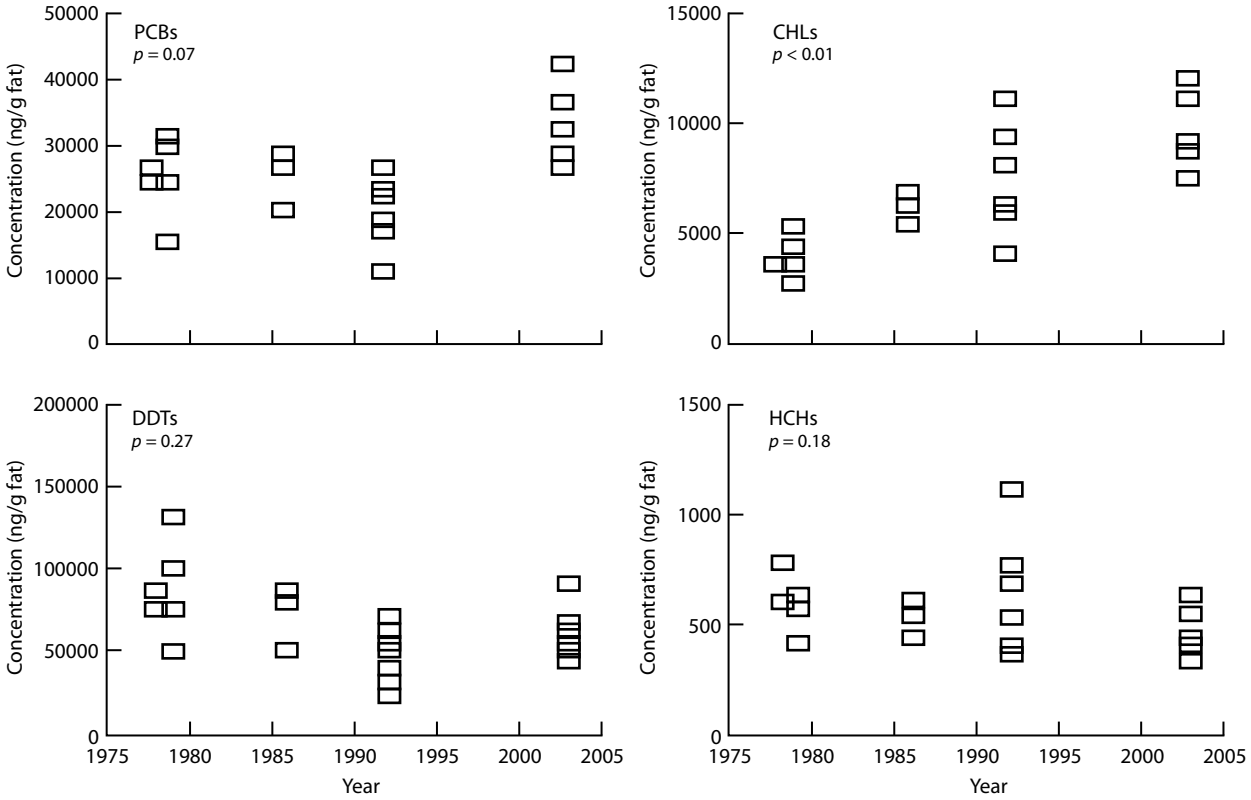


Figure 3.20. Temporal trends in concentrations (ng/g fat) of abundant organohalogen compounds in blubber of stranded striped dolphins from Japan, 1979-2003 (data from Isobe et al., 2009). PCBs = Total PCB based on technical Kanechlor mixtures; 4,4'-DDTs = 4,4'-DDT + 4,4'-DDE + 4,4'-DDD; CHLs = trans/cis-nonachlor, trans/cis-chlordane, oxychlordane; HCHs = α-, β-, γ-HCH.

(e.g. Barra et al., 2006) and Africa (e.g. UNEP, 2009a), there are no temporal trends of PCBs and organochlorine pesticides such as DDT in wildlife yet available.

PBDEs and HBCDD

Extensive time trend data are now available for PBDEs and HBCDD as a result of ongoing collections and analyses by various monitoring programmes in the Baltic, the Great Lakes, the Arctic and in Asia Pacific, as well as by retrospective analysis of samples from wildlife specimen banks. In the Baltic, the significant increasing concentrations of BDE-47 and BDE-99 and other congeners related to PentaBDE products that were found in guillemot eggs from the late sixties until the early nineties are followed by decreasing values during the period 1998-2008 (Bignert et al., 2010) (**Figure 3.21**). Similar trends for pentaBDEs were observed in a retrospective study of peregrine falcon eggs from southern Sweden (Johansson et al., 2011). In contrast HBCDD showed an increase in guillemot eggs averaging 2.9% per year from the late 1970s to 2008 (**Figure 3.21**).

No significant time trends in ΣPBDE concentrations in Indo-Pacific humpback dolphins and finless porpoises from Hong Kong Harbor were observed from 1997 to 2008 (**Figure 3.22**)(Isobe et al., 2009; Lam et al., 2009). The dolphins and porpoises have resident populations distributed in the northwestern and eastern waters of Hong Kong. ΣPBDE concentrations may have increased over the period 1997-2003 in both species, although sampling years are limited. These results were consistent with the temporal patterns of PBDEs observed in California sea lions stranded between 1994 and 2003 near San Francisco (Stapleton et al., 2006). In contrast, significantly increasing concentrations of HBCDD were found in dolphins from Hong Kong (**Figure 3.22**). This increase in HBCDD was consistent with several other studies on marine mammals from California (Stapleton et al., 2006), Japan (Isobe et al., 2009) and the UK (Law et al., 2008a).

ΣPBDEs in juvenile polar bears from East Greenland achieved maximum concentrations in 2004-05 (**Figure 3.23**) (Dietz et al., 2012), which is about 10 years later than for top predators in the Baltic (Bignert et al., 2010; Johansson et al., 2011) and in UK coastal waters (Law et al., 2010). This presumably reflects delayed exposure of the Greenland polar bears due to long-range atmospheric and oceanic transport. Similar to Baltic guillemots and marine mammals in the UK, the USA and east Asia, HBCDD was found to be increasing in polar bears, with highest median values in samples from 2010 (Dietz et al., 2012).

PFCs

Houde et al. (2011) have reviewed temporal trends of PFSAs and PFCAs in wildlife to 2010. Between 1999 and 2008, a decline of PFSAs (mainly PFOS) and PFCAs (PFNA, PFDA, PFUnA) was found in harbour seal liver samples collected in the German Bight (**Figure 3.24**). With this decline was a large

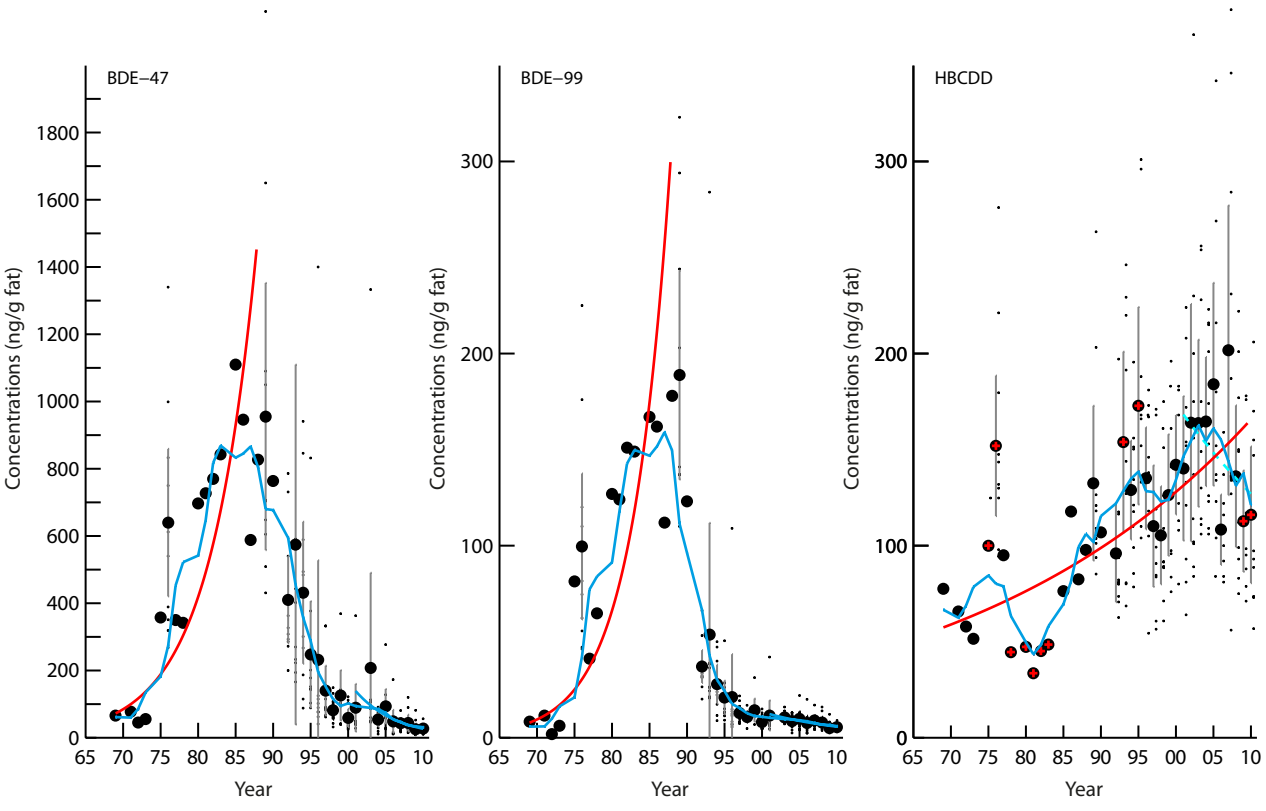


Figure 3.21. Temporal trends in concentrations (ng/g fat) of the PBDE congeners, BDE-47 and BDE-99, and of HBCDD in guillemot eggs from southern Sweden from Gotland (Baltic Sea), from 1968 to 2008 (Bignert et al., 2010).

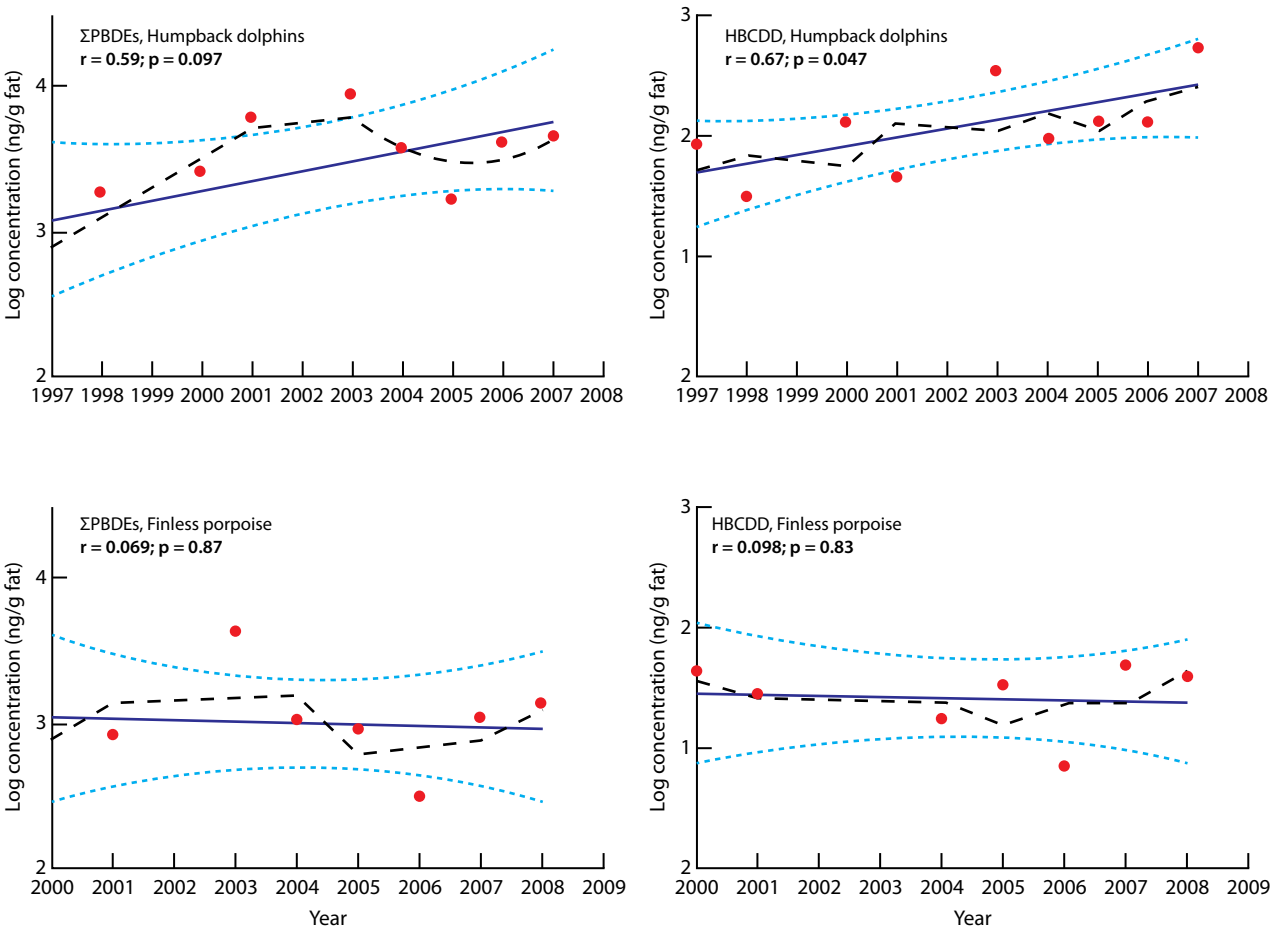


Figure 3.22. Temporal trends in concentrations (ng/g fat) of PBDEs (sum of 14 congeners) and of HBCDDs (sum of 3 congeners) in blubber of Indo-Pacific humpback dolphins (1997-2007) and finless porpoises (2000-2008) from Hong Kong Harbour. Longer dashed lines represent three-year moving averages. Shorter dashed lines represent 95% confidence intervals. (based on figure form from Lam et al., 2009).

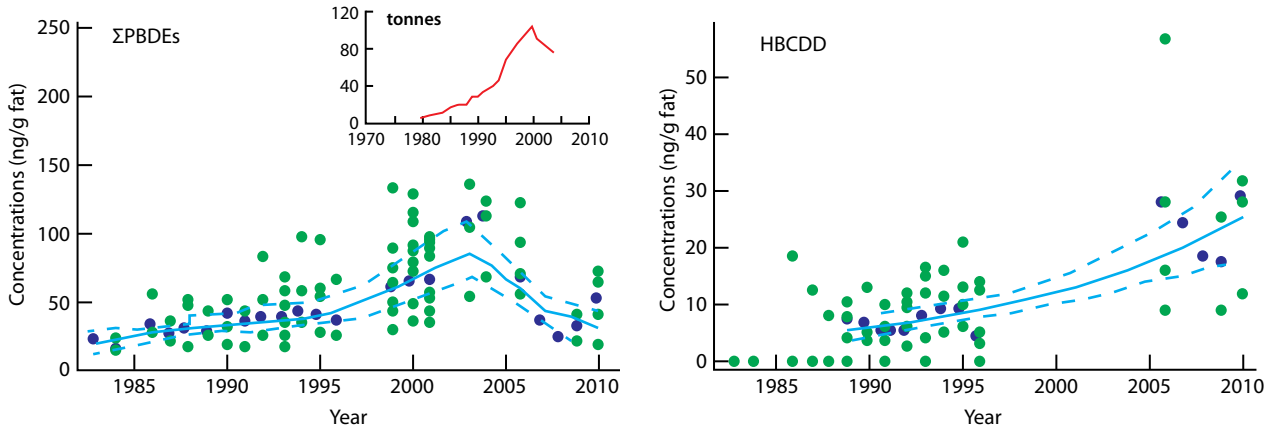


Figure 3.23. Temporal trends in concentrations (ng/g fat) of PBDEs and HBCDD in fat from juvenile polar bears from East Greenland. The filled red dots are median values, the red lines indicate significant trends, and the dotted lines show the 95% confidence intervals**.** Inset graphic: The estimated time trend of BDE-47 emissions in North America based on Alcock et al. (2003). (based on figure from Dietz et al., 2012).

decrease (~95%) in the PFOS precursor, PFOSA (Ahrens, Siebert & Ebinghous, 2009). Other marine mammals showed more variable trends, although these data are not as recent (**Figure 3.24**). Increasing concentrations of both PFSAs and PFCAs were observed in East Greenland polar bears up to 2005 and in melon-headed whale in Japan up to 2000. Northern sea otters did not show a distinctive trend in PFCs over the period 1986 to 2000 (**Figure 3.24**). Rüdel et al. (2011)

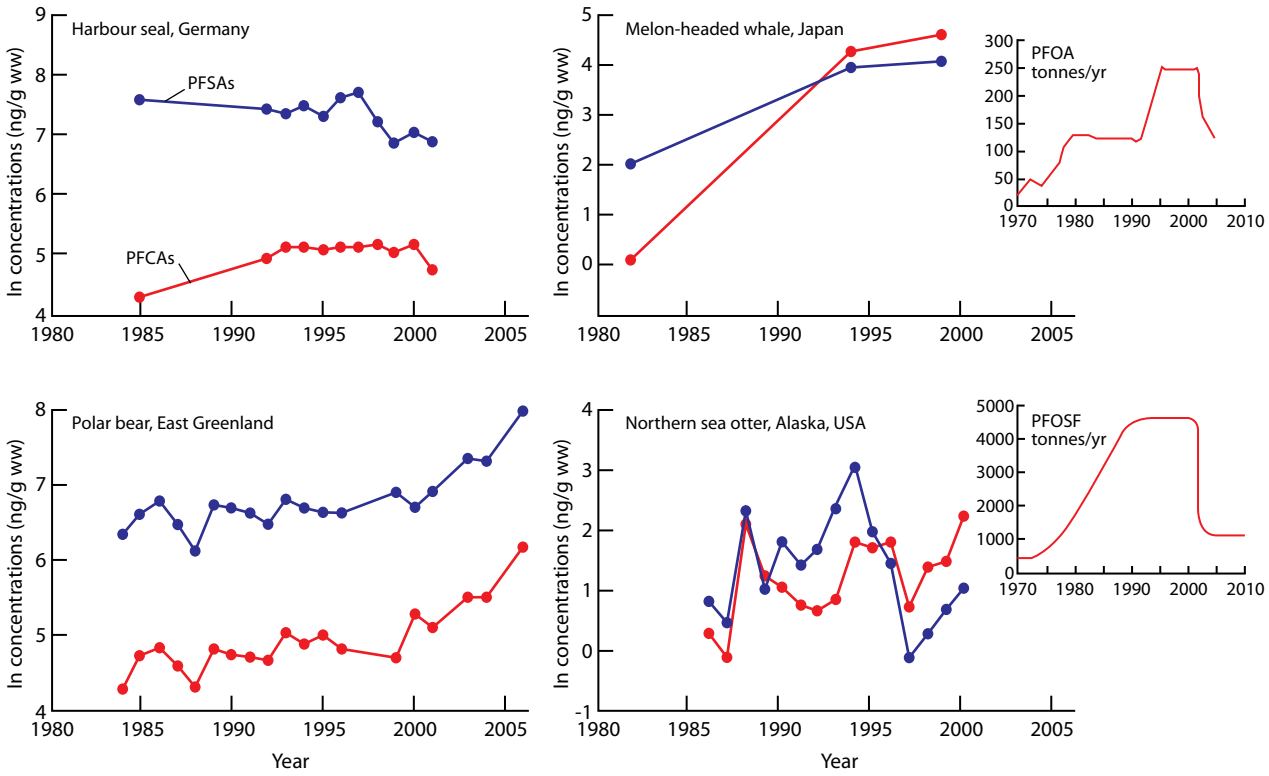


Figure 3.24. Temporal trends in concentrations (ng/g wet weight) of perfluorinated carboxylates (PFCAs) and perfluorinated sulfonates (PFSAs) in tissues of marine mammals (Houde et al., 2011). Inserted graphs: Estimated maximum global emissions of PFOA (Armitage et al., 2006) and estimated total global production of PFOSF, the major PFOS starting material (Paul, Jones & Sweetman, 2009). Composite figure based on the references given.

noted a decline of PFOSA over the period 1993 to 2008 in pooled samples of blue mussels from the sites in the North Sea and southern Baltic Sea while PFOS showed no trend. In the same region where the mussels were sampled, PFOS and PFOA in herring gull eggs showed a slow decline from maxima in the late 1990s except in the Baltic where increasing levels in eggs were observed. Bignert et al. (2011) also found highest mean concentrations of PFOS in guillemot eggs from Gotland (southern Baltic) in the late 1990s and continuing elevated levels to 2009, despite the phase out of most PFOS use in Europe as of 2001. A review of temporal trend studies of PFOS in Arctic biota show generally increasing levels of PFCs from the 1970s, although some studies from the Canadian Arctic show declines in PFOS levels from the mid-2000s (Butt et al., 2010). In general, PFOS concentrations appear to be declining in biota from sites nearer sources of PFOS and its many precursors and not in remote sites where exposure occurs via long-range ocean transport.

Mercury

Many time series for mercury in aquatic biota exist for local pollution issues and for national and regional assessments. At the regional level, there are trends for 20+ years available from biomonitoring programmes in the Baltic (Bignert et al., 2010; 2011), the North Sea (OSPAR, 2009; 2010), the Arctic (Rigét et al. 2011), and the Great Lakes (SOLEC, 2009). These programmes typically summarize trends in multiple species, e.g. Rigét et al., (2011) assessed 83 times series studies for mercury in Arctic fish, seabirds and marine mammals. No equivalent assessments of local time series studies appear to be available for East Asia, Africa or South America. In general, regional assessments have shown mercury/methylmercury trends to be variable among species within the same area; more specifically, it can be increasing in one species and declining in another. For example, mercury concentrations in guillemot eggs (Gotland, central Baltic) have decreased significantly, whereas mercury concentrations in herring from the southern Baltic were unchanged over 25+ years (**Figure 3.25**) (Bignert et al., 2011).

In the Arctic, mercury concentrations in eggs of thickbilled murres (Brünnich's guillemot) have increased about 3-fold over 35 years, while in northern fulmars the increase has been less pronounced (~50%) over the same period (Braune et al., 2010; **Figure 3.26**). In ringed seals from the same region, mercury concentrations have remained similar over this same period, although data are more limited for the 1980s (Gaden et al., 2009; Muir et al., 2011). A key factor influencing mercury trends is the production and availability of methylmercury, which is dependent upon ecosystem-specific variables and the form of the mercury loaded (Munthe et al., 2007). Gaden et al. (2009) concluded that summer environmental conditions, such as timing of the ice free season, can influence the composition of

prey (mercury exposure) available to ringed seals. Finally, Rigét et al. (2011) found no overall consistent trend for mercury across 83 time series for tissues and species from the circumpolar Arctic during the period from the early 1970s to 2007. However, they did note a west-to-east gradient in the temporal trends, with larger numbers and a higher proportion of biotic datasets in the Canadian and Greenland region of the Arctic showing significant increases than in the North Atlantic and European Arctic.

In addition, special studies incorporating museum specimens have provided insights into the long term trends of mercury and methylmercury in marine biota. Vo et al. (2011) showed that black footed albatross collected between 1880 and 2002 in the north Pacific basin exhibited a 3.8 fold increase in methylmercury levels in feathers consistent with historical global and recent regional increases in anthropogenic emissions. Dietz et al. (2011) showed that mercury has been increasing slowly in Northwest Greenland polar bear hair from preindustrial times to the present. They used hair samples dated from 1300 A.D. (n = 2), museum specimens from the period 1892-1960, and contemporary collections up to 2008. They found an increasing trend between 1892 and 2008 of ~ 1.6%/year that represented a 23- to 27-fold increase from the baseline level in 1300 A.D. Thus it appears that over the past 100 years methylmercury exposure of marine biota in the northern hemisphere has increased substantially compared to pre-industrial times (Dietz, Outridge & Hobson, 2009).

3.2.2 Humans

3.2.2.1 Internal exposure

Bioaccumulation – External exposures to EDCs for humans have been discussed above in section 3.1.5. For EDCs to have an effect though, they must be taken up into the body, and transported to sites where they interfere with endocrine system processes (Chapter 1). The tissues in the body where EDCs are found depend on the properties of the EDCs; some are

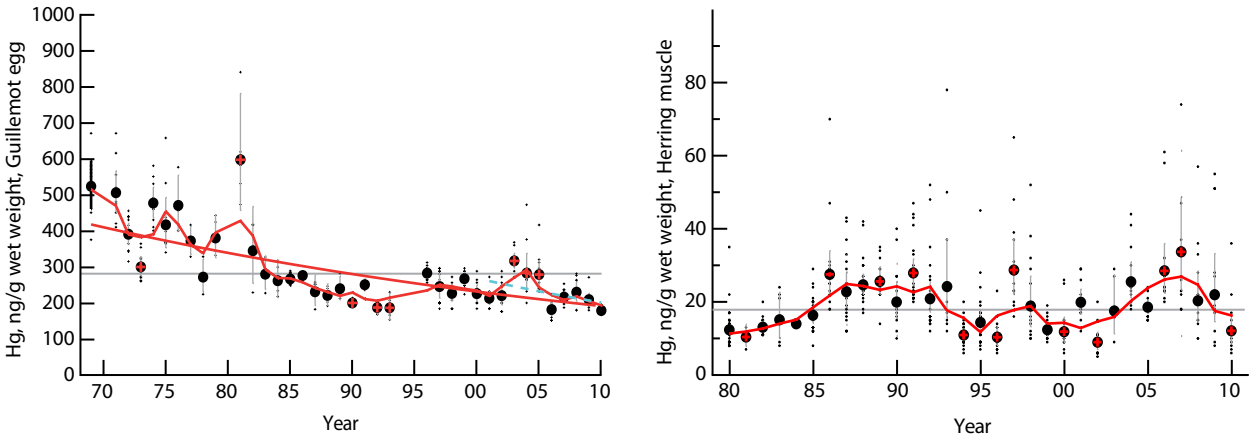


Figure 3.25. Temporal trends in concentrations (ng/g wet weight) of mercury in guillemot eggs and herring muscle from the Baltic Sea (Bignert et al. 2011).

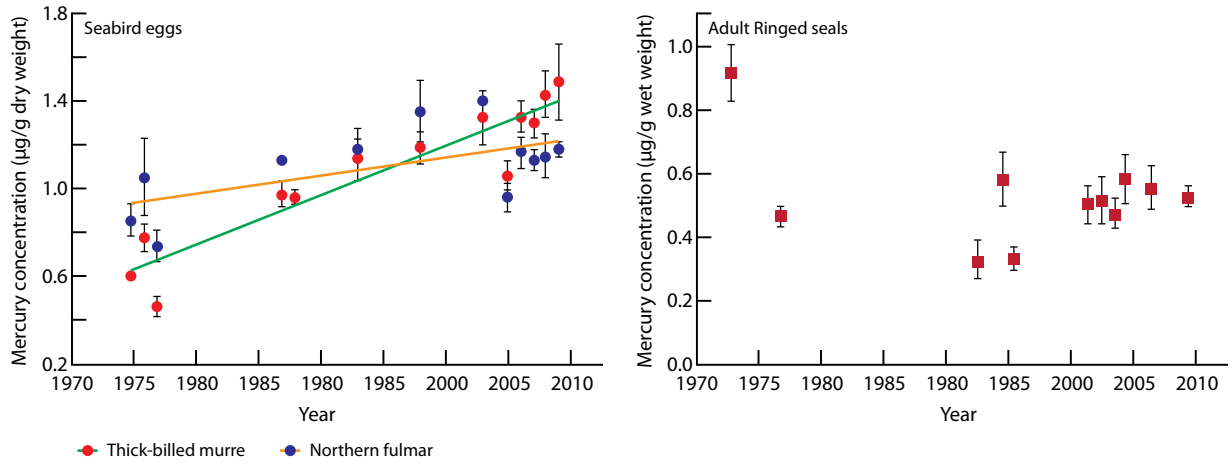


Figure 3.26. Temporal trends in concentrations of mercury in seabird eggs (µg/g dry weight; Braune et al. 2010) and ringed seal muscle (µg/g wet weight; Gaden et al. 2009; Muir et al. 2011) from the Canadian Arctic archipelago.

bioaccumulated in adipose tissue or in muscle whereas others are found in blood or tissues such as lungs or adrenals (Letcher, Klasson-Wehler & Bergman, 2000).

Animal studies have demonstrated that many EDCs are able to cross the placental barrier and thereby expose the fetus. In humans, several studies have reported detectable concentrations of EDCs in cord blood, and correlations between levels in cord blood and maternal blood (Needham et al., 2011). Transfer of chemicals between the mother and the embryo, fetus and thereafter the newborn baby is a matter of concern for humans. The similar levels of some POPs in human maternal serum, cord serum and in mothers' milk are shown in the diagram in **Figure 3.27**, emphasizing the transfer of EDCs to the fetus and the newborn nursing child (Needham et al., 2011). In contrast to the lipophilic POPs (e.g. DDTs, PCBs and PBDEs), perfluorinated alkyl acids (such as PFOS and PFOA) are proteinophilic. Concentrations of PFOS and PFOA in mothers' milk are therefore much lower than in blood (Kärrmann et al., 2007). Nevertheless, it has been demonstrated that mothers' milk is a significant exposure source for the nursing infant (Thomsen et al., 2010a) and the estimated dietary intake for a nursing infant is similar to that of an adult.

Metabolism - Metabolites of several EDCs exhibit stronger endocrine disrupting potential than their parent compounds, e.g. phenolic metabolites of PCBs and PBDEs (Dingemans et al., 2008; Hamers et al., 2008; Meerts et al., 2004). In some cases, the metabolism even leads to more persistent compounds that accumulate in tissues (Letcher, Klasson-Wehler & Bergman, 2000). Polyfluorinated telomer alcohols can be metabolized in the organism to perfluorinated carboxylic acids with half-lives of several years (Lau et al., 2007). Decabromodiphenyl ether (decaBDE), with a half-life of about two weeks in human blood (Thuresson et al., 2006), is debrominated in the body to more persistent nona- and octabrominated diphenyl ethers as experimentally shown in rats (Huwe & Smith, 2007).

Excretion - EDCs can be excreted from the body in multiple ways, and excretion rates are highly dependent on chemical properties. Nonpersistent EDCs are rapidly metabolized, primarily via the liver, and excreted through urine or faeces. More persistent EDCs can be excreted, but because they tend to accumulate in different parts of the body, such as fat, they are released much more slowly. One pathway that is unique to mothers and infants is excretion via breast milk. Persistent, lipophilic EDCs are excreted in mothers' milk, leading to the exposure of the breast fed infant (WHO, 2010c). Studies on the elimination rates of EDCs during lactation have demonstrated a significant transfer of EDCs to the breast fed child. In many cases, the exposure of the infant to POPs during breast feeding exceeds the tolerable daily intake defined for lifelong exposure (Polder et al., 2008a; 2008b). However, for optimal infant feeding, WHO recommends "exclusive breastfeeding for 6 months" (WHO, 2001). "Breastfeeding is an important source of nutrients for an infant and numerous health benefits from breastfeeding have been documented" (American Academy of Pediatrics, 2005).

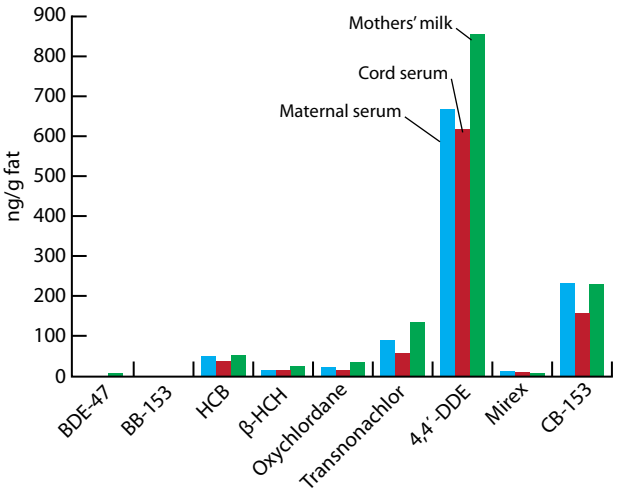


Figure 3.27. Concentrations (ng/g fat) of nine selected POPs in maternal serum, cord serum and mothers' milk from the same individual from the Faroe Islands (diagram prepared on basis of data from Needham et al., 2011). The selected abbreviated compounds are: BDE-47 = 2,2',4,4'-tetrabromodiphenyl ether; BB-153 = 2,2',4,4',5,5'-hexabromobiphenyl; HCB = hexachlorobenzene; β-HCH = β-hexachlorocyclohexane; 4,4'-DDE = 1,1-bis(4-chlorophenyl)-2-dichloroethene; CB-153 = 2,2',4,4',5,5'-hexachlorobiphenyl.

3.2.2.2 What has been measured in humans

Biomonitoring using suitable human tissues can give an integrated measure of the internal exposure from different exposure pathways. It has been widely used for monitoring exposure to persistent halogenated EDCs ever since Laug, Kunze & Pitchett (1951) detected DDT in mothers' milk from non-occupationally exposed mothers. From the 1970s and onwards, much effort was focussed on biomonitoring POPs (UNEP, 2009c; Konishi, Kuwabara & Hori, 2001; Norén & Meironyté, 2000). More recently, human biomonitoring has also been applied to more easily metabolizable compounds, such as phthalates, bisphenol A and PAHs (e.g. NHANES (USA); Swedish monitoring programme; Knudsen & Merlo, 2012).

Blood is commonly used to assess internal exposures for persistent and bioaccumulative chemicals in human populations. As the lipophilic chemicals (e.g. PCBs, PBDEs and organochlorine pesticides) are associated with blood fats, analysis is performed on serum or plasma. However, due to the low fat content of human serum and plasma, the detection can be difficult at low concentrations. Also for breast milk analyses several ml are used. The challenge with blood analysis is that sample volumes are often limited especially for biobank samples.

Several of the halogenated phenols, original compounds or metabolites of POPs accumulate in blood due to their proteinophilic properties. Both the POPs and the halogenated phenols are also efficiently transferred through the placenta (Park et al., 2008; Wan et al., 2010). The concentrations in

human blood of, e.g. OH-PCBs can reach about 20% of the PCB levels in the fats (Linderholm et al., 2007; Park et al., 2008). Further, pentachlorophenol levels in blood are in the high µg/g range on a fat weight basis (Zheng et al., 2011), which is far higher than concentrations of individual PCB congeners (Glynn et al., 2011). Perfluorinated alkyl acids (PFAAs) are likewise proteinophilic, and as little as 150 mL of serum is sufficient to measure a wide range of PFAAs (Haug, Thomsen & Becher, 2009).

Blood is also used to determine effective internal doses of non-persistent chemicals which are *per se* reactive or form reactive intermediates in humans and thereby bind to proteins or DNA. Examples are DNA adducts of BaP and haemoglobin adducts of acrylamide (Knudsen & Merlo, 2012). For non-persistent chemicals with an elimination half-life of a few hours, concentrations in blood decrease quickly after the exposure and are usually lower than those in urine (Needham & Sexton, 2000). As a result, concentrations of the chemicals in blood will be highly dependent on the time elapsed since exposure and, in many cases, below the concentration that can be measured (below the limit of detection). Determination of blood concentrations of rapidly excreted chemicals may therefore have limited value for biomonitoring purposes in epidemiological investigations.

Urine is used for human biomonitoring of compounds that are rapidly metabolized and excreted such as phthalates, bisphenol A and PAHs. When considering chemicals like these with short half-lives in the body, it is very important to regularly assess exposure since urine levels can vary over short periods and do not reflect long-term exposures (Preau et al., 2010). Spot (one time) urine samples do not give a representative picture of the internal exposure, as excretion of the chemical and urine dilution vary during a day. Corrections for dilution have been made by normalizing concentrations to creatinine content or specific weight. Twenty four hour urine samples would give the best picture of recent exposure, however this is not always feasible in large scale epidemiological studies.

While blood and urine are the most common biological matrices used for biomonitoring, many other tissues or fluids have been used, for example hair, toenails, and saliva. In addition, a number of other biological matrices are used to assess internal exposures in pregnant women and infants, including amniotic fluid, placenta, cord blood, and meconium (Koch & Calafat, 2009).

Further, mothers' milk biomonitoring has been used to determine both internal doses to the lactating woman and exposures to the developing baby. Mothers' milk biomonitoring is most amenable for measuring POPs such as organochlorine pesticides, PCBs, dioxins and PBDEs. The non-invasive sample collection and the high fat content make mothers' milk an ideal human sample for these EDCs.

Autopsies and biopsies have been performed in certain cases to obtain human tissues for analysis, but this is not feasible for large scale monitoring. For example, lipophilic POPs have been analysed in adipose tissue as this is a depot for these compounds (Guvenius, Bergman & Norén, 2001; Yu et al., 2010). The liver is a target for PBDEs and higher concentrations have been found in human liver than in adipose tissue (Doucet et al., 2009).

Availability of biomonitoring data has greatly accelerated since the 1990s, driven by both government investment and enhanced technical capabilities. The most comprehensive studies include the National Reports on Human Exposure to Environmental Chemicals conducted by the USA government, the German Environmental Survey conducted by the German Federal Government, and the Arctic Monitoring and Assessment Programme, an international programme to implement components of the Arctic Environmental Protection Strategy and administered by eight countries with land mass above the Arctic Circle (Porta et al., 2008). For example, the USA has made large investments in their national biomonitoring programme, which is administered by the Centers for Disease Control and Prevention (CDC). The programme collects blood and urine from a representative sample of the USA population as part of the National Health and Nutrition Examination Survey (NHANES) and analyses the NHANES participants' blood and urine for various chemicals (Centers for Disease Control and Prevention 2008). The number of chemicals the government has measured and reported has increased from a little over 100 to well over 200 persistent and non-persistent compounds between late 1990 and 2011 (Centers for Disease Control and Prevention 2008). Many chemicals are found ubiquitously in the USA population. Some of the chemicals found in virtually all the population include a number of POPs. Some of these chemicals have been banned in the U.S. for over 30 years (such as DDT, and PCBs), as well as in many other parts of the world. Others include persistent and bioaccumulative chemicals that have been more recently phased out. These include several PFCs (PFOS, PFOA, PFHxS, and PFNA have been found in over 98% of the USA population) and PBDEs (BDE-47, BDE-100, and BDE-153 found in over 95% of the USA population) (Calafat et al., 2007; Centers for Disease Control and Prevention, 2008). A number of less persistent compounds are also found in virtually all of the USA population including certain phthalates, PAHs, phenols, such as bisphenol A, and perchlorate (Centers for Disease Control and Prevention, 2008). While levels of lead in the USA have declined greatly, due to the removal of lead from gasoline, paint, food cans and other products, there are populations both within and outside the USA with high blood lead levels, often from exposure to lead contaminated paint (US EPA, 2010b). In addition, exposure to many compounds, such as organophosphate pesticides, is still found in relatively large segments of the population (e.g. 16 to 89% of children) (Payne-Sturges et al., 2009). Other studies have also found multiple chemicals measured in human tissues in Europe and in populations in the Arctic – indicating a global distribution of pollution exposure (Porta 2004; Porta et al., 2008).

Consequently, there is simultaneous exposure to multiple chemicals in the population. In particular, some exposures are common during sensitive periods of development. A study published by Woodruff et al. found that virtually all pregnant women in the USA are exposed to at least 43 different chemicals, though there were some chemicals that were detected in few to no pregnant women (Woodruff et al.,

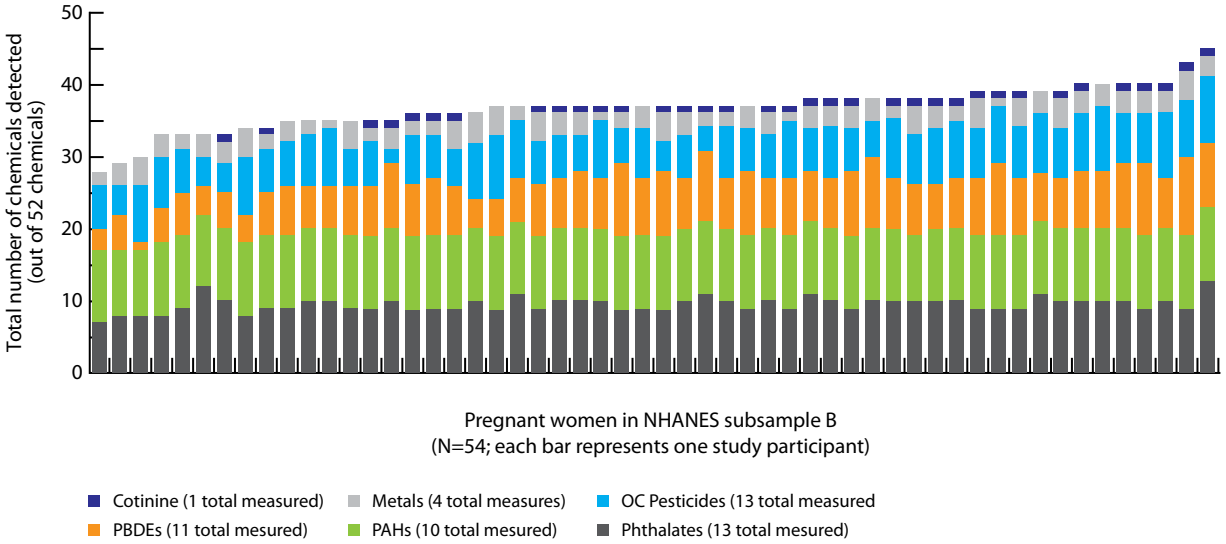


Figure 3.28. Number of chemicals detected by chemical class in pregnant women in the USA (National Health and Nutrition Examination Survey subsample B (metals, cotinine, organochlorine pesticides, phthalates, PBDEs, and PAHs), 2003-2004. (Figure from Woodruff, Zota & Schwartz (2011), redrawn; Used with publisher's permission).

2008) (**Figure 3.28**). While the authors did not evaluate the observed levels with potential adverse health consequences, many of the chemicals measured were similar to levels measured in epidemiologic studies finding an association with adverse reproductive and developmental outcomes. These include: phthalates and an increased risk of adverse male reproductive outcomes when exposure occurs prenatally (Swan et al., 2005; Chapter 2.3); mercury and developmental neurological outcomes (Lederman et al., 2008; Chapter 2.6), PBDEs and neurodevelopmental outcomes (Herbstman et al., 2009; Chapter 2.6); and PCBs and maternal thyroid hormone disruption during pregnancy (National Toxicology Program, 2006; Chevrier et al., 2008; Chapter 2.5 & 2.6).

Exposures can also be higher in certain populations, including children, and in certain types of exposure situations such as occupational settings. For example, women living in the agricultural Salinas Valley of California had higher measurable levels of several pesticides compared to a representative sample of pregnant women in the USA (Castorina et al., 2010). Children can have higher exposure because of unique behaviours, i.e. smaller children have more exploratory behaviour, and put their hands in their mouth more often, which can lead to increased exposure to chemicals that are prevalent in objects they come in contact with, such as toys or dust (US EPA, 2008). For example, limited data on PFCs found higher levels in children ages 3 to 11 years when compared with other age groups (Kato et al., 2009; Toms et al., 2009). Another example is for PBDEs (e.g. hand-to-mouth behaviour or particular diets); these data also indicate that children younger than 7 can have the highest exposures. A large study conducted in Australia found that the levels of PBDEs in blood were greatest for children ages 2 to 6 years, compared with older children and adults (Toms et al., 2009). A study of 20 young children (ages 1.5 to 4 years) in various locations throughout the USA found that their PBDE blood levels were about 3 times higher than those of their mother (Lunder et al., 2010). In California, children 2 to 5 years of age had PBDE blood levels that were greater than those in adults and in children of a similar age in other parts of the USA; this difference is likely due to California's unique regulatory requirement for foam to meet certain standards for flammability that require the use of chemicals, and the ubiquitous presence of PBDEs in the house, particularly house dust (Zota et al., 2008; Rose et al., 2010).

Exposures to multiple chemicals has implications for assessing risks, as studies find that exposures to several chemicals that adversely affect the same common health outcome can result in a greater risk than exposure to an individual chemical (National Research Council, 2008). The USA National Academy of Sciences recommends that health risk assessments incorporate simultaneous exposures to multiple chemicals (National Research Council, 2008).

3.2.2.3 Spatial trends for humans

PBDEs

Biomonitoring of PBDEs has been used to identify geographic differences in exposures. On a global scale, the USA population has higher PBDE body burdens than people in Europe or Asia (Toms et al., 2011; **Figure 3.29)**, except for those regions of developing countries where people have been exposed to, e.g. e-waste. Blood levels of the dominant congener BDE-47 in people in North America (0.63-46 ng/g fat) are approximately one order of magnitude higher than those observed in Europe (0.24-2.4 ng/g fat) (Fredriksen et al., 2009), likely due to higher use of PentaBDE, OctaBDE and DecaBDE in North America. Zota et al. (2008) found that serum levels of PBDEs were about two times higher in California compared to

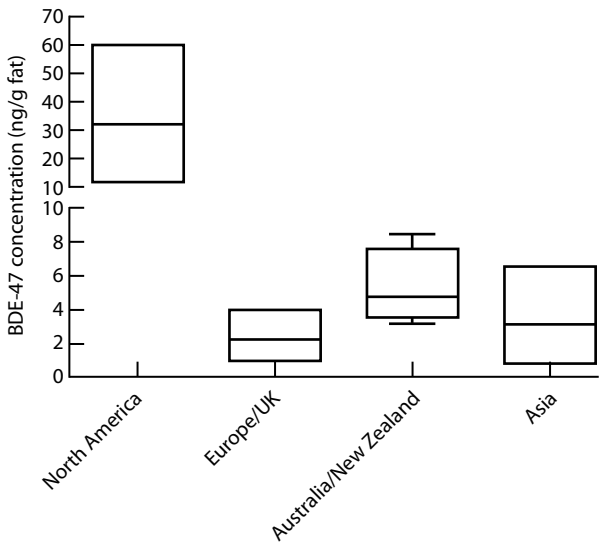


Figure 3.29. Range (min. to max.) of BDE-47 concentrations (ng/g fat) in human serum by continent (Toms et al., 2011). (Figure redrawn; Used with publisher's permission).

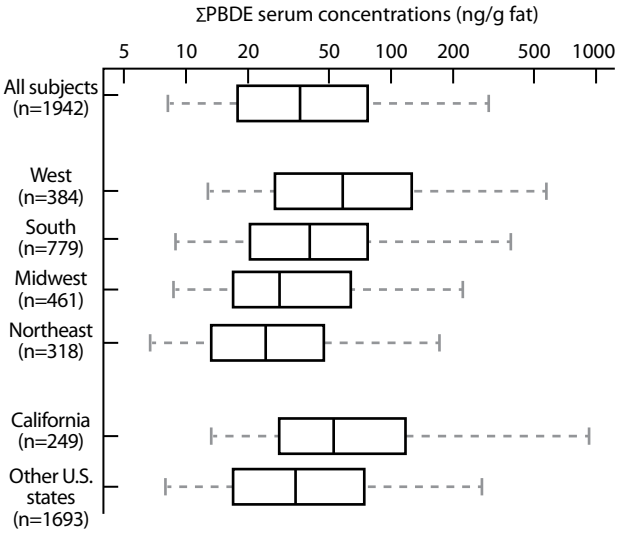


Figure 3.30. Differences in PBDE serum concentrations (ng/g fat) by geographic region within the USA using data from the 2003-2004 National Health and Nutrition Examination Survey. (Figure from Zota et al. (2008), redrawn; Used with publisher's permission).

the rest of the USA (**Figure 3.30**). Further, a small study of low income pregnant women in California found the highest levels of PBDEs measured in pregnant women in the world (Zota et al., 2011). When compared to similar-aged children in other countries or parts of the USA, children in California (2-5 years of age) had PBDE concentrations that were 5-1000 times higher and were related to the presence of new furniture or mattresses in the home and their dietary habits (Rose et al., 2010). Other subpopulations at risk from PBDEs also include those that have unique exposure sources. For example, Thomsen et al. (2008) found significant associations between the serum levels of PBDEs in hobby anglers and the self-reported consumption of trout and pike from a BFR contaminated lake in Norway. Among the hobby anglers, the median for the sum of seven PBDEs was 18 and 8.4 ng/g fat for men and women, respectively. In the reference group eating only food with background contamination, the corresponding median was 3.7 ng/g fat for both men and women.

Phthalates

Measurement of urinary phthalate metabolites is used to estimate internal exposure to these chemicals. Most biomonitoring data on phthalate exposure have been collected for the German and the USA populations. In general, the data from both countries are in good accordance, with the highest levels found for metabolites of diethyl phthalate, DBP and DEHP (Wittasek et al., 2011). However some differences were observed; the metabolites monobutyl phthalates (MnBP and MiBP) were highest in Germany whereas the concentrations of monoethyl phthalates and monobenzyl phthalate were highest in the USA. Different patterns of phthalate use, e.g. in personal care products, may explain these different exposure levels. Data from other countries are scarce but emerging over the last years. In 36 Japanese volunteers urinary concentrations of DEHP metabolites were comparable to concentrations from the German studies (Itoh, Yoshida & Masunaga, 2007). The daily exposure back-calculated from the urinary metabolite levels showed for DEHP medians of 0.9 µg/kg body weight (bw)/ per day for the USA studies (n=6), 4.1 µg/kg bw/per day for the German studies (n=6), and 1.8 µg/kg bw/per day for the Japanese studies (n=2).

3.2.2.4 Temporal trends for humans

DEHP and DiNP

As an example of how exposure to phthalates follows the production volumes, results from investigations using the German Environmental Specimen Bank for Human Tissues are presented. Wittasek et al. (2007) measured DEHP metabolites in 24h urine samples collected from a total of 634 university students between 1988 and 2003. The calculated daily intakes of DEHP varied between 0.19 and 39.8 µg/kg body weight but showed a significant decrease over the 15 year time period from

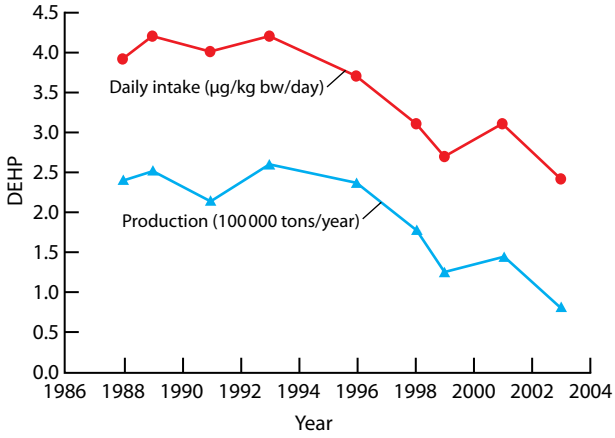


Figure 3.31. Time course of industrial DEHP production in Germany and median daily intake of DEHP in university students. (Figure from Helm (2007), redrawn; Used with publisher's permission).

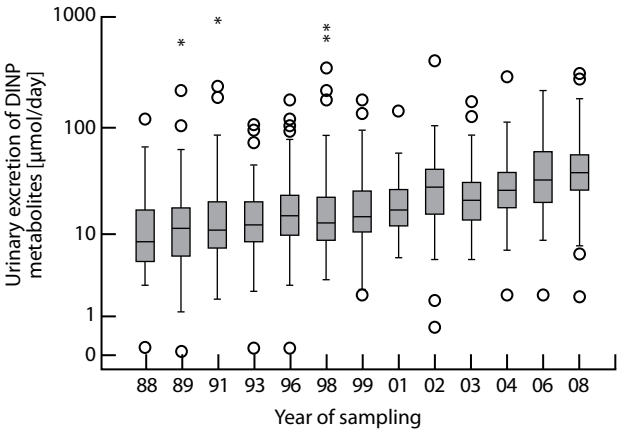


Figure 3.32. Trend of daily urinary excretion (µmol/day) of diisononylphthalete ester (DiNP) metabolites, presented as the sum of 7-hydroxy-monoisononyl phthalate ester (OH–MiNP) and 7-oxo -monoisononyl phthalate ester (oxo-MiNP), for the period 1988–2008 shown as box plots with median and central fifty percentile range. (Figure from Göen et al. (2011), redrawn; Used with publisher's permission).

a median of 4.2 µg/kg body weight/day in 1998 to 2.4 µg/kg body weight/day in 2003. Helm (2007) compared these intake data with the production data for DEHP in Germany and found a nearly perfect correlation (**Figure 3.31**). In contrast to DEHP, a continuous increase in the urinary excretion of metabolites of di-iso-nonyl phthalate (DiNP) was observed (**Figure 3.32**), starting from 10 µmol/day in 1988 to 40 µmol/day in 2008 (Göen et al., 2011). These temporal trends are explained by the restriction in use of DEHP for several applications and the substitution with DiNP.

Persistent organic pollutants

To investigate the temporal trends in human exposures to POPs, retrospective time trend studies have been performed in Norway (Haug, Thomsen & Becher 2009; Thomsen, Liane & Becher, 2007). The studies determined PCBs, PBDEs and PFCs in archived serum samples (from men 40-50 years old), pooled according to year of collection, from more than 25 years in the period 1976 up to the early or mid-2000s. For PCBs, blood levels continuously decreased over that time, corresponding to the 1980 ban on new uses of PCBs in Norway. In the same period, blood levels of PBDEs were increasing until around 2000, after which they seem to reach a plateau and possibly decrease. This may be related to voluntary actions among downstream users of PBDEs and, subsequently, the phase-out of PentaBDE and OctaBDE, due to EU legislative measures (Cox & Efthymiou, 2003). Similarly, for PFOS, a nine-fold increase in the serum concentrations of this PFC was observed from 1977 until the mid-1990s, when the concentrations reached a plateau before starting to decrease around year 2000 (see **Figure 3.33**). This may be related to the voluntary phase-out of perfluorooctylsulfonyl chemicals by the main manufacturer at that time.

Similar trends in POPs concentrations have been found in Germany. Each year since 1985 the German Environmental Specimen Bank for Human Tissues has collected and stored human specimens (mainly urine, blood and scalp hair) from about 500 volunteers at four universities in Germany. Temporal trends in concentrations of inorganic elements and a wide range of organic pollutants have been measured

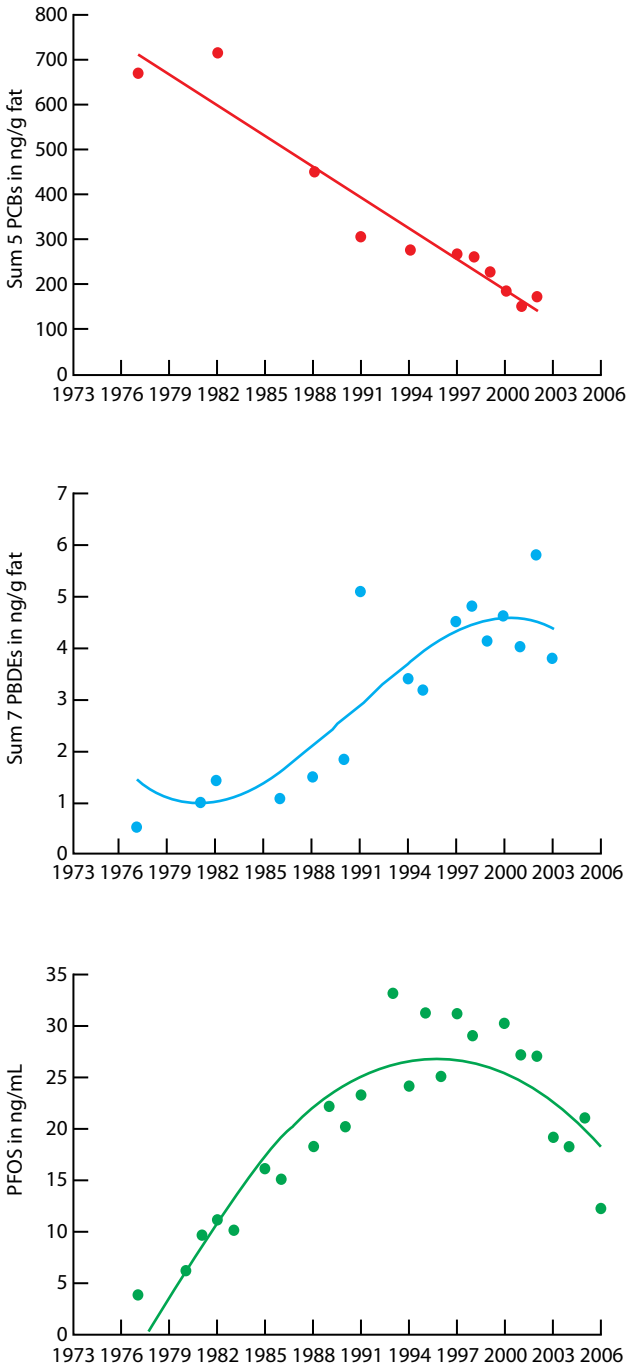


Figure 3.33. Temporal trends in concentrations of PCBs (sum of five congeners, ng/g fat; top graph), PBDEs (sum of seven congeners, ng/g fat; middle graph), and PFOS (ng/mL; bottom graph) in pooled (n>20) serum samples from Norwegian men. PCB, PBDE and PFOS concentrations were measured in the same group of men (Thomsen, Liane & Becher, 2007; Haug, Thomsen & Becher, 2009).

either in real-time or retrospectively (Wiesmüller & Gies, 2011). Plasma concentrations of chlorinated pollutants, such as pentachlorophenol, HCB, PCDDs/PCDFs and PCBs, are clearly declining in the time periods investigated, while they are increasing for PBDEs (until 1999). These trends seem to reflect the changes in production and usage patterns.

3.2.2.5 Prenatal exposure

It is well recognized that transfer of metals and xenobiotic chemicals, including EDCs, from mother to child occurs through the placenta during pregnancy (Tan, Meiller & Mahaffey, 2009; Winneke 2011; Barr, Bishop & Needham, 2007). Such in utero exposures have become an important public health concern because of the possible impact of EDCs on sensitive development and programming of organ function (Grandjean et al., 2008; see Chapter 2, all sections). Many current studies are focusing on the association between in utero exposures to a variety of environmental chemicals, in particular EDCs, and birth, developmental and neurocognitive outcomes (Herbstman et al., 2009; Suzuki et al., 2010; Tan, Meiller & Mahaffey, 2009; Chapter 2.6). The concept of "developmental origins of health and adult disease" hypothesizes that fetal and early life exposures can induce adverse effects in adulthood (Newbold et al., 2008; Fox et al., 2012).

Assessment of fetal exposures to EDCs can typically be achieved by measuring chemicals in maternal tissues as a surrogate for fetal exposure, or by measuring chemicals in cord blood, amniotic fluid or neonatal meconium (Barr, Wang & Needham, 2005). Fetal exposure to POPs and bioaccumulative metals can be assessed by making maternal blood measurements. For the non-persistent chemicals, i.e. chemicals with short environmental and biological half-lives, a single maternal measure likely does not accurately reflect total fetal exposure during gestation (Barr, Wang & Needham, 2005). Further, some EDCs can bioaccumulate in the fetus resulting in greater exposure during this very sensitive period of development. For example, fetal methylmercury levels have been shown to be about 1.7 times greater than maternal levels (Stern & S Smith, 2003). Other chemicals may not accumulate in the fetus; for example PBDEs are lower in umbilical cord blood than in maternal blood (Frederiksen et al., 2010b).

The transfer of chemicals across the placenta has also been investigated in ex vivo human placenta perfusion systems. Frederiksen et al. (2010a) studied the kinetics and the extent of placental transfer of BDE-47, -99 and -209 by adding these PBDEs to maternal circulation and monitoring the chemicals in the maternal and fetal compartments. Placental transfer was dependent on the degree of bromination. The transport of BDE-47 occurred much faster and to a greater extent than for BDE-99, while the transport of BDE-209 seemed to be very limited. Using the same approach, Balakrishnan et al. (2010) investigated the placental transfer of the non-persistent bisphenol A at environmentally relevant concentrations. About 27% of bisphenol A was detected in the fetal compartment within 3 hours, demonstrating that low concentrations can cross the human placenta, mainly in its original, unconjugated form.

Umbilical cord blood has often been used for assessing the exposure to a variety of halogenated POPs and metals. It has the advantage of being a non-invasive sample, but the sample amount is limited and the fat content is lower than in maternal blood. Thus, the detection of low levels of lipophilic POPs can be difficult (Barr, Wang & Needham, 2005). A comprehensive review of concentrations of xenobiotic chemicals in the maternal-fetal compartments has been presented by Barr, Bishop & Needham (2007). In the following, only a few examples are presented to demonstrate the pervasiveness of fetal exposures to a mixture of environmental chemicals.

Needham et al. (2011) measured the concentrations of 87 environmental chemicals in paired mother-child samples (cord serum and tissue, placenta, maternal serum and mothers' milk) from a birth cohort on the Faroe Islands, where exposures to marine contaminants is high. Virtually all substances found in mothers were also present in fetal tissues and cord blood, demonstrating that transplacental passage had occurred. For organohalogen compounds detectable in all tissues, a high correlation between concentrations in maternal serum and the other tissues investigated was generally observed. Concentrations of chlorinated POPs in cord serum were 20% of what was found in the mothers' sera; after adjusting for differences in the fat content of the sera, cord blood had slightly more than half of the POPs levels of maternal serum. In addition, mercury levels showed excellent correlations among the different sample types, suggesting that all of the tissues, including the easily collected umbilical cord, are useful for biomonitoring fetal exposure to this metal (Grandjean et al., 2005).

Park et al. (2008) investigated in detail the placental transfer of PCBs and their hydroxylated metabolites in a birth cohort from a PCB contaminated area. It was demonstrated that PCBs were transferred on a 1:1 basis between the fat compartments in maternal and fetal blood, but concentrations on fresh weight basis were lower due to the lower fat content of the fetal serum. Hydroxylated PCBs, in contrast, were not associated with fats but instead were bound to serum proteins. Thus, concentrations of hydroxylated PCBs in cord serum were quite similar to those in maternal serum on fresh weight basis. This suggests either a higher placental transfer rate of the hydroxylated PCBs compared to the original PCB compounds or a higher metabolism of PCBs in the fetus.

Frederiksen et al. (2010b) determined PBDEs in 51 pairs of maternal and cord plasma from a cohort of Copenhagen women. The concentrations observed in maternal (median 1.77 ng/g fat) and fetal (median 0.96 ng/g fat) plasma were highly correlated with each other, but the placental transport was found to decrease with increasing degree of PBDE bromination. Furthermore, positive correlations were found for the sum of PBDEs in cord blood and house dust indicating that house dust is a significant source of human exposure, including *in utero*, to PBDEs in Denmark.

Gützkow et al. (2011) determined PFAAs in 123 paired samples of maternal and fetal plasma from a subcohort of the Norwegian Mother and Child Cohort Study. Strong and highly significant correlations were found between maternal and cord blood concentrations on fresh weight basis for PFOA, PFOS and three other PFAAs. Compared to the maternal samples, cord plasma had a 1.4-4 fold lower median concentration of all the PFAAs measured. Placental transfer was found to be selective, with a higher proportion of shorter chained PFAAs in cord blood together with a higher amount of branched isomers of PFOS, indicating specific fetal exposures to some EDCs.

3.2.2.6 Case study of post-natal exposure - POPs in mothers' milk

Mothers' milk is an excellent matrix for the analysis of fat soluble pollutants, i.e. POPs and other persistent and bioaccumulative chemicals. Several reviews have addressed the issue over the last decade (e.g. Fuerst 2006; LaKind, Amina & Berlin, 2004; Norén & Meironyté, 2000; Solomon and Weiss, 2002; Tanabe & Kunisue, 2006). It is, however, possible to use mothers' milk to trace other chemicals (such as PFOS and PFOA) that have primary accumulation in blood and liver, and to assess their time trends (as shown in **Figure 3.34;** Sundström et al., (2011) and those of pentachlorophenol (Norén & Meironyté, 2000). The increasing concentrations of PFOS and PFOA stopped around year 2000 and then declined in the most recent years, likely due to legislative measures and changes in production of these chemicals (3M Company, 2000; US EPA, 2006b). For other chemicals with shorter half-lives, mothers' milk would not be an appropriate tissue to monitor for exposure assessments.

The general affinity of chlorinated and brominated POPs for fats has led to the identification of 22 POPs, HBCDD and chlorinated paraffins in mothers' milk. For some of these the dataset is very limited, e.g. chlorinated paraffins were reported in mothers' milk in Germany for the first time in 2005 (Reth et al., 2005). The year after, levels of both short- and medium-chained chlorinated paraffins (SCCPs and MCCPs) were reported for UK mothers' milk, the former in sum concentrations between 50 and 800 ng/g fat and the latter at lower concentrations (6-300 ng/g fat) (Thomas et al., 2006). The data indicate that it is primarily chain lengths of C10-C14 of the chlorinated paraffins that accumulate in the milk fat.

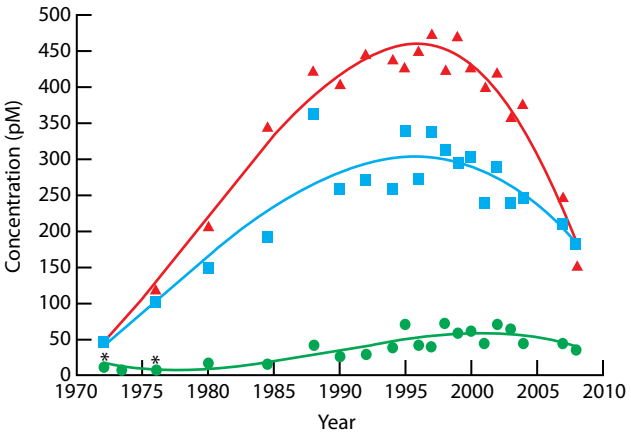


Figure 3.34. Temporal trends of PFOS (▲), PFOA (■) and PFHxS (●) (pmol) in mothers' milk from Stockholm, Sweden, from 1972 -2008 (Sundström et al., 2011).

For many of EDCs, the global coverage of their concentrations in mothers' milk is poor. For example, only a few reports from Europe and one from Canada have been published for pentachlorobenzene, showing median levels of 1 ng/g fat or less. Median toxaphene levels are higher and in the range of 10-60 ng/g fat. Some typical concentrations of the least studied POPs in mothers' milk are shown in **Table 3.2**. The WHO human milk survey included several POPs in their analysis and results from one country in each of South America, Africa and Asia are shown in **Figure 3.35** (UNEP, 2009c). Levels of ΣDDTs dominated across all of the countries.

Data on PBDEs, PFOS and HCHs in mothers' milk exist from around the world. Two of the chemicals were either just emerging or not considered 10 years ago, (i.e. PBDEs and PFOS, respectively), while HCHs were reported but had only limited data available for assessing human exposure and transfer to nursing children. Still, there are some countries from which no data have been reported. The longest temporal trend study on POPs (BDE-47, BDE-153 and HBCDD) in mothers' milk comes from Stockholm (Bergman et al., 2010; Fängström et al., 2008) and results are shown in **Figures 3.36** and **3.37**. In addition, BDE-47, BDE-153, β-HCH and γ-HCH (Lindane) levels in mothers' milk show some variability across the globe (**Figures 3.38** and **3.39**). It is important to note that almost no data were obtained from South America, while other continents are well represented.

Data on POPs in mothers' milk are dominated by reports on DDT (including DDE) and PCBs, with more than 50 reports on DDE from 1995 until today. Similarly, PCB reports number more than 100 during this period. However, it is not possible to compare all the data because different congeners were measured or the results were calculated and presented differently from one study to another (i.e. concentrations can be given in fresh weight or fat weight for one or several congeners and either as mean or median concentrations). This is a problem for the POPs most frequently reported in mothers' milk, but also occurs for any other human or wildlife tissue.

Temporal trends of 4,4'-DDE and CB-153 in Swedish mothers' milk are shown in **Figures 3.40** and **3.41**, respectively, from 1972 until 2010 (Bergman et al., 2010). Comparing these data to international levels indicate that 4,4'-DDE concentrations may be from 50 up to more than 10,000 ng/g fat in some mothers' milk, with high concentrations from Zimbabwe (Chikuni et al., 1997), India (Devanathan et al., 2009), and Vietnam (Haraguchi et al., 2009). Levels of 4,4'-DDE are more commonly between 50–1000 ng/g fat. For comparison, PCB levels (as mirrored by CB-153) range over two orders of magnitude 5–500 ng/g fat, with the lowest and highest levels in Vietnam (Tue et al., 2010a; 2010b; Nguyen

Table 3.2. Examples of mean or median concentrations (ng/g fat) for the least well studied POPs in mothers' milk worldwide. The countries from where the samples originate are given in the table, with references as footnotes.

Country	Heptachlor (mean)	Mirex (median)	∑PBB[8]* (mean)	PCBz (median)	Toxaphene (mean)	HBCDD (mean)
Australia		0.2[1]				
Canada		2[2]		1[2]	7[2]	
China					1[3]	
Denmark		0.2[4]	0.3[4]	0.3[4]		
Finland		0.3[4]	0.2[4]	0.2[4]		
Germany	20[5]					
Japan						1[6]
Jordan	500[7]					
Mexico	600[8]			200[8]		
Norway		0.6[9]				2[10]
Russia		0.5[11]			10[11]	0.5[11]
Spain						5[12]
Sweden						0.4[13]
Taiwan	3[14]					
The Philippines						0.9[15]
USA		2[16]				
USA		1[17]				

*BB-31, -49, -52, -77, -80, -101, -153 and -155.

[1] Mueller et al., 2008, [2] Newsome & Ryan, 1999, [3] Hedley et al., 2010, [4] Shen et al., 2008, [5] Schlaud et al., 1995, [6] Kakimoto et al., 2008, [7] Nasir, 1998, [8] Rodas-Ortíz, 2008, [9] Polder et al., 2008a, [10] Thomsen, 2010b, [11] Polder et al., 2008b, [12] Eljarrat et al., 2009, [13] Fängström et al., 2008, [14] Chao et al., 2006, [15] Malarvannan et al., 2009, [16] Greizerstein et al., 1999, [17] Madden & Makarewicz, 1996.

et al., 2010) and the Czech Republic (Cerná et al., 2010), respectively.

PCDDs/Fs in mothers' milk have been recently and extensively studied through the WHO human monitoring programme (UNEP, 2011b). The levels in mothers' milk vary between a few to almost 25 pg WHO-PCDD/F-TEQ/g fat in the 34 countries from which milk was obtained. A snapshot of TEQs (PCDD/F and DL-PCBs) in a few Eastern Asian countries is shown in **Figure 3.42** (Zheng et al., 2008a).

3.2.2.7 HPCs and Non-HPCs in mothers' milk

Whereas the contamination of mothers' milk by POPs is well documented, data on the presence of other EDCs are scarce. Ye et al. (2006) reported the presence of free and total (free plus those conjugated to endogenous molecules such as glucuronic acid or sulfate) selected environmental phenols in 20 mothers' milk samples. Bisphenol A was found at median concentrations of 0.4 ng/mL and 1.1 ng/mL for free and total species, respectively, indicating that the conjugated forms of

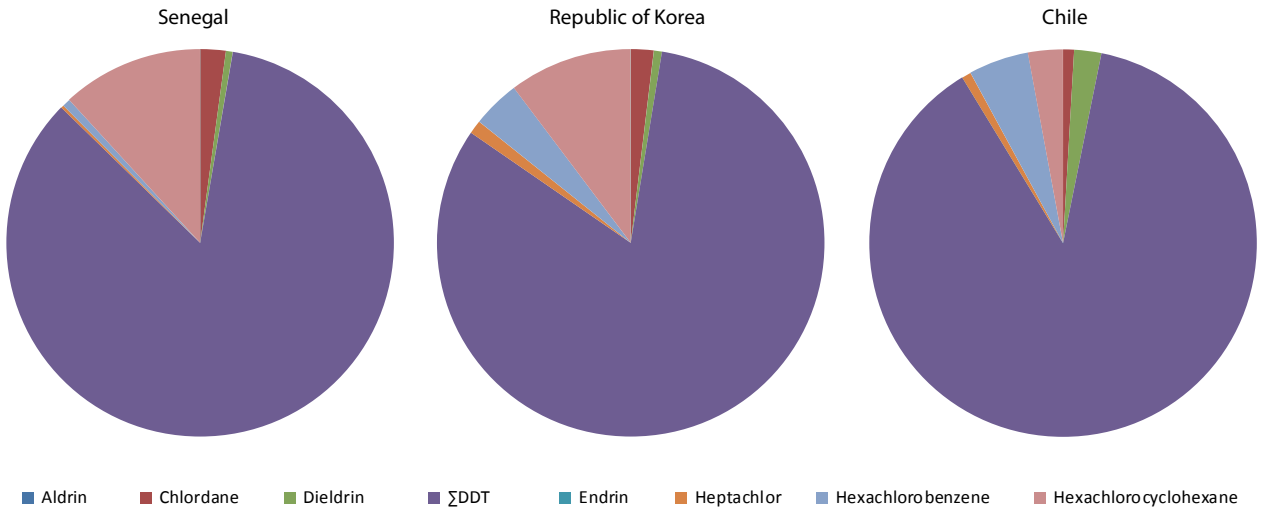


Figure 3.35. Relative content of different organochlorine pesticides in mothers' milk from three countries in three continents around the globe (diagram prepared on basis of UNEP, 2009c).

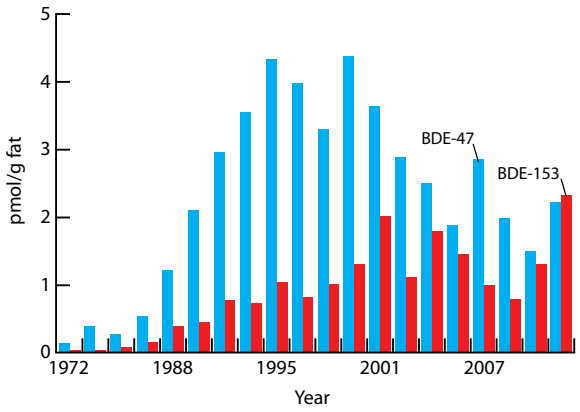


Figure 3.36. Temporal trends of the concentrations (pmol/g fat) of two PBDE congeners, BDE-47 and BDE-153, in mothers' milk from Stockholm, Sweden, as assessed 1972- 2010 (Norén and Meironyté, 2000; Bergman et al., 2010).

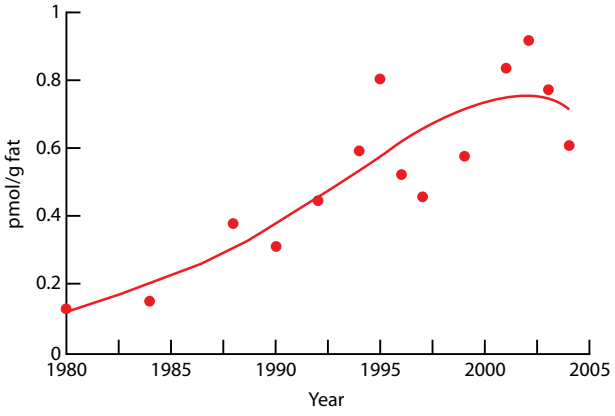


Figure 3.37. Temporal trends of HBCDD concentrations (pmol/g fat) in mothers' milk in Stockholm, Sweden, from 1980 – 2004 (Fängström et al., 2008).

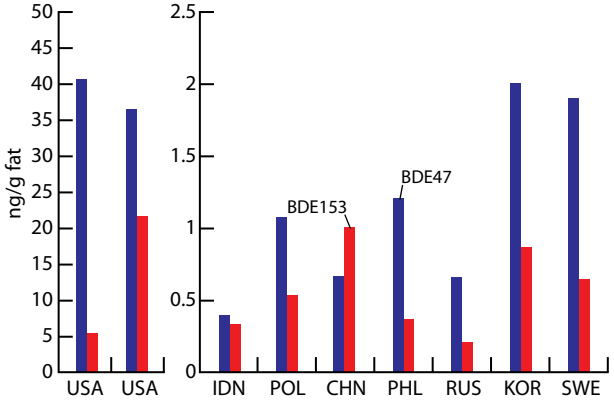


Figure 3.38. Examples of concentrations (ng/g fat) reported for two PBDE congeners, BDE-47and BDE-153, in mothers' milk from eight different countries (Country codes according to ISO 3166/MA Alpha-3-code). The two USA studies represent concentrations of the two PBDE congeners in milk from mothers in Massachusetts, USA (left USA bar) (Johnson-Restrepo et al., 2007) and from mothers in the USA in general (USA right bar) (Schecter et al., 2010).

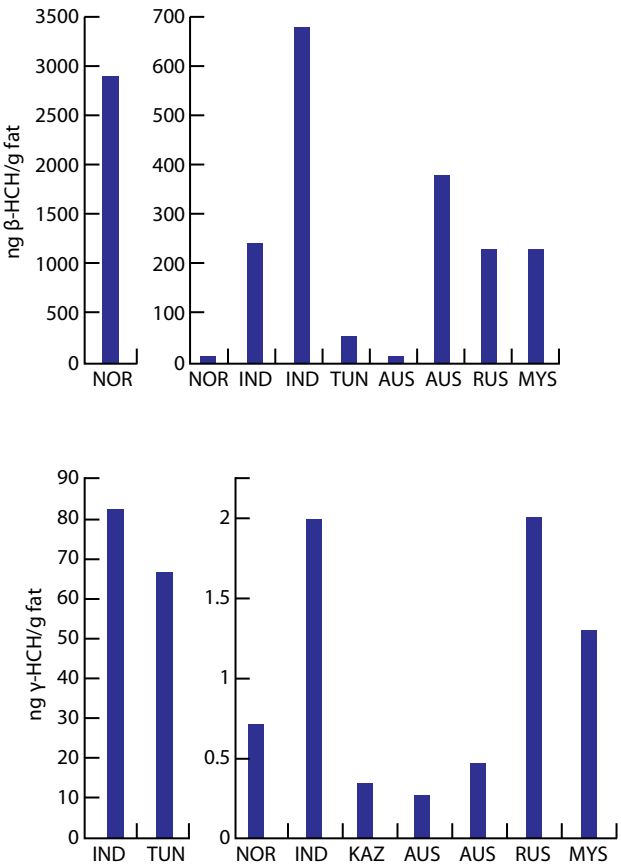


Figure 3.39. Concentrations (ng/g fat) of β-HCH (upper diagram) and γ-HCH (Lindane) (lower diagram) in mothers' milk from selected countries worldwide (Country codes according to ISO 3166/MA Alpha-3-code). Data are from WHO mothers'milk programme (UNEP, 2009c; 2011b).

this compound appear to be prevalent in milk. Conjugation (e.g. glucuronidation, sulfation) is a defense mechanism able to reduce the potential toxicity of compounds when only the free species is bioactive, as it is the case for bisphenol A. Accordingly, only free bisphenol A is usually measured in monitoring programmes used for human exposure assessment. However, most of the ingested conjugates are hydrolyzed during the digestive process, including in infants, although the phenomenon occurs to a limited extent in babies as compared with adults (Franke et al., 2006). The hydrolysis can take place in the stomach, due to the action of hydrochloric acid, but requires mainly the action of gut microflora to cleave conjugated bisphenol A (and other conjugates) to the free bisphenol A, prior to its intestinal absorption. For these reasons, exposure of breast fed infants should be based on total bisphenol A concentrations in milk and not only on free species.

Cariou et al. (2008) analyzed tetrabromobisphenol A (TBBPA) in mothers' milk samples collected in France and found levels varying from 0.062 to 37.000 ng/g fat (median value = 0.5 ng/g fat). These values are approximately one or two orders of magnitude higher than concentrations observed by other authors in various countries (Abdallah & Havrad, 2011;

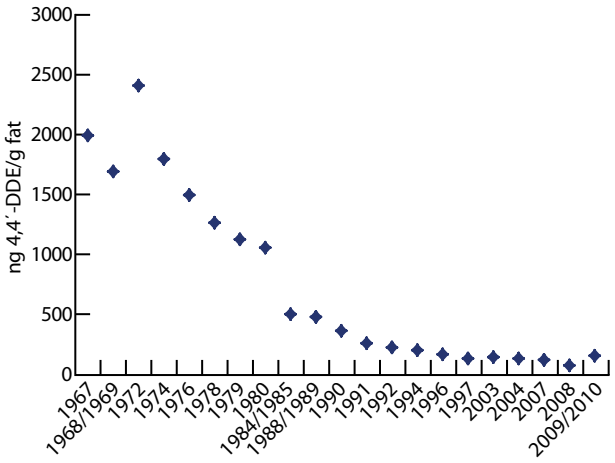


Figure 3.40. Temporal trends of 4,4'-DDE concentrations (ng/g fat) in Swedish mothers' milk (Norén & Meironyté, 2000; Bergman et al., 2010).

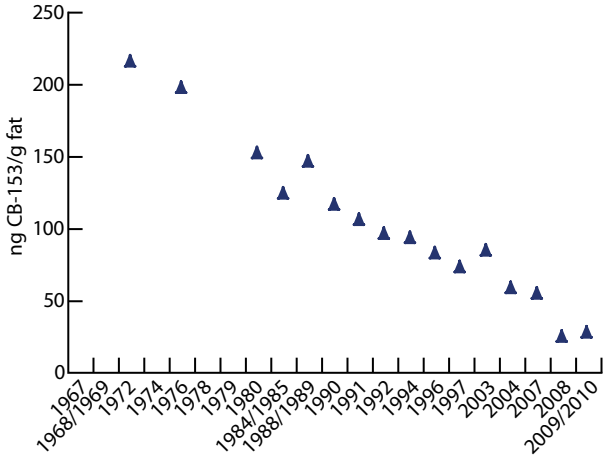


Figure 3.41. Temporal trends of CB-153 concentrations (ng/g fat) in Swedish mothers' milk 1972 - 2010 (Norén & Meironyté, 2000; Bergman et al., 2010).

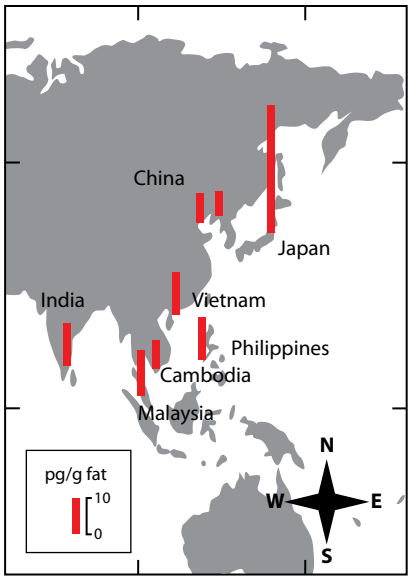


Figure 3.42. PCDD/PCDF and dioxin-like PCBs TEQ concentrations (pg/g fat) in mothers' milk from seven Asian countries (Zheng et al., 2008a) (Graph redrawn; Used with publisher's permission).

Shi et al., 2009; Thomsen, Lundanes & Becher, 2002). The apparent discrepancy between these studies is probably due to the fact that Cariou and co-workers included a hydrolysis step in the sample preparation procedure that was not done by other groups. This also suggests, as previously described for bisphenol A, that a major fraction of TBBPA found in mothers' milk is most likely present as TBBPA conjugates.

3.2.3 Conclusions

EDCs in wildlife and humans

- Exposure assessments of POPs and mercury in wildlife cover more of the globe today than a decade ago. This indicates increased monitoring of EDC exposures in wildlife. However, there are still major data gaps for POPs and mercury, particularly in tropical and subtropical areas.
- Newer information shows that wildlife are being exposed to a much greater diversity of chemicals in the environment than was documented ten years ago. In particular, pharmaceutical and personal care product ingredients and halogenated phenolic compounds are now commonly reported in wildlife.
- High levels of several POPs are still found in top predators in polar regions due to long-range transport and deposition of these chemicals and food web biomagnification.
- Several brominated flame retardants and perfluorinated surfactants have also become focal issues over this decade and are found globally in many different wildlife species. Unlike other POPs, PBDEs and PFOS are generally highest in wildlife near urban areas around the globe.
- Mussels have been widely used for POPs and metals monitoring and show promise for determining spatial and temporal trends of EDCs that are less persistent like bisphenol A, alkyl phenols and PAHs.
- Monitoring of abiotic media such as surface waters, soils, dust, and sediments near sources is also needed for assessing wildlife and human exposure to less persistent EDCs.
- The primary biological matrices in humans for measuring POPs are blood and mothers' milk, for POPs metabolites is blood, and for less persistent and less bioaccumulative chemicals is urine, due to the short half-lives in humans.
- Concentrations of EDCs in humans are strongly affected by activities, diet, nutritional status, and the places where people live, work and play. Concentrations of some EDCs (e.g. PBDEs) are higher in young children because of their high hand-to-mouth activities.
- Human and wildlife exposure to EDCs prior to and during pregnancy (prenatal exposure) is of particular concern due to the vulnerability of the developing fetus. Pregnant females are exposed to multiple chemicals. Most chemicals can cross the placenta, leading to fetal exposure.

- Wildlife and human infants can be exposed to EDCs via mothers' milk.

Changes in EDCs over time

A decade ago, the majority of data on human internal exposure to EDCs was on legacy POPs such as DDTs, PCBs and HCB. Since then, numerous reports on other POPs (e.g. PBDEs, PFOS), and chemicals used in materials and consumer products (e.g. phthalates, triclosan, siloxanes, bisphenol A, parabens) in human samples have been published for some countries. It is clear that humans are being exposed to a diverse mixture of EDCs.

- Global data on human concentrations of some POPs (e.g. chlorinated paraffins, mirex, toxaphene and endosulfan) and the less persistent, less bioaccumulative chemicals (e.g. phthalates, bisphenol A) are lacking.
- Long-term temporal trends of EDCs in wildlife and humans are available only for some POPs and mercury, and only in very few areas of the world.
- Over time, several POPs (e.g. PCBs, PBDEs and PFOS) have increased and then more recently decreased in most areas where concentrations in wildlife were measured. These decreases are due to restrictions or bans on their use in many countries.
- Temporal trend data for POPs are available only from a few human populations in the world. The data suggest that levels of the POPs that have been banned, or restricted in their use, are declining.
- There are very limited exposure and temporal trend data for less persistent, non-bioaccumulative EDCs in wildlife and humans.

3.3 Emerging issues and EDCs of concern

3.3.1 Identification of EDCs from chemicals in commerce

Identifying endocrine active chemicals from among the chemicals in commerce worldwide is a major challenge (Phillips et al., 2008). The EDCs identified so far (e.g. in TEDX, 2011) have a wide diversity of molecular structures. Selection of chemicals for detailed screening has been based on expert judgment using available toxicology data. However, as the USA EPA Endocrine Disrupting Screening Program (EDSP) website has noted, with the exception of food-use and consumer pesticides with regulatory mandates requiring prenatal developmental and two-generation reproductive toxicity testing, substantial endocrine effects data are lacking for most chemicals (US EPA, 2011c). Selection of potential EDCs from European chemical lists has also used a combination of exposure modeling and expert judgment to identify about 550 substances (Petersen, Rasmussen & Gustavsen, 2007).

There are over 143,000 chemicals in commerce based on the preregistrations done as part of the REACH legislation in the European Union (ECHA, 2012), although early estimates suggested that only about 30,000 produced or imported in quantities of one metric ton would require full registration (European Commission, 2003). In the USA there are 84,000 registered chemicals (including polymers) under the Toxic Substances Control Act (TSCA) inventory (>4.5 t/yr), although not all of these chemicals may be currently in production (USA EPA, 2011b). The identities of 17,000 inventory listings do not appear on the public version of the TSCA inventory because manufacturers have claimed the chemicals' identities as confidential business information (Denison, 2007). The Chinese "Inventory of Existing Chemical Substances in China" includes about 45,000 substances (http://www.crc-mep.org.cn/iecscweb/IECSC.aspx?La=1); however, this is thought to be only 30-50% of the chemicals likely imported or produced in China (http://www.rsc.org/chemistryworld/News/2010/April/01041001.asp). India has an inventory of hazardous chemicals (Government of India, 2008); however, no firm number of chemicals in commerce is yet available from this country. The large and growing production of chemicals in China and India, and their incorporation into consumer and industrial materials and goods for global export add to the challenge of defining the full numbers of chemicals in commerce.

Based on present knowledge, it is possible to trace high production volume chemicals to their application areas, but that is not the case for numerous additives and process chemicals. Adding greatly to the complexity are the unknown or unintended by-products that are formed during chemicals manufacturing, during combustion processes, and via environmental transformations, thus adding to the number of chemicals present in the environment. While the active ingredients in pharmaceuticals and pesticides have to be documented on the final product, this is not the case in materials and goods for construction, down-stream manufacturing and consumption.

The physical-chemical characteristics and reactivity of the individual chemicals in commerce range from low molecular masses that are highly water soluble and volatile to molecular masses up to around 1000 Daltons that are poorly water soluble, non-volatile, and either neutral or ionizable. Their reactivity goes from highly reactive to almost inert. All organic chemicals have a certain half-life in each of the environmental compartments and in vivo, due to metabolism. Further, almost all chemicals are transformed abiotically and biologically to numerous other chemicals (transformation products (TPs)). Accordingly, the chemosphere consists of compounds with the characteristics listed in **Table 3.1**, being persistent and bioaccumulative and undergoing long-range transport, or being stable enough to expose humans and wildlife to them even though their half-lives are short. Finally, there are the most reactive chemicals with very short half-lives in the

abiotic environment and in biota. These compounds will form more stable TPs than their parent compounds and adducts to biomacromolecules.

The range of properties of known EDCs based on the TEDX list (TEDX, 2011) is illustrated in **Figure 3.41** and compared against their environmental bioaccumulation potential to humans (EBAP) (Czub & McLachlan, 2004). EBAP is the ratio of the quantity of a chemical in a human to the quantity of the chemical present in 1 m^2 of the environment. Highly bioaccumulative chemicals have a maximum EBAP at log K_{OW} ~7 and log K_{OA} ~9. The majority of known EDCs (555 of 792 organic chemicals with known structures) are found within the chemical space of >10% maximum EBAP (log K_{OW} >3 and <10; log K_{OA} >6), indicating that they can accumulate in humans and in the agricultural and marine food webs. However, a significant fraction of the TEDX list (30%) consists of chemicals with predicted properties that are outside of this range (**Figure 3.43**).

Thus, selecting all chemicals with some potential to accumulate in human and wildlife food webs, along with knowledge of toxicology, is one approach to identify chemicals for further assessment. The screening of hundreds of thousands of chemicals in commerce has been done largely with a focus on persistent and bioaccumulative chemicals (Howard & Muir, 2010; Brown & Wania, 2008). As quantitative structure-activity relationships (QSARs) are developed for various endocrine endpoints, it will be possible to screen these lists again and again for various structures with known biological activity. However, with limited or no toxicological data on the vast majority of the chemicals, the development and validation of suitable QSARs is a challenge (Cronin and Worth, 2008). For example for the TEDX list of 850 chemicals with known effects on the endocrine system, 30% are halogenated phenolic compounds, indicating the importance of testing chemicals with these structures for estrogenic and thyroidogenic effects. These structures can be readily identified (Walker et al., 2003) and QSARs models have been developed for several endocrine receptor-related in vitro endpoints (reviewed by Schmieder et al., 2003). A greater challenge is the identification of novel EDCs and novel modes of action given the limited domains of existing QSAR training sets (Phillips et al., 2008); some chemicals may not be identified as EDCs using the current QSAR approaches given the diverse modes of actions that these chemicals can have on the endocrine system (Chapter 1).

The most obvious approach to initiate measurements of potential novel EDCs is a better global declaration of chemicals in materials and goods. Since the knowledge is, or should be, in place on the manufacturing side, it is reasonable to not only apply that to, e.g. pharmaceuticals, pesticides and cosmetics, but also to the chemicals going into construction materials, textiles, electronics and other consumer materials and goods. As such, structure-based selections of chemicals and preparation of highly pure standards for their analysis and testing can be done. Until proper documentation is available on all products, the scientific and regulatory communities need to work both ways, using the information available on chemicals in products and the well-designed and directed chemical analytical and bio analytical pathways.

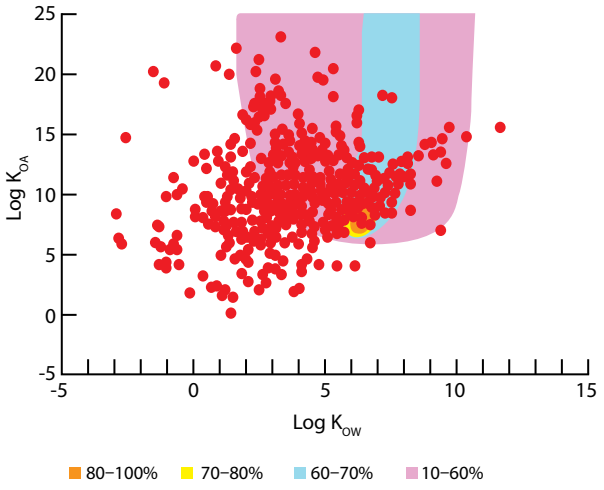


Figure 3.43. Log K_{OW} and log K_{OA} values for 792 EDCs from the TEDX (2011) EDC list are overlaid on the environmental bioaccumulation potential (EBAP; coloured areas) of persistent chemicals for a marine and agricultural diet (Czub & McLachlan, 2004). The coloured regions represent the percentage of the maximum EBAP obtained within the chemical partitioning space. The log K_{OA} values were estimated using EPISuite 4.1 KOAWIN software (US EPA, 2011a). The K_{OW} values were based on measured values for 421 EDCs and values predicted using EPISuite KOWIN for the others.

3.3.2 Analytical challenges in identifying, quantifying and reporting EDCs

Wildlife and humans are exposed to a wide variety of EDCs that differ greatly in their physical-chemical properties. Further, these compounds are generally present at very low levels and in complex matrices requiring highly selective and sensitive methods. It is clear that very low exposures to chemicals can have an effect on the endocrine system (see Chapter 1) and it is critical to be able to quantify these exposures. The wide range of different compound classes requires a variety of analytical approaches and techniques. In general, complicated and time-consuming extraction and purification steps are required, followed by chromatographic techniques often coupled to mass spectrometry. The majority of established analytical methods focus on the specific classes listed in **Table 3.1** and closely related compounds, e.g. dioxins and dioxin-like PCBs, indicator PCBs, organochlorine pesticides, BFRs, PFCs, phthalates, bisphenol A, and a large number of halogenated phenolic and non-phenolic compound classes known to exert ED effects (TEDX, 2011). Indeed, large numbers of halogenated phenols have been identified both in wildlife and in humans (Athanasiadou et al., 2008; Letcher, Klasson-Wehler & Bergman, 2000; Hovander et al., 2002). For routine quantitative analysis, however, analytical standards are required and, while they are available for most pesticides,

pharmaceuticals, PAHs, metals and many halogenated organics with POPs characteristics, they are typically not available for TPs nor for the vast array of organic chemicals in commerce (Howard & Muir, 2010). Thus targeted analyses based on analytical standards provide the necessary sensitivity and selectivity, but lead to a fragmented picture of the occurrence of EDCs in the environment, wildlife and humans. Consequently, there is a high demand for developing screening analytical methods that will accommodate a wide variety of analytical functional groups at low detection levels.

Successful chemical analyses are built on methods development, and should not rely only on instruments with lower and lower detection limits. Since POPs have been target analytes for a long time it is here the most advanced techniques for detection and quantification are available. Better methodologies are required for chemicals with variable persistence and short half-lives in vivo. These are adding up to a very large number of chemicals with highly different structures. These are the chemicals for which new methods need to be developed and the analytes need to be characterized in detail to promote methodological advances. Hence pure compounds are required. HBCDD may serve as an example of how analysis of a simple molecule has become increasingly complex with time (EFSA, 2011b; Law et al., 2005). Without detailed chemical characterization, it would not have been possible to improve the quality of the analyses and exposure assessments. While commercial chemicals can be obtained and purified, there is a major demand for chemical synthesis of metabolites and abiotically transformed products.

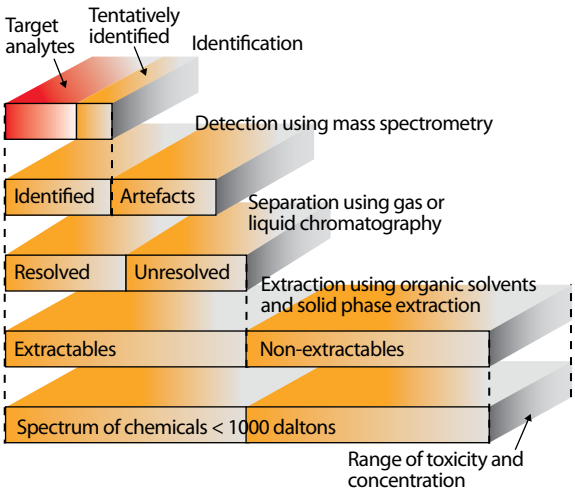


Figure 3.44. An illustration of the complexity of measuring chemicals, including potential EDCs, in environmental media. While the "spectrum of chemicals" (not isomers or congeners) is very large, only a subset can be extracted and separated by chromatography, and even fewer identified. [redrawn from Daughton, 2005; GC – gas chromatography, LC- liquid chromatography, MS – mass spectrometry, SPE – solid phase extraction]

The analysis of target analytes can be viewed as a top-down approach that only scratches the surface of the number of chemicals that can be measured as illustrated by **Figure 3.44**. Larger numbers can be tentatively identified based on mass spectra, but authentic standards are ultimately needed. Even larger numbers are isolated by the conventional extraction and separation technology widely employed in trace organics analysis laboratories, but they cannot be readily identified. Furthermore, the analyst has to make a decision about how to best allocate analytical resources for this task. The use of effects directed analysis (EDA) and QSAR directed non-target analyses are two emerging techniques that are helping to address this challenge (Hecker & Hollert, 2009; Helbling et al., 2010; Schymanski et al., 2009).

EDA involves bioassay-directed fractionation techniques to decrease the complexity of the sample matrix before identification of the endocrine active compounds. Estrogenic and androgenic in vitro assays are typically employed to screen fractions of sediment or wastewater extracts that were previously separated by liquid chromatography (Hecker & Hollert, 2009; Weiss et al., 2009; 2011). QSAR directed analysis involves identification of transformation products from structural information and identification from full scan high resolution mass spectra using post-acquisition data processing. Non-target analyses of candidate chemicals for which no standards are available can sometimes be accomplished by very high resolution mass spectrometry. However improved prediction systems for theoretical fragmentation patterns, retention times, and ionization behaviour are needed to widely apply this technology (Krauss, Singer & Hollender, 2010).

Even though wildlife and human matrices are available for extraction and analysis, the question remains on how measured levels are to be translated into an internal dose. Since chemical analyses and exposure assessments still lack standardization, it is only possible to do limited comparisons between studies. This lack of standardization is also hampering assessments of effects of mixtures. In this context, it is necessary to point out that it is not the mass-based concentrations that count when mixture doses are assessed, but the number of molecules. Hence, levels of contaminants in humans and wildlife need to be compared on a molar basis.

Human biomonitoring is a valuable tool in exposure assessment but it is usually performed at a single time point that disregards the variation in exposures throughout life and critical time periods. Recently, the concept of "exposome" has been introduced in the investigation of human exposure to environmental contaminants, representing the total exposure from conception onwards being of critical interest for understanding the environmental causes for disease (Rappaport, 2011; Wild, 2005). The comprehensive measurements of all exposure events during a lifetime requires ongoing, longitudinal sampling, particularly during critical life stages such as fetal development, early childhood and the reproductive years.

3.3.3 Conclusions

- While large lists of chemicals in commerce are now available and can be searched electronically, it is difficult to identify potential EDCs from among these chemicals because there is a lack of endocrine effects data on which to build suitable QSARs.
- Even for known or potential EDCs there is still a lack of data on where these chemicals are produced and used in products, materials and goods. This limits our ability to identify where and how much EDC might be in the environment or in wildlife and humans.
- Only a few of all potential EDCs are measured in the environment and in people and wildlife. Further, there is little known about metabolites of EDCs and how EDCs are transformed in products, which limits our ability to identify and measure them in wildlife, humans and the environment.
- The majority of identified EDCs have properties that suggest they will accumulate in humans and in agricultural/ aquatic food webs. However these EDCs may not be representative of the full range of molecular structures and properties of potential and known EDCs, due to the previous narrow focus on testing only halogenated chemicals for their estrogenic and thyroidogenic effects.
- The lack of appropriate methods for measuring many industrial chemicals, pesticides, pharmaceuticals, etc., is a major obstacle for exposure assessments of potential EDCs.
- The use of effects directed analysis as well as non-target analysis with high resolution mass spectrometry are emerging techniques that are helping to address the issue of lack of knowledge of specific chemicals or transformation products.
- Since there are no standards for how concentrations are to be reported for either wildlife or human matrices, this is strongly hampering comparisons between studies and further exposure assessments on a general basis.

3.4 Main messages

1. **EDCs are everywhere** - EDCs are chemically diverse, primarily include human-made chemicals, and are used in a wide range of materials and goods. EDCs are present in food, nature and human beings. They can also be formed in the environment and in humans, wildlife and plants.
2. **Increasing number of EDCs** – Unlike ten years ago, it is better understood that humans and wildlife are exposed to far more EDCs than just persistent organic pollutants (POPs). However, only a fraction of the potential EDCs in the environment are currently understood.
3. **Exposed to mixtures** - Humans and wildlife are also exposed to multiple EDCs at the same time, and there is justifiable concern that different EDCs can act together and result in an increased risk of adverse effects on human health and wildlife.
4. **Still measuring only a few** - Right now only a narrow spectrum of chemicals and a few classes of EDCs are measured, making up the tip of the iceberg. More comprehensive assessments of human and wildlife exposures to diverse mixtures of EDCs are needed. It should be a global priority to develop the abilities to measure any potential EDCs. Ideally, an "exposome" should be developed, i.e. a highly detailed map of environmental exposures that might occur throughout a lifetime.
5. **Exposure occurs at early life stages** - Exposures to EDCs occur during vulnerable periods of human and wildlife development – from fertilization through fetal development and through nursing of young offspring - and raises particular concern.
6. **Important routes of exposure -** New routes of exposure to EDCs, in addition to food intake, have been identified and include indoor environments and electronics recycling and dumpsites in developing countries. Children can have higher exposures due to their hand-to-mouth activities. For some EDCs, all of the routes of exposure are not understood.
7. **All sources of EDCs not known** – All sources of exposure to EDCs are not understood because of the lack of chemical constituent declarations for materials and goods.
8. **Importance of biotic and abiotic environmental monitoring** – Spatial and temporal monitoring is critical for understanding trends and levels of exposure. This monitoring should include both tissues from humans and wildlife (representing a range of species), as well as water or other environmental compartments to capture the less persistent EDCs.
9. **Changes in use lead to changes in levels** – Levels in humans and wildlife are related to how much a chemical is used. Bans on several POPs have led to declines in environmental levels and human body burdens. In contrast, there are increasing levels of some newer EDCs such as perfluorinated alkyl compounds and replacements for banned brominated flame retardants.
10. **Global movement of EDCs** - There is global transport of EDCs through natural processes (ocean and air currents) as well as through commerce, leading to worldwide exposure of humans and wildlife to EDCs**.**

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Appendix I. Common names and Latin names of species mentioned in the present document.

African clawed frog	*Xenopus laevis*
African darter	*Anhinga rufa*
American alligator	*Alligator mississippiensis*
American kestrel	*Falco sparverius*
American robin	*Turdus migratorius*
American toad	*Bufo americanus*
American white ibis	*Eudocimus albus*
Antartic fur seal	*Arctocephalus gazella*
Arctic char	*Salvelinus alpines*
Arctic fox	*Vulpes lagopus*
Arctic glaucous gull	*Larus hyperboreus*
Atlantic croaker	*Micropogonias undulates*
Atlantic white–sided dolphin	*Lagenorhynchus acutus*
Atlantic white–sided dolphin	*Lagenorhynchus acutus*
Atlantic(bottlenose) dolphin	*Tursiops truncatus*
Bacteria	*Aeromonas hydrophila*
Bacteria	*Aeromonas salmonicida*
Baikal seal	*Pusa sibirica*
Bald Eagle	*Haliaeetus leucocephalus*
Bearded seal	*Erignathus barbatus*
Beluga or White whale	*Delphinapterus leucas*
Beluga whales	*Delphinapterus leucas*
Black deer (Sitka black tail deer)	*Odocoileus hemionus sitkensis*
Black-crowned night heron	*Nycticorax nycticorax*
Blackfooted albatross	*Phoebastria nigripes*
Blue mussel	*Mytilus edulis*
Bluegill	*Lepomis macrochirus*
Brown bullhead	*Ameiurus nebulosus*
Brown shrimp	*Crangon crangon*
Bull frog	*Rana catesbeiana*
Burmeister's porpoise	*Phocoena spinipinnis*
Buzzard	*Buteo buteo*
Californian sea lion	*Zalophus californianus*
Caribou	*Rangifer tarandus*
Carp (common)	*Cyprinus carpio*
Cat	*Felis catus*
Cattle	*Bos primigenius*
Chicken	*Gallus domesticus*
Chinook salmon	*Oncorhynchus tshawytcha*
Clam (soft-shelled)	*Mya arenaria*
Clapper rail	*Rallus longirostris*
Colonial ascidian	*Botryllus schlosseri*
Common bottlenose dolphin	*Tursiops truncatus*
Crow	*Corvus brachyrhynchos*
Deer mice	*Peromyscus maniculatus*
Dog	*Canis lupus*
Double crested cormorant	*Phalacrocorax auritis*
Dusky dolphin	*Lagenorhynchus obscurus*
Earthworm	*Lumbricus terrestris*
Eastern bluebird	*Sialia sialis*
Eastern oyster	*Crassostrea virginica*
Echinoid	*Paracentrotus lividus*
Eland	*Tragelaphus oryx*
Eland(common)	*Taurotragus oryx*
Elephant	*Loxodonta africana/ Elephas maximus*
Estuarine bivalve	*Scrobicularia plana*
European harbor porpoise	*Phocoena phocoena*
European shag	*Phalacrocorax aristotelis*
False killer whale	*Pseudorca crassidens*
Fathead minnow	*Pimephales promelas*
Finless porpoise	*Neophocaena phocaenoides*
Flounder	*Paralichthys olivaceus*
Franciscana dolphin	*Pontoporia blainvillei*
Fraser's dolphin	*Lagenodelphis hosei*
Freshwater amphipod	*Gammarus pulex*
Freshwater hydroid	*Hydra vulgaris*
Freshwater water flea	*Daphnia magna*
Fulmar	*Fulmarus glacialis*
Galapagos sea lion	*Zalophus wollebaeki*
Ganges river dolphin	*Platanista gangetica gangetica*
Gizzard shad(American)	*Dorosoma cepedianum*
Goat (domestic)	*Capra aegagrus hircus*
Goldfish	*Carassius auratus auratus*
Great blue heron	*Ardea herodias*
Great cormorant	*Phalacrocorax carbo*
Great egret	*Ardea alba*
Grey seal	*Halichoerus grypus*
Guillemot	*Uria aalge*
Guinea pig	*Cavia porcellus*
Harbour seal	*Phoca vitulina*
Herring	*Clupea harengus*
Herring Gull (American)	*Larus smithsonianus*
Herring gull(European)	*Larus argentatus*
Horse	*Equus caballus*
Housefly	*Musca domestica*
Human	*Homo sapiens*
Humpback dolphin (Chinese)	*Sousa chinensis*
Humpback dolphin (Indian)	*Sousa plumbea*
Humpback dolphin(Atlantic)	*Sousa teuszi*
Indo-pacific dolphin	*Tursiops aduncus*
Indo-pacific humpback dolphin	*Sousa chinensis*
Isopod	*Porcellio scaber*

Ivory gull	*Pagophila eburnea*
Jaguar	*Panthera onca*
Japanese medaka	*Oryzias latipes*
Killer whale	*Orcinus orca*
Lesser black-backed gull	*Larus fuscus fuscus*
Lion	*Panthera leo*
Lobster	*Homarus americanus*
Long beaked common dolphin	*Delphinus capensis*
Loon (common)	Gavia immer
Mallard	*Anas platyrhynchos*
Mediterranean mussel	*Mytilus galloprovincialis*
Melon headed whale	*Peponocephala electra*
Mink	*Mustela vison*
Minke whale	*Balaenoptera acutorostrata*
Mosquitofish	*Gambusia affinis holbrooki*
Mouse	*Mus musculus*
Mudpuppy (common)	*Necturus maculosus*
Mummichog	*Fundulus heteroclitus*
Mussel	*Elliptio complanata*
Neogastropod	*Thais clavigera*
Northern fur seal	*Callorhinus ursinus*
Northern pike	*Esox lucius*
Osprey	*Pandion haliaetus*
Otter	*Lutra lutra*
Owl limpet (sea snail)	*Lottia gigantean*
Pacific white-sided dolphin	*Lagenorhynchus obliquider*
Perch	*Perca fluviatilis*
Peregrine falcon	*Falco peregrinus peregrinus*
Pinnipeds	Seals, sea lions and walrus
Polar bear	*Ursus maritimus*
Polar seastar	*Leptasterias polaris*
Pumpkinseed sunfish	*Lepomis gibbosus*
Rabbit	*Oryctolagus cuniculus*
Rainbow trout	*Oncorhynchus mykiss*
Ramshorm snails	*Marisa cornuarietis*
Rat	*Rattus norvegicus*
Ringed seal	*Pusa hispida*
Risso's dolphin	*Grampus griseus*
River otter	*Lutra canadensis*
Roach	*Rutilus rutilus*
Rock shell	*Thais clavigera*
Rough woodlouse	*Porcellio scaber*
Sand dollar	*Echinodermata: Echinoidea*
Sand goby	*Pomatoschistus minutus*
Sea otter	*Enhydra lutris*
Sea slug	*Aplysia*
Sea star	*Asterias rubens*
Sea turtle (green)	*Chelonia mydas*
Sea urchin	*Phylum echinodermata*
Sharptooth catfish	*Clarias gariepinus*
Sheep	*Ovis aries*
Short-beaked common dolphin	*Delphinus delphi*
Skipjack tuna	*Katsuwonus pelamis*
Southern catfish	*Silurus meridionalis*
Spotted seal	*Phoca largha*
Star ascidian	*Botryllus schlosseri*
Steller sea lion	*Eumetopias jubatus*
Striped dolphin	*Stenella coeruleoalba*
Subantartic fur seal	*Arctocephalus tropicalis*
Tasmanian devil	*Sarcophilus harrisii*
Thickbilled murre (Brünnich's Guillemot)	*Uria lomvia*
Tiger	*Panthera tigris*
Tree sparrow	*Passer montanus*
Tree swallow	*Tachycineta bicolour*
Tucuxi dolphin	*Sotalia fluviatilis*
Tunicate	*Styela plicata*
Vase tunicate	*Ciona intestinalis*
Viviparous blenny	*Zoarces viviparus*
Walrus	*Odobenus rosmarus*
Water flea	*Daphnia magna* or *Daphnia pulex (another species)*
White stork	*Ciconia ciconia*
White sucker	*Catostomus commersoni*
White tailed deer	*Odocoileus virginianus*
White-beaked dolphin	*Lagenorhynchus albirostris*
Yellow perch	*Perca flavescens*
Zebra mussel	*Dreissena polymorpha*
Zebrafish	*Danio rerio*

Appendix II. The table includes common names, abbreviations, when applicable, chemical names of those chemicals that are mentioned in the text of the Chapters 1-3. Chemical Abstract System numbers (CAS #) are given for further information on each of the chemicals as well as class of compound and/or major use.

Common names	Chemical name; or other common name	CAS #	Class or use	Abbreviation
Acetochlor	2-Chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide	34256-82-1	Herbicide	
Alachlor	2-Chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide	15972-60-8	Herbicide	
Amitrole	1,2,4-Triazol-3-amine aminotriazole	61-82-5	Herbicide	
Anthracene	Paranaphthalene	120-12-7	PAH	
Aroclor 1254	Polychlorinated biphenyls	11097-69-1	PCB mixture	
Arsenic (As)	-	7440-38-2	Heavy metal	
Atrazine	1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine	1912-24-9	Herbicide	ATR
Benzo(a)anthracene	Benzanthracene; Benzanthrene; 1,2-Benzanthracene; Benzo[b] phenanthrene; Tetraphene	56-55-3	PAH	BaA
Benzo(a)pyrene	3,4-Benz[a]pyrene	50-32-8	PAH	BaP
BB-153	2,2′,4,4′,5,5′-Hexabromobiphenyl	59080-40-9	PBB	
Benzyl butyl phthalate	n-Benzyl butyl phthalate	85-68-7	Phthalate	BBP
BDE-209	Decabromodiphenyl ether	1163-19-5	PBDE	decaBDE
BDE-47	2,2′,4,4′-Tetrabromodiphenyl ether	5436-43-1	PBDE	
BDE-99	2,2′,4,4′,5-Pentabromodiphenyl ether	60348-60-9	PBDE	
Benzene	1,3,5-Cyclohexatriene	71-43-2	Aromatic solvent	
Benzylidene camphor	(3*E*)-1,7,7-Trimethyl-3-[(4-methylphenyl methylene]-2-norbornanone	36275-29-3	UV filter	
Bisphenol A	2,2-Bis(4-hydroxyphenyl)propane	80-05-7	Plastics monomer	BPA
Bisphenol A diglycid ether	2-[[4-[2-[4-(Oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy] methyl]oxirane	1675-54-3	Plastics monomer	
Bisphenol F	Bis(4-hydroxydiphenyl)methane	87139-40-0	Plastics monomer	BPF
Bisphenol S	4,4′-Sulfonylbisphenol	80-09-1	Plastics monomer	BPS
Bromacil	5-Bromo-3-(butan-2-yl)-6-methylpyrimidine-2,4(1H,3H)-dione	314-40-9	Herbicide	
Butylate	S-Ethyl diisobutyl(thiocarbamate)	2008-41-5	Herbicide	
Butylated hydroxyanisole	2(3)-tert-Butyl-4-hydroxyanisole	25013-16-5	Antioxidant	BHA
Cadmium (Cd)	Cadmium chloride	10108-64-2	Heavy metal	
Carbamazepine	5H-Dibenzo[b,f]azepine-5-carboxamide	298-46-4	Pharmaceutical	
Carbaryl	1-Naphthyl methylcarbamate	63-25-2	Insecticide	
CB-15	4,4′-Dichlorobiphenyl	2050-68-2		
CB-77	3,3′,4,4′-Tetrachlorobiphenyl	32598-13-3	Planar PCB	
CB-118	2,3′,4,4′,5-Pentachlorobiphenyl	31508-00-6		
CB-126	3,3′,4,4′,5-Pentachlorobiphenyl	57465-28-8	Planar PCB	
CB-132	2,2′,3,3′,4,6′-Hexachlorobiphenyl	38380-05-1		
CB-138	2,2′,3,4,4′,5′-Hexachlorobiphenyl	35065-28-2		
CB-153	2,2′,4,4′,5,5′-Hexachlorobiphenyl	35065-27-1		
CB-169	3,3′,4,4′,5,5′-Hexachlorobiphenyl	32774-16-6	Planar PCB	
CB-180	2,2′,3,4,4′,5,5′-Heptachlorobiphenyl	35065-29-3		
Chlordane	cis/trans-Chlordane	5103-71-9, 5103-74-2	Organochlorine Insecticide	
Chlordibromomethane	Chlordibromomethane	124-48-1	Trihalomethane	
Chlorinated Paraffins	Polychlorinated alkanes		Flame retardants, Lubricants, Plasticizers	CPs or PCAs
Chlorpyrifos	O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate	2921-88-2	Insecticide	
Citalopram	(RS)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile	59729-33-8	Pharmaceutical	
Clofentezine	3,6-Bis(2-chlorophenyl)-1,2,4,5-tetrazine	74115-24-5	Pesticide/Acaracide	
Coumaphos	O,O-Diethyl O-3-chloro-4-methyl-2-oxo-2H-chromen-7-yl phosphorothioate	56-72-4	Pharmaceutical	

Coumestrol	3,9-Dihydroxy-6-benzofurano[3,2-c]chromenone	479-13-0	Coumestans/ Phytestrogen	
D4	Octamethylcyclotetrasiloxane	556-67-2	Cyclic siloxane	
D5	Decamethylcyclopentasiloxane	541-02-6	Cyclic siloxane	
D6	Dodecamethylcyclohexasiloxane	540-97-6	Cyclic siloxane	
Daidzein	7-Hydroxy-3-(4-hydroxyphenyl) chromen-4-one	486-66-8	Isoflavones/ phytestrogen	
Dibromochloropropane	1,2-Dibromo-3-chloropropane	96-12-8	Pesticide/Soil Fumigant	DBCP
Desethylatrazine	4-Amino-2-chloro-6-isopropylamino-s-triazine	6190-65-4	Herbicide Metabolite	DEA
2,4-D	2,4-Dichlorophenoxy)acetic acid	94-75-7	Herbicide	2,4-D
2,4-Dichlorophenol	1,3-Dichloro-4-hydroxybenzene	120-83-2	Chlorophenol	2,4-DCP
3-Diltiazem	cis-(+)-[2-(2-Dimethylaminoethyl)-5-(4-methoxyphenyl)-3-oxo-6-thia-2-azabicyclo[5.4.0]undeca-7,9,11-trien-4-yl] ethanoate	42399-41-7	Pharmaceutical	
2,4′-DDD (o,p′-DDD)	2-(2-Chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane	53-19-0	Organochlorine Insecticide	o,p′-DDD 2,4′-DDD
2,4′-DDT (o,p′-DDT)	2-(2-Chlorophenyl)-2-(4-chlorophenyl-1,1,1-dichloroethane	789-02-6	Organochlorine Insecticide	o,p′-DDT 2,4′-DDT
4,4′-DDD (p,p′-DDD)	2,2-Bis-(4-chlorophenyl)--1,1-dichloroethane	72-54-8	Organochlorine Insecticide	p,p′-DDD 4,4′-DDD
4,4′-DDE (p,p′-DDE)	2,2-Bis-(4-chlorophenyl)-1,1-dichloroethene	72-55-9	Organochlorine Insecticide	p,p′-DDE 4,4′-DDE
4,4′-DDT (p,p′-DDT)	2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethane	50-29-3	Organochlorine Insecticide	p,p′-DDT 4,4′-DDT
Di-(2-ethylhexyl)adipate	Bis(2-ethylhexyl)adipate	103-23-1	Plasticizer	DEHA
Dehydroepiandrosterone	(3S,8R,9S,10R,13S,14S)-3-Hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a] phenanthren-17-one	53-43-0	Natural hormone	DHEA
Dexamethasone	(8S,9R,10S,11S,13S,14S,16R,17R)-9- Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16- trimeth-yl-6,7,8,9,10,11,12,13,14,15,16,17- dodecahydro-3H-cyclopenta[a]phenanthren-3-one	50-02-2	Synthetic steroid	
Dibutyl phthalate	Di-n-butyl phthalate	84-74-2	Phthalate	DBP
Dibutyltin	Di-n-butyltin dichloride	683-18-1	Plastics stabilizer	DBT
Dicofol	2,2-Bis(4-chlorophenyl)-1,1,1-trichloroethanol	115-32-2	Organochlorine Insecticide	
Dieldrin	(1aR,2R,2aS,3S,6R,6aR,7S,7aS)-3,4,5,6,9,9-Hexa-chloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphtho[2,3-b]oxirene	60-57-1	Organochlorine Insecticide	
Diethyl hexyl phthalate	Bis(2-ethylhexyl)phthalate	117-81-7	Phthalate	DEHP
Mono-2-ethylhexyl phthalate	Phthalic acid mono-2-ethylhexyl ester	4376-20-9	DEHP Hydrolysis product	MEHP
Mono-n-butyl phthalate	Phthalic acid mono-2-n-butyl ester	131-70-4	DBP Hydrolysis product	MnBP
Diethylstilbestrol	4,4′-(3E)-Hex-3-ene-3,4-diyldiphenol	56-53-1	Synthetic estrogen	DES
Diisononyl phthalate	Bis(7-methyloctyl) phthalate	28553-12-0	Phthalate	DiNP
Diphenhydramine	2-(Diphenylmethoxy)-N,N-dimethylethanamine	58-73-1	Antihistamine	DPH
Dimethylbenz(a)anthra-cene	7,12-Dimethylbenz(a)anthracene	57-97-6	PAH	DMBA
Endosulfan (alpha/beta)	6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro- 6,9-meth-ano-2,4,3-benzodioxathiepine-3-oxide	115-29-7	Organochlorine Insecticide	
Endrin	(1aR,2S,2aS,3S,6R,6aR,7R,7aS)-3,4,5,6,9,9-Hexa-chloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphtho[2,3-b]oxirene	72-20-8	Organochlorine Insecticide	
Estradiol	17B-Estradiol, (17β)-estra-1,3,5(10)-triene-3,17-diol	50-28-2	Natural hormone	
Estrone	3-Hydroxy-13-methyl- 6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren- 17- one	53-16-7	Natural hormone	E1
Ethinylestradiol	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol	57-63-6	Synthetic hormone	EE2
Ethylene thiourea	Imidazolidine-2-thione	96-45-7	Herbicide	

Ethylparaben	Ethyl 4-hydroxybenzoate	120-47-8	Antifungal Preservative	
Fadrozole	4-(5,6,7,8-Tetrahydroimidazo[1,5-a]pyridin-5-yl)benzonitrile	102676-31-3	Pharmaceutical	
Fenbuconazole	(RS)-4-(4-Chlorophenyl)-2-phenyl-2-(1H-1,2,4-triazol-1-ylmethyl)butyronitrile	114369-43-6	Fungicide	
Fenitrothion	O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate	122-14-5	Organophosphate Insecticide	
Fenoxycarb	Ethyl N-[2-(4-phenoxyphenoxy)ethyl]carbamate	72490-01-8	Insecticide	
Finasteride	N-(1,1-Dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-ene-17-carboxamide	98319-26-7	Pharmaceutical	
Fipronil	(RS)-5-Amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile	120068-37-3	Insecticide	
Fluoxetine	(RS)-N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine	54910-89-3	Pharmaceutical	
Flutamide	2-Methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]-propanamide	13311-84-7	Pharmaceutical	
Fonofos	(RS)-(O-ethyl S-phenyl ethylphosphonodithioate)	944-22-9	Organophosphate Insecticide	
Formaldehyde	Methana, Formol, Methyl aldehyde, Methylene glycol	50-00-0	Solvent	
Furan	Oxole, Furfuran, 1,4-Epoxy-1,3-butadiene	110-00-9	Solvent	
Galaxolide	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-Hexamethylcyclopenta[g]-2-benzopyran	1222-05-5	Synthetic musk	HHCB
Genistein	5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	446-72-0	Isoflavones, Phytestrogen	
Hexabromocyclododecane	1,2,5,6,9,10-Hexabromocyclododecane	25637-99-4	BFR	HBCDD
Hexachlorobenzene		118-74-1	Chlorinated Aromatic	HCB
Heptachlor	1,4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indene	76-44-8	Organochlorine Insecticide	
Heptachlor epoxide	1,4,5,6,7,8,8a-Heptachloro-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindene	1024-57-3	Organochlorine Insecticide Metabolite	
Hexachlorobutadiene	Hexachloro-1,3-butadiene	87-68-3	Solvent	
Heptachlorodibenzo-dioxin	eg 1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	35822-46-9	Dioxin	HpCDD
HPTE	2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane	2971-36-0	Methoxychlor Metabolite	
Iodine (I)	-	7553-56-2	Halogen; Essential element	
Kepone	Chlordecone; 1,1a,3,3a,4,5,5,5a,5b,6-Decachlorooctahydro-2H-1,3,4-(methanetriyl)cyclobuta[cd]pentalen-2-one	143-50-0	Organochlorine Insecticide	
Lead (Pb)	-	7439-92-1	Heavy metal	
Levonorgestrel	13-Ethyl-17-ethynyl-17-hydroxy- 1,2,6,7,8,9,10,11,12,13,14,15,16, 17- tetradecahydrocyclopenta[a] phenanthren-3-one	797-63-7	Synthetic Estrogen	
Lindane	gamma-Hexachlorocyclohexane	58-89-9	Organochlorine Insecticide	γ-HCH
Linuron	3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea	330-55-2	Herbicide	
Malathion	Diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate	121-75-5	Organophosphate Insecticide	
Mancozeb	Manganese ethylenebis(dithiocarbamate)	8018-01-7	herbicide	
Manganese		7439-96-5	Heavy metal	
Methylsulfonyl-DDE	3-Methylsulfonyl-2,2′-bis(4-chlorophenyl)-1,1′-dichloroethene	62938-14-1	DDE Metabolite	$MeSO_2$-DDE
Methoxychlor	2,2-Bis(4-methoxyphenyl)-1,1,1-trichloroethane	72-43-5	Organochlorine Insecticide	
Methyl bromide	Monobromomethane, 1-bromomethane	74-83-9	Fumigant, Pesticide	
Methyl farnesoate	Methyl (2E,6E)-3,7,11-trimethyl-2,6,10-dodecatrienoate	10485-70-8	Juvenile Hormone	
Methyl triclosan	2,4-Dichloro-1-(4-chloro-2-methoxyphenoxy)benzene	4640-01-1	Triclosan Transformation Product	
Methylbenzylidene camphor	(3E)-1,7,7-Trimethyl-3-[(4-methylphenyl)methylene]-2-norbornanone	36861-47-9	UV filter	

Methylcholanthrene	20–Methylcholanthrene	56-49-5	PAH	
Mirex	1,1a,2,2,3,3a,4,5,5,5a,5b,6-dodecachlorooctahydro-1H-1,3,4-(methanetriyl)cyclobuta[cd]pentalene	2385-85-5	Organochlorine Insecticide	
Monosodium glutamate	Sodium 2-Aminopentanedioate	142-47-2	Food Additive	
n-Butylbenzene	n-Butylbenzene;Butylbenzene;1-Phenylbutane	104-51-8	Chemical Synthesis Intermediate	
Nicotine	3-[(2S)-1-Methylpyrrolidin-2-yl]pyridine	54-11-5	Alkaloid	
Nonachlor	trans-Nonachlor; (1S,7R)-1α,2β,3β,4,5,6,7,8,8-Nonachloro-2,3,3aα,4,7,7aα-hexahydro-4,7-methano-1H-indene	39765-80-5	Organochlorine Insecticide	
Nonylphenol	p-Nonylphenol; 4-Nonylphenol	104-40-5	Surfactant	NP
Norfluoxetine	Seproxetine; S)-3-phenyl-3-[4-(trifluoromethyl)phenoxy] propan-1-amine	126924-38-7	Pharmaceutical	
Octachlorodibenzo-p-dioxin	1,2,3,4,6,7,8,9-Octachlorodibenzo-p-dioxin	3268-87-9	Dioxin	OCDD
Octachlorostyrene	1,2,3,4,5-Pentachloro-6-(trichlorovinyl)benzene	29082-74-4	Chlorinated Aromatic	OCS
Octyl-methoxycinnamate	2-Ethylhexyl (2E)-3-(4-methoxyphenyl)prop-2-enoate	5466-77-3	UV filter	
Octylphenol	p-Octylphenol; 4-n-Octylphenol	1806-26-4	Surfactant	OP
Oxychlordane	2-Endo,4,5,6,7,8,8-octachloro-2,3-exo-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-1-ex 3a,4,7,7a-tetrahydro-1,2-epoxy-4,5,6,7,8,8-hexachloro-4,7-methanoindan	27304-13-8	Chloridane Metabolite	
Parathion	O,O-Diethyl O-(4-nitrophenyl) phosphorothioate	56-38-2	Organophosphate Insecticide	
Pendimethalin	3,4-Dimethyl-2,6-dinitro-N-(1-ethylpropyl)aniline	40487-42-1	Herbicide	
Pentachlorobenzene	1,2,3,4,5-Pentachlorobenzene	608-93-5	Chlorinated Aromatic	
Pentachloronitrobenzene	2,3,4,5,6-Pentachloronitrobenzene; Quintozene	82-68-8	Herbicide	PCNB
Pentachlorophenol	2,3,4,5,6-Pentachlorophenol	87-86-5	Herbicide, fungicide	PCP
Perchlorate	Perchloric acid, ion(1-)	14797-73-0	Oxidizer	
Permethrin	3-Phenoxybenzyl (1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	52645-53-1	Insecticide	
PFDS	Perfluorodecanesulfonate/-sulfonic acid	67906-42-7, 335-77-3	PFAS	
PFHxS	Perfluorohexanesulfonate	108427-53-8	PFAS	
PFNA	Perfluorononanoate/-nonanoic acid	375-95-1	PFAS	
PFOA	Perfluorooctanoate/-octanoic acid	335-67-1	PFAS	
PFOS	Perfluorooctane sulfonate/-sulfonic acid	2795-39-3, 1763-23-1	PFAS	
PFOSF	Perfluorooctane sulfonyl fluoride	307-35-7	PFAS	
Phorate	O,O-Diethyl S-[(ethylsulfanyl)methyl] phosphorodithioate	298-02-2	Organophosphate Insecticide	
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid; Tordon 101	1918-02-1	Herbicide	
Polyvinylchloride	-	9002-86-2	Polymer; PVC	
8-Prenylnaringenin	(2S)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-en-1-yl)-3,4-dihydro-2H-1-benzopyran-4-one; 8-Isopenenyl-naringenin	53846-50-7	Prenylflavonoid	
Procloraz	N-Propyl-N-[2-(2,4,6-trichlorophenoxy)ethyl]-1H-imidazole-1-carboxamide	67747-09-5	Fungicide	
Procymidone	3-(3,5-Dichlorophenyl)-1,5-dimethyl-3-azabicyclo[3.1.0] hexane-2,4-dione	32809-16-8	Fungicide	
Prodiamine	5-Dipropylamino-α,α,α-trifluoro-4,6-dinitro-o-toluidine	29091-21-2	Herbicide	
Propylthiouracil	6-Propyl-2-sulfanylpyrimidin-4-one	51-52-5	Pharmaceutical	
Pyrene	Benzo[def]phenanthrene	129-00-0	PAH	
Pyrimethanil	4,6-Dimethyl-N-phenylpyrimidin-2-amine	53112-28-0	Fungicide	
Pyriproxyfen	4-Phenoxyphenyl (RS)-2-(2-pyridyloxy)propyl ether	95737-68-1	Juvenile hormone	
Resorcinol	1,3-Dihydroxybenzene	108-46-3	Disinfectant, Chemical intermediate	
Sertraline	(1S,4S)-4-(3,4-Dichlorophenyl)-N-methyl-1,2,3,4-tetrahydro-naphthalen-1-amine	79617-96-2	Pharmaceutical	

Short chain chlorinated paraffins	Polychlorinated n-alkanes, C10 to C13, 50-60% chlorine	63449-39-8, 85535-84-8	Flame Retardant; plasticizer	SCCP
2,4,5-T	2,4,5 Trichlorophenoxyacetic acid	93-76-5	Herbicide	2,4,5,-T
Tamoxifen	(Z)-2-[4-(1,2-Diphenylbut-1-enyl)phenoxy]-N,N-dimethyle-thanamine	10540-29-1	Pharmaceutical	
Tetrabromobisphenol A	2,2′,6,6′-Tetrabromo-4,4′-isopropylidenediphenol	79-94-7	BFR	TBBPA
Testosterone	(8R,9S,10R,13S,14S,17S)- 17-Hydroxy-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15,16,17- dodecahydrocyclopenta[a] phenanthren-3-one	58-22-0	Natural hormone	
Tetrachlorodibenzofuran	eg 2,3,7,8-Tetrachlorodibenzofuran	51207-31-9	Chlorinated dioxin	TCDF
Tetrachlorodibenzo-*p*-dioxin	eg 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	1746-01-6	Chlorinated dioxin	TCDD
PCB methyl sulfones	4-Methylsulfonyl-2,2′,3,4′,5′,6-hexachlorobiphenyl	116806-76-9	PCB metabolite	$MeSO_2$-PCB
Tetraiodothyronine	(2S)-2-Amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodo-phenyl]propanoic acid; Levothyroxine	51-48-9	Natural hormone	T4
Thiazopyr	Methyl 2-difluoromethyl-5-(4,5-dihydro-1,3-thiazol-2-yl)-4-isobutyl-6-trifluoromethylnicotinate	117718-60-2	Herbicide	
Toxaphene	Polychlorinated bornanes	8001-35-2	Organochlorine Insecticide	
2,4,6-Tribromophenol	1,3,5-Tribromo-2-hydroxybenzene	118-79-6	BFR, Natural product	2,4,6-TBP
Trenbolone	17β-Hydroxyestra-4,9,11-trien-3-one	10161-33-8	Anabolic Steroid	
Tributyltin	Bis(tri-n-butyltin)oxide	56-35-9	Fungicide	TBT
Trichloroethylene	1,1,2-Trichloroethene	79-01-6	Chlorinated Solvent	TCE
Trichlorophenate	2,4,5-Trichlorophenol; 2,4,6-Trichlorophenol; Trichlorophenol	95-95-4, 88-06-2	Fungicide	
Triclocarban	3-(4-Chlorophenyl)-1-(3,4-dichlorophenyl)urea	101-20-2	Microbicide	
Triclosan	5-Chloro-2-(2,4-dichlorophenoxy)phenol	3380-34-5	Microbicide	
Tri-iodothyronine	(2S)-2-Amino-3- [4-(4-hydroxy-3-iodo-phenoxy)- 3,5-diiodo-phenyl]propanoic acid	6893-02-3	Natural hormone	T3
Triphenyl phosphate	Phosphoric acid, triphenyl ester	115-86-6	Flame retardant	TPP
Triphenyltin	Fentin; (acetoxy)(triphenyl)stannane; hydroxytriphenylstan-nane	900-95-8, 668-34-8	Fungicide	
Venlafaxine	1-[2-Dimethylamino-1-(4-methoxyphenyl)-ethyl]cyclohexanol	93413-69-5	Pharmaceutical	
Vinclozolin	RS)-3-(3,5-Dichlorophenyl)-5-methyl- 5-vinyloxazolidine-2,4-dione	50471-44-8	Fungicide	
Zearalenone	(3S,11E)-14,16-Dihydroxy-3-methyl-3,4,5,6,9,10-hexa-hydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione	17924-92-4	Mycotoxins	

Abbreviations and Acronyms

AAARD	American Autoimmune Related Disease Association
ACC	Adrenocortical carcinoma
ACTH	Adrenocorticotropic hormone
AD	Autistic disorder
A.D.	Anno Domini
ADD	Attention deficit disorders
ADH	Antidiuretic hormone
ADHD	Attention deficit hyperactivity disorder
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
AHS	Agricultural Health Study
AIDS	Acquired immunodeficiency syndrome
AITD	Autoimmune thyroid diseases
ALSPAC	Avon longitudinal study of parents and children
AMAP	Arctic Monitoring and Assessment Programme
AMH	Anti-Mullerian hormone
ANP	Atrial natriuetic peptide
ANSES	Agence national de sécurité sanitaire de l'alimentation, de l'environnement et du travail (French Agency for Food, Environmental and Occupational Health and Safety)
AR	Androgen receptor
ART	Assisted reproduction techniques
ASRM	American Society for Reproductive Medicine
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCERC	Breast Cancer and the Environment Research Centers
BFRs	Brominated flame retardants
BMI	Body mass index
BMPs	Bone morphogenetic proteins
BPH	Benign prostatic hyperplasia
BSEF	Bromine Science and Environmental Forum
BW	Body weight
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CEBPs	CCAAT enhancer-binding proteins
CH	Congenital hypothyroidism
CHD	Coronary heart disease
CI	Confidence interval
CIS	Carcinoma *in situ*
COC	Combined oral contraceptives
COUP-TFII	Chicken ovalbumin upstream promoter-transcription factor II
COX-2	Cyclooxygenase-2
CPP	Central precocious puberty
CPs	Chlorinated paraffins
CRH	Corticotropin-releasing hormone
CRH-BP	Corticotropin-releasing hormone-binding protein
CT	Calcitonin
CVDs	Cardiovascular diseases
CYP	Cytochrome P
CXorf6	Chromosome X open reading frame
D1	Iodothyronine deiodinase type 1
D2	Iodothyronine deiodinase type 2
D3	Iodothyronine deiodinase type 3
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X-chromosome, gene-1
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DIT	Developmental immunotoxicity
DMRT-1	Doublesex and mab-3 related transcription factor 1
DN	Disseminated neoplasia
DNA	Deoxyribonucleic acid
DOC	Deoxycorticosterone
E	Epinephrine
E1	Estrone
E2	Estradiol
EBAP	Environmental bioaccumulation potential
EC50	Half maximal effective concentration
EC90	90% maximal effective concentration
ECHA	European Chemicals Agency
ED	Endocrine disrupting
EDA	Effects directed analysis
EDCs	Endocrine disrupting chemicals
EDSP	Endocrine Disrupting Chemical Screening Program
EE2	Ethinylestradiol
EFSA	European Food Safety Authority
EGF	Epidermal growth factor
EPA	Environmental Protection Agency
EPO	Erythropoietin
ER	Estrogen receptors
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EROD	Ethoxyresorufin o-deethylase
ERRγ	Estrogen related receptor gamma
ERT	Estrogen therapy
ESHRE	European Society of Human Reproduction and Embryology
esr-1 (ER alpha)	Estrogen receptor alpha
esr-2 (ER beta)	Estrogen receptor beta
ESPE	European Society for Pediatric Endocrinology
EU	European Union
FGF	Fibroblast growth factor
FGF 10	Fibroblast growth factor 10

FGF receptor 2	Fibroblast growth factor receptor 2
FSH	Follicle-stimulating hormone
FW	Fresh weight
GA	Gestational age
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GH	Growth hormone
GI	Gastrointestinal
GLOBOCAN	Cancer incidence and mortality worldwide
GLP	Good laboratory practice
GLP-1	Glucagon-like peptide-1
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRADE	Grading of recommendations assessment, development and evaluation
H295R	Human adrenocortical H295R cell line
HDL	High density lipoproteins
HELCOM	Helsinki Commission
HFA-DB	Health For All Database
HIV	Human immunodeficiency virus
HNF4A	Hepatocyte nuclear factor 4 alpha
Hox A10	Homeobox A10 gene
HOXA13	Homeobox A13 gene
HPA	Hypothalamic-pituitary-adrenal
HPCs	Halogenated phenolic chemicals
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
HRT	Hormone replacement therapeutics
HSD	Hydroxysteroid dehydrogenase
IARC	International Agency for Research on Cancer
IDDM	Insulin dependent diabetes mellitus
ICCM	International Conference on Chemicals Management
ICo	Intercollicular complex
IFCS	Intergovernmental Forum on Chemical Safety
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IGF	Insulin-like growth factor (IGF-1, IGF-II)
IgG	Immunoglobulin G
IGL	Internal granule layer
IgM	Immunoglobulin M
IKK	IκB kinase
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-6	Interleukin-6
ILO	International Labour Organisation
IMT	Intimal medial thickness
INSL3	Insulin-like peptide 3
IPCC	Intergovernmental Panel on Climate Change
IPCS	International Programme on Chemical Safety
IQ	Intelligence quotient
IUCN	International Union for Conservation of Nature
IUGR	Intrauterine growth restriction
K_{OA}	Octanol-air partition coefficient
KOWIN	Model for prediction of octanol-water partition coefficients
K_{ow}	Octanol-water partition coefficient
KOAWIN	Model for prediction of octanol-air partition coefficients
LC	Leydig cell
LC	Liquid chromatography
LDL	Low density lipoproteins
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LPI	Living planet index
LPD	Living planet database
LPUEs	Landings per unit effort
LXRs	Liver X receptors
MAMLD1	Mastermind-like domain containing 1
MAPK	Map kinase
MCF7	Breast cancer cell line Michigan cancer foundation
MCTB	Myobacterial copper transport protein B
mDCs	Myeloid dendritic cells
MHC-I	Major histocompatibility complex-class I
MIPS	Morphologically intermediate papilla syndrome
MOFs	Multi-oocyte follicles
MBP	Myeline basic protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSH	Melanocyte-stimulating hormone
NAS	National Academy of Science
NCOA3	Nuclear receptor coactivator 3
NCHS	National Center for Health Statistics
NE	Norepinephrine
NHIS	National Health Interview Survey
NF-κB	Nuclear factor kappaB
NGO	Non-Governmental Organization
NHANES	National Health and Nutrition Examination Survey
NIEHS	National Institute of Environmental Health Sciences
NIH	National Institutes of Health
NIS	Sodium/iodide symporter
NK	Natural killer
NOAEL	No-observed-adverse-effect level
Nr4a1	Nuclear receptors 4a1
Nr4a3	Nuclear receptors 4a3
NRC	National Research Council
NR1I2	Nuclear receptor subfamily 1, group I, member 2

NSP-A	Neuroendocrine specific protein A
NYS	New York State
OATP	Organic anion-transporting polypeptide
OCs	Organochlorines
OECD	Organization for Economic Cooperation and Development
OVA	Ovalbumin
OSPAR	Oslo/Paris convention
PAC	Polycyclic aromatic compounds
PACE	Partnership for Action on Computing Equipment
PAHs	Polycyclic aromatic hydrocarbons
PCOS	Polycystic ovary syndrome
PES	Pediatric Endocrine Society
PFCs	Perfluorinated compounds
PGCs	Primordial germ cells
PGE2	Prostaglandin E2
PM2.5	Particulate matter 2.5
POF	Premature ovarian failure
POPs	Persistent organic pollutants
PPAR	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor alpha
PPAR(gamma)	Peroxisome proliferator-activated receptor gamma
PPARG	Peroxisome proliferator-activated receptor gamma
PPARγ	Proliferator-activated receptor gamma
PR	Progesterone receptor
PRL	Prolactin
PROD	Penthyloxyresorufin o-depenthylase
PROS	Pediatric Research in Office Settings
PSA	Prostate-specific antigen
PTH	Parathyroid hormone
PXR	Pregnane X receptor
PYY	Pancreatic peptide YY
QSAR	Quantitative structure-activity relationship
RC 3	Rat cortex clone 3
REACH	Registration, evaluation, authorization and restriction of chemicals
RfD	Reference dose
RLI	Red list index
ROPME	Regional Organization for the Protection of the Marine Environment
ROR	Retinoid-related orphan receptor
ROS	Reactive oxygen species
RXFP2	Relaxin/insulin-like family peptide receptor 2
RXR	Retinoid-X receptor
SAICM	Strategic Approach to International Cemicals Management
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SGA	Small for gestational age
Shh	Sonic hedgehog
SOLEC	State of the lakes ecosystem conference
SPE	Solid phase extraction
SRKW	Southern resident killer whales
SSRIs	Selective serotonin reuptake inhibitors
StAR	Steroidogenic acute regulatory
STAT3	Signal transducer and activator of transcription 3
STW	Sewage treatment works
SXR	Steroid and xenobiotic receptor
T	Testosterone
T1DM	Type 1 diabetes mellitus
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroxine binding globulin
TDI	Tolerable daily intake
TDS	Testicular dysgenesis syndrome
TEDX	The endocrine disruption exchange
TEQ	Toxic equivalent
TGC	Testicular germ cell cancers
TH	Thyroid hormone
Th2	T helper cell 2
TNFα	Tumor necrosis factor alpha
TPs	Transformation products
TPO	Thyroperoxidase
TR	Thyroid hormone receptor
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta
TRH	Thyrotropin-releasing hormone
TSCA	Toxic substances control act
TSH	Thyroid-stimulating hormone
TTR	Transthyretin
UDPGT	Uridine diphosphate glucuronyltransferase
UGP	Urogenital papillae
UK	United Kingdom
UN	United Nations
UNEP	United Nations Environment Programme
US	United States
USA	United States of America
US EPA	United States Environmental Protection Agency
US NAS	United States National Academy of Sciences
US NTP	US National Toxicology Program
UV	Ultraviolet
VTG	Vitellogenin
WBI	Wild bird index
WHO	World Health Organization
WOE	Weight of evidence
WPSI	Water bird population status index

Endocrine Disrupting Chemicals have many sources

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:11 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 7 of 9
Attachments: 64 Brausch and Rand Chemosphere 82t.pdf; 65 HEL data on file.docx; 66 Necasova New Probabilistic Risk Assessment.pdf; 67 Rachon Immunopharmacol Immunotoxicol.docx; 68 Duale Octyl Methoxycinnamate modulates gene expr.pdf; 69 Xu Photochem Photobiol.docx; 70 Broniowska The effect of UV-filters.pdf; 71 Jang Sequential assessment in daphna.pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

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Review

A review of personal care products in the aquatic environment: Environmental concentrations and toxicity

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ABSTRACT

Considerable research has been conducted examining occurrence and effects of human use pharmaceuticals in the aquatic environment; however, relatively little research has been conducted examining personal care products although they are found more often and in higher concentrations than pharmaceuticals. Personal care products are continually released into the aquatic environment and are biologically active and persistent. This article examines the acute and chronic toxicity data available for personal care products and highlights areas of concern. Toxicity and environmental data were synergized to develop a preliminary hazard assessment in which only triclosan and triclocarban presented any hazard. However, numerous PCPs including triclosan, paraben preservatives, and UV filters have evidence suggesting endocrine effects in aquatic organisms and thus need to be investigated and incorporated in definitive risk assessments. Additional data pertaining to environmental concentrations of UV filters and parabens, *in vivo* toxicity data for parabens, and potential for bioaccumulation of PCPs needs to obtained to develop definitive aquatic risk assessments.

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1. Introduction

Personal care products (PCPs) are a diverse group of compounds used in soaps, lotions, toothpaste, fragrances, and sunscreens, to name a few. The primary classes of PCPs include disinfectants (e.g. triclosan), fragrances (e.g. musks), insect repellants (e.g. DEET), preservatives (e.g. parabens) and UV filters (e.g. methylbenzylidene camphor). Unlike pharmaceuticals which are intended for internal use, PCPs are products intended for external use on the human body and thus are not subjected to metabolic alterations; therefore, large quantities of PCPs enter the environment unaltered through regular usage (Ternes et al., 2004). Many of these compounds are used in large quantities, and recent studies have indicated many are environmentally persistent, bioactive, and have the potential for bioaccumulation (Peck, 2006; Mackay and Barnthouse, 2010).

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PCPs are among the most commonly detected compounds in surface water throughout the world (Peck, 2006); however, in comparison to pharmaceuticals, relatively little is known about PCP toxicity (Daughton and Ternes, 1999). Numerous reviews have been published examining pharmaceutical toxicity and occurrence (Halling-Sorensen et al., 1998; Crane et al., 2006; Fent et al., 2006; among others), but less attention has been placed on determining potential risk of PCP release into aquatic environments. The objective of this review was to summarize recent publications regarding environmental concentrations (surface water) and aquatic toxicity of PCPs in order to identify research needs and to estimate hazard as a result of PCP release into the aquatic environment.

2. Disinfectants

Triclosan (TCS) and triclocarban (TCC) are biphenyl ethers used as antimicrobials in soaps, deodorants, skin creams, toothpaste and plastics (McAvoy et al., 2002). TCS and TCC are among top 10 most commonly detected organic wastewater compounds for frequency and concentration (Kolpin et al., 2002; Halden and Paull, 2005). TCS has been identified in wastewater treatment plant (WWTP) effluent at concentrations greater than 10 $\mu g\,L^{-1}$ (Lopez-Avila and Hites, 1980). A USGS study monitoring 95 compounds in surface water throughout the United States, found TCS to be one of the most frequently detected compounds with surface water concentrations as high as 2.3 $\mu g\,L^{-1}$ (Kolpin et al., 2002). For all published studies conducted to-date TCS has been detected in 56.8% of surface water samples with a median concentration of about 50 $ng\,L^{-1}$ (Table 1). TCS has been detected in surface water worldwide (United States-Boyd et al., 2004; Loraine and Pettigrove, 2006; Benotti et al., 2009; Dougherty et al., 2010; Romania – Moldovan, 2006; United Kingdom – Kasprzyk-Horden et al., 2008; South Korea – Kim et al., 2009; Yoon et al., 2008, to name a few) with a number of studies in Switzerland having identified TCS and also its methyl derivative methyl triclosan (M-TCS) in WWTP effluent, surface water, and fish tissue. In this study, TCS was detected in surface water at concentrations higher than M-TCS and found as high as 74 $ng\,L^{-1}$ (Lindstrom et al., 2002) whereas concentrations in WWTP effluent were considerably higher for both compounds (up to 650 $ng\,L^{-1}$ TCS and 11 $ng\,L^{-1}$ M-TCS). M-TCS is relatively stable and lipophilic and thus is likely to bioaccumulate in biota. The highest reported concentrations of M-TCS were found in fish with concentrations as high as 2100 $ng\,g^{-1}$ of lipid (Buser et al., 2006). Interestingly, numerous studies investigating bioaccumulation have found that M-TCS is bioaccumulated whereas TCS is not based on data with semi-permeable membrane devices (Poiger et al., 2001; Lindstrom et al., 2002). Furthermore, bioaccumulation of TCS has not been observed in aquatic plants although M-TCS has been observed to bioaccumulate after 28 d in *Sesbania herbacea* (Stevens et al., 2009). However, other studies have contradicted these findings demonstrating TCS bioaccumulates to a much greater degree in algae than M-TCS (Coogan et al., 2007). It is currently unknown whether phylogenetic differences in physiology across trophic groups can influence bioaccumulation and what factors are most important for uptake and accumulation to occur. One possible explanation for differences in TCS bioaccumulation is due to potential ionization of TCS. In typical environmental conditions TCS ranges from completely protonated (pH = 5.4) to totally deprotonated (pH = 9.2) based on a p*K*a values of 7.8 at normal pH ranges in surface waters (Young et al., 2008). These variations in ionization cause differences in *Dow* values relating to differences in bioaccumulation. Although this has not been investigated for TCS, studies with the pharmaceutical fluoxetine (Prozac®) indicate vast differences in bioaccumulation at different pH's (Nakamura et al., 2008). Based on these results at higher pH's TCS would be expected to accumulate more due to its pKa value of 7.8 whereas at lower pH's M-TCS would be expected to accumulate to higher levels.

TCC has been used in PCPs since 1957 and has been observed in surface water at concentrations up to 6.75 $\mu g\,L^{-1}$ (Halden and Paull, 2005). It is believed that TCC occurs as frequently in WWTP effluent and surface water as TCS; however, until 2004 TCC could not be detected at low levels ($ng\,L^{-1}$) (Halden and Paull, 2004; Coogan et al., 2007). However, TCC has been detected at higher concentrations and more frequently in WWTP effluent and surface water than TCS or M-TCS over the last 5 years (Coogan et al., 2007). Additionally, TCC has demonstrated a propensity to bioaccumulate more than either TCS or M-TCS in aquatic organisms. Other disinfectants (phenol, 4-metyhl phenol, and biphenylol) are also commonly used in households and have the potential to be released into aquatic environments. These compounds have been identified in surface water or WWTP effluent (Kolpin et al., 2002, 2004; Glassmeyer et al., 2005) with phenol found more often than 4-methyl phenol and biphenylol, as well as in greater concentrations (as high as 1.3 $\mu g\,L^{-1}$) (Kolpin et al., 2002).

Acute toxicity of TCS and biphenylol has been examined in invertebrates, fish, amphibians, algae, and plants. TCS is more toxic to similarly studied trophic groups in comparison to other disinfectants. For all disinfectants studied, invertebrates are only slightly more sensitive than fish for acute time periods (Table 2). Additionally for longer-term exposure, fish and vascular plants appear to be less sensitive to TCS exposure whereas algae and invertebrates are more sensitive (Table 3). High TCS sensitivity in algae is likely due to TCS antibacterial characteristics, through disruption of lipid synthesis through the FabI (fatty acid synthesis) and FASII (enoyl acyl carrier protein reductase) pathways (McMurry et al., 1998; Lu and Archer, 2005), membrane destabilization (Lyrge et al., 2003; Franz et al., 2008), or uncoupling of oxidative phosphorylation (Newton et al., 2005) which are similar between algae and bacteria (Coogan et al., 2007). Toxicity of TCS to animal species is likely due to non-specific narcosis as no common receptors are known to exist (Lyndall et al., 2010). Acute toxicity to amphibians has been studied in four different amphibians using a modified FETAX assay (Palenske et al., 2010). Amphibians were more sensitive than fish however they were not as sensitive as algae during short-term exposures. For longer exposure duration algae appears to be the most sensitive trophic group. Algal growth was the most sensitive endpoint and was affected at concentrations less than 1 $\mu g\,L^{-1}$ (Orvos et al., 2002). Aquatic plants, invertebrates, and fish were not highly sensitive to chronic exposure of TCS (Table 3). Only minimal aquatic toxicity data exists for TCC, but recent studies indicate TCC is slightly more toxic to aquatic invertebrate and fish for both

Table 1
Summary of measured concentration of personal care products in surface water ($ng\,L^{-1}$).

Compound	Class	n[a]	Range ($ng\,L^{-1}$)	Median ($ng\,L^{-1}$)
Triclosan	Disinfectant	710	<0.1–2300	48
Methyltriclosan	Disinfectant	4	0.5–74	–
Triclocarban	Disinfectant	29	19–1425	95
Musk ketone	Fragrance	178	4.8–390	11
Musk xylene	Fragrance	93	1.1–180	9.8
Celestolide	Fragrance	73	3.1–520	3.2
Galaxolide	Fragrance	282	64–12 470	160
Tonalide	Fragrance	245	52–6780	88
DEET	Insect repellant	188	13–660	55
Paraben[b]	Preservative	6	15–400	–
4MBC	UV filter	19	2.3–545	10.2
BP3	UV filter	18	2.5–175	20.5
EHMC	UV filter	21	2.7–224	6.1
OC	UV filter	22	1.1–4450	1.9

[a] *n* = Number of samples.
[b] Includes all parabens.

Table 2
Acute toxicity data for personal care products.

Compound	Category	Species	Trophic group	Endpoint/duration	LC50 (mg L^{-1})	Additional tox. values	References
Biphenylol	Antimicrobial	*Daphnia magna*	Invert.	48 h Mobility	3.66		1
		D. magna	Invert.	48 h Survival	3.66		2
		Tetrahymena pyriformis	Invert.	48 h Survival	5.7–8.26		3
		T. pyriformis	Invert.	60 h Survival	5.7–8.26		4
		Cyprinus carpio	Fish	44 h Survival	157–292		5
Triclosan	Antimicrobial	*D. magna*	Invert.	48 h	0.39		6
		Ceriodaphnia dubia	Invert.	24, 48 h (pH = 7.0)	0.2, ~125		6
		Pimephales promelas	Fish	24, 48, 72, 96 h	0.36, 0.27, 0.27, 0.26		6
		Lepomis macrochirus	Fish	24, 48, 96 h	0.44, 0.41, 0.37		6
		Oryzias latipes	Fish	96 h	0.602 (larvae), 0.399 (embryos)		7
		Xenopus laevis	Amphibian	96 h	0.259		8
		Acris blanchardii	Amphibian	96 h	0.367		8
		Bufo woodhousii	Amphibian	96 h	0.152		8
		Rana sphenocephala	Amphibian	96 h	0.562		8
		Pseudokirch-neriella subcapitata	Algae	72 h Growth	0.53 (μg L^{-1})		9
Triclocarban	Antimicrobial	*D. magna*	Invert.	48 h	0.01		10
		C. dubia	Invert.	48 h	0.0031		10
		Mysidopsis bahia	Invert.	48, 96 h	0.015, .01		10
		Salmo gairdneri	Fish	96 h	0.120		10
		L. macrochirus	Fish	96 h	0.097		10
		Scenedesmus subspicatus	Algae	72 h Growth	0.02		10
		P. subcapitata	Algae	72 h Growth	0.017 (μg L^{-1})		9
Benzophenone	Fixative	*Caenorahbditis elegans*	Nematode	24 h	56.8		11
		P. promelas	Fish	96 h	10.89		12
1,4-dichlorobenzene[a]	Insect repellant	*D. magna*	Invert.	24, 48 h Immobilization	1.6, 0.7		13, 14
		Artemia salina	Invert.	24 h	14		15
		Palaemonetes pugio	Invert.	96 h	60		16
		M. bahia	Invert.	96 h	1.99		17
		Danio rerio	Fish	24 h, 96 h	4.25, 2.1		18,19
		Jordanella floridae	Fish	96 h	2.05		20
		P. promelas	Fish	96 h	4.2		21
		O. mykiss	Fish	24 h	1.18		19
		L. macrochirus	Fish	96 h	4.3		22
		Cyprinodon variegatus	Fish	96 h	7.4		23
		Selenastrum capricornutum	Algae	96 h Growth	0.57		14
		Scenedesmus pannonicus	Algae	72 h Growth	31		13
		S. subspicatus	Algae	48 h Growth, Biomass	38, 28		22
		Skeletonema costatum	Algae	96 h Growth	59.1		17
N,N-diethyl-*m*-toluamide (DEET)[b]	Insect repellant	*D. magna*	Invert.	48 h, 96 h	160, 108		25
		Gammarus fasciatus	Invert.	96 h	100		26
		P. promelas	Fish	96 h	110		27
		Gambusia affinis	Fish	24–48 h	235		28
		Oncorhynchus mykiss	Fish	96 h	71.3		29
		Chlorella protothecoides	Algae	24 h Photosynthesis	388		30
Musk ambrette (MA)	Nitro musk	*Vibrio fischeri*	Bacteria	Microtox	>Sol.[c]		31
		Pseudokirch-neriella subcapitata	Algae	72 h	>Sol.		31
Musk ketone (MK)	Nitro musk	*V. fischeri*	Bacteria	Microtox	>Sol.		31
		Nitocra spinipes	Invert.	96 h	>1.0		32
		Acartia tonsa	Invert.	48 h	1.32	LC10 = 0.40	33
		D. magna	Invert.	24, 48 h	>Sol., 5.6		28
		D. magna	Invert.	48 h	>0.46		31
		D. rerio	Fish	96 h Survival, Hatching	>0.4		28
		P. subcapitata	Algae	72 h	>Sol.		31
Musk moskene (MM)	Nitro musk	*V. fischeri*	Bacteria	Microtox	>Sol.		31
		D. magna	Invert.	24 h	>Sol.		31
		Danio rerio	Fish	96 h Survival, Hatching	>0.4		34
		P. subcapitata	Algae	72 h	>Sol.		31
Musk Tibetene (MT)	Nitro musk	*V. fischeri*	Bacteria	Microtox	>Sol.		31
		P. subcapitata	Algae	72 h	>Sol.		31

Table 2 (*continued*)

Compound	Category	Species	Trophic group	Endpoint/duration	LC50 (mg L^{-1})	Additional tox. values	References
Musk xylene (MX)	Nitro musk	*V. fischeri*	Bacteria	Microtox	>Sol.		31
		D. magna	Invert.	24, 48 h Mobility	EC50 ⩾ Sol.		35
		Oncorhynchus. mykiss	Fish	96 h	>1000		36
		L. macrochirus	Fish	96 h	1.2		37
		D. rerio	Fish	96 h Survival, Hatching	>0.4		34
		P. subcapitata	Algae	72 h	>Sol.		31
Celestolide (ADBI)	Polycyclic musk	*N. spinipes*	Invert.	96 h	>2.0		38
		A. tonsa	Invert.	48 h	>2.0	LC10 > 2.0	39
		D. rerio	Fish	96 h Survival, Hatching	>1.0		38
		D. rerio	Fish	96 h Malformation	LOEC∼0.65		39
		O. latipes	Fish	96 h Survival	1.97		40
Galaxolide (HHCB)	Polycyclic musk	*N. spinipes*	Invert.	96 h	1.90		28
		A. tonsa	Invert.	48 h	0.47	LC10 = 0.12	39
		Lampsilis cardium	Benthic invert.	24, 48 h	1.0, 0.99		41
		D. rerio	Fish	96 h Survival, Hatching	>0.67		39
		D. rerio	Fish	96 h Malformations	LOEC ∼ 0.45		39
		O. latipes	Fish	96 h Survival	0.95		40
Tonalide (AHTN)	Polycyclic musk	*N. spinipes*	Invert.	96 h	0.61		31
		A. tonsa	Invert.	48 h	0.71	LC10 = 0.45	32
		L. cardium	Benthic invert.	24, 48 h	0.45, 0.28		41
		D. rerio	Fish	96 h Malformation	LOEC ∼ 0.1		39
		D. rerio	Fish	96 h Survival, Hatching	>0.67		38
		O. latipes	Fish	96 h Survival	1.00		40
Traseolide (ATII)		*O. latipes*	Fish	96 h Survival	0.95		40
Phantolide (AHMI)		*O. latipes*	Fish	96 h Survival	1.22		40
Cachmeran (DPMI)		*O. latipes*	Fish	96 h Survival	11.6		40
Benzylparaben	Preservative	*T. thermophila*	Protozoa	24 h, 28 h	4.3, 5.7	LOEC = 0.48	42
		V. fisheri	Bacteria	15 min, 30 min Illuminescence	0.11, 0.11	LOEC = 0.02	42
		Photobacterium leiognathi	Bacteria	15 min, 30 min Illuminescence	1.3, 1.6	LOEC = 0.25	42
		D. magna	Invert.	48 h	4.0		43
		D. magna	Invert.	24 h, 48 h Mobility	5.2, 6	LOEC = 1.2	42
		P. promelas	Fish	48 h	3.3		43
Butylparaben	Preservative	*T. thermophila*	Protozoa	24 h, 28 h	5.3, 7.3	LOEC = 2.5	42
		V. fisheri	Bacteria	15 min, 30 min Illuminescence	2.5, 2.8	LOEC = 0.7	42
		P. leognathi	Bacteria	15 min, 30 min Illuminescence	3.7, 4.3	LOEC = 1.12	42
		D. magna	Invert.	48 h	5.3		43
		D. magna	Invert.	24 h, 48 h Mobility	6.2, 6	LOEC = 3.2	42
		P. promelas	Fish	48 h	4.2		43
Ethylparaben	Preservative	*T. thermophila*	Protozoa	24 h, 28 h	25, 30	LOEC = 10.7	42
		V. fisheri	Bacteria	15 min, 30 min Illuminescence	2.5, 2.7	LOEC = 0.55	42
		P. leognathi	Bacteria	15 min, 30 min Illuminescence	19, 24	LOEC = 5.5	42
		D. magna	Invert.	48 h	18.7		43
		D. magna	Invert.	24 h, 48 h Mobility	25,23	LOEC = 12	42
		P. promelas	Fish	48 h	34.3		43
Isobutylparaben	Preservative	*D. magna*	Invert.	48 h	7.6		43
		P. promelas	Fish	48 h	6.9		43
Isopropylparaben	Preservative	*D. magna*	Invert.	48 h	8.5		43
		P. promelas	Fish	48 h	17.5		43
Methylparaben	Preservative	*T. thermophila*	Protozoa	24 h, 28 h	54, 58	LOEC = 11.5	42
		V. fisheri	Bacteria	15 min, 30 min Illuminescence	9.6, 10	LOEC = 2.9	42
		P. leognathi	Bacteria	15 min, 30 min Illuminescence	31,35	LOEC = 8.5	42
		D. magna	Invert.	48 h	24.6		42
		D. magna	Invert.	24 h, 48 h Mobility	32, 21	LOEC = 15	42
		P. promelas	Fish	48 h	>Sol.		43

(*continued on next page*)

Table 2 (*continued*)

Compound	Category	Species	Trophic group	Endpoint/duration	LC50 (mg L^{-1})	Additional tox. values	References
Propylparaben	Preservative	*T. thermophila*	Protozoa	24 h, 28 h	9.7, 12.5	LOEC = 2.6	42
		V. fisheri	Bacteria	15 min, 30 min Illuminescence	2.5, 2.6	LOEC = 0.9	42
		P. leognathi	Bacteria	15 min, 30 min Illuminescence	21, 25	LOEC = 4.5	42
		D. magna	Invert.	48 h	12.3		43
		D. magna	Invert.	24 h, 48 h Mobility	13, 7	LOEC = 6	42
		P. promelas	Fish	48 h	9.7		43
Benzophenone-3	UV filter	*D. magna*	Invert.	48 h Immobility	1.9		44
Benzophenone-4	UV filter	*D. magna*	Invert.	48 h Immobility	50		44
4-Methylbenzy-lidene camphor	UV filter	*D. magna*	Invert.	48 h Immobility	0.56		44
2-Ethyl-hexyl-4-trimethoxy-cinnamate	UV filter	*D. magna*	Invert.	48 h Immobility	0.29		44

References: (1) Kopperman et al. (1974), (2) Carlson and Caple (1977), (3) Schultz et al. (1989), (4) Schultz and Riggin (1985), (5) Loeb and Kelly (1963), (6) Orvos et al. (2002), (7) Ishibashi et al. (2004), (8) Palenske et al. (2010), (9) Yang et al. (2008), (10) TCC Consortium (2002), (11) Ura et al. (2002), (12) Marchini et al. (1992), (13) Canton et al. (1985), (14) Calamari et al. (1982), (15) Abernathy et al. (1986), (16) Curtis and Ward (1981), (17) USEPA (1978), (18) Roederer (1990), (19) Calamari et al. (1983), (20) Smith et al. (1990), (21) Carlson and Kosian (1987), (22) Buccafusco et al. (1981), (23) Heitmuller et al. (1981), (24) Kuhn and Pattard (1990), (25) Seo et al. (2005), (26) Mayer and Ellersieck (1986), (27) Brooke et al. (1984), (28) Michael and Grant (1974), (29) Office of Pesticides Program (2000), (30) Costanzo et al. (2007), (31) Schramm et al. (1996), (32) Breitholtz et al. (2003), (33) Wollenberger et al. (2003), (34) Tas et al. (1997), (35) Hughes and Krishnaswami (1985), (36) MITI (1992), (37) Adema and Langerwerf (1985a,b), (38) Van der Plassche and Balk (1997), (39) Dietrich and Chou (2001), (40) Yamauchi et al. (2008), (41) Gooding et al. (2006), (42) Bazin et al. (2010), (43) Dobbins et al. (2009), (44) Fent et al., 2009.

[a] 1,4-dichlorobenzene table is modified from Boutonnet et al. (2004).

[b] DEET information is modified from Table presented by Costanzo et al. (2007).

[c] No effects found at concentrations exceeding water solubility.

Table 3
Chronic toxicity data for personal care products.

Compound	Category	Species	Trophic level	Endpoint/duration	LOEC (μg L^{-1})	NOEC (μg L^{-1})	References
Triclosan	Antimicrobial	*D. magna*	Invert.	21 d Survival, Reproduction	Repro. = 200 (LOEC)	Surv. = 200 (NOEC)	1
		C. dubia	Invert.	7 d Survival, Reproduction		50, 6	1
		C. dubia	Invert.	7 d Survival, Reproduction	IC25 = 170		2
		Chironomus riparius	Invert.	28 d Survival, Emergence		440	3
		Chironomus tentans	Invert.	10 d Survival, Growth	LC25 = 100		4
		Hyalella azteca	Invert.	10 d Survival, Growth	LC25 = 60		4
		O. mykiss	Fish	96 d ELS[c] Hatching, Survival	No Effect, 71.3		1
		O. latipes	Fish	14 d Hatching	213		5
		O. latipes	Fish	21 d Growth, Fecundity, HSI and GSI[d], VTG[e]	200, No Effect, 200, 20		5
		O. latipes	Fish	14 d Hatchability	IC25 = 290		2
		Gambusia affinis	Fish	35 d Sperm Count, VTG	101.3		6
		Danio rerio	Fish	9 d Hatchability	IC25 = 160		2
		Xenopus laevis	Amphibian	21 d Metamorphosis	No effect (200)		7
		Rana catesbeiana	Amphibian	18 d Development	300		8
		Rana pipiens	Amphibian	24 d Survival, Growth	230, 2.3		9
		Bufo americanus	Amphibian	14 d Survival, Growth	No effect (230)		10
		S. capricornutum	Algae	96 h Growth	EC50 = 4.46	EC25 = 2.44	1
		S. subspicatus	Algae	96 h Biomass, Growth Rate	EC50 = 1.2, 1.4	EC50 = 0.5, 0.69	1
		S. costatum	Algae	96 h Growth Rate	EC50 ⩾ 66	EC25 > 66	1
		A. flos-aquae	Algae	96 h Biomass	EC50 = 0.97	EC25 = 0.67	1
		P. subcapitata	Algae	72 h Growth	EC25 = 3.4	0.2	2, 11
		N. pelliculosa	Algae	96 h Growth Rate	EC50 = 19.1	EC25 = 10.7	1
		Natural algal assemblage	Algae	96 h Biomass	0.12		12
		Closterium ehrenbergii	Algae	96 h Growth		250	13
		Dunaliella tertiolecta	Algae	96 h Growth		1.6	14
		L. gibba	Plant	7 d Growth	EC50 ⩾ 62.5	EC25 ⩾ 62.5	1
		S. herbacea	Plant	28 d Seed Germination, Morphology	100 germination, 10 morphology		15
		E. prostrata	Plant	28 d Seed Germination, Morphology	No effect, 1000		15
		B. frondosa	Plant	28 d Seed Germination, Morphology	100, 10		15

Table 3 (*continued*)

Compound	Category	Species	Trophic level	Endpoint/duration	LOEC ($\mu g\ L^{-1}$)	NOEC ($\mu g\ L^{-1}$)	References
Triclocarban	Antimicrobial	*D. magna*	Invert.	21 d Growth	4.7	2.9	16
		M. bahia	Invert.	28 d Reproduction	0.13	0.06	16
		P. subcapitata	Algae	14 d Growth	10 000	EC50 = 36 000	16
Benzophenone	Fixative	*Pimephales promelas*	Fish	7 d Survival, Growth	9240, 3100	5860, 2100	17
		P. promelas	Fish	7 d ELS (Survival, Growth)	6400, 1800	3300, 1000	17
1,4-dichlorobenzene	Insect repellant	*D. magna*	Invert.	28 d Growth		0.22	18
		D. magna	Invert.	21 d Reproduction		0.3	19
		Jordanella floridae	Fish	28 d Growth		>0.35	20
		O. mykiss	Fish	60 d Growth		>0.122	18
		P. promelas	Fish	33 d Growth		0.57	21
		D. rerio	Fish	28 d Growth		1.0	22
Musk ketone (MK)	Nitro musk	*D. magna*	Invert.	21 d Development, Reproduction	340		23
		D. magna	Invert.	21 d Survival	LC50 = 338–675		24
		A. tonsa	Invert.	5 d Developmental Rate	EC50 = 66	EC10 = 10	25
		A. tonsa	Invert.	5 d Juvenile Survival	2000	800	25
		N. spinipes	Invert.	7 d Developmental Rate, Survival	30		23
		N. spinipes	Invert.	26 d Population Growth Rate	100		23
		D. rerio	Fish	ELS 24–48 h Tail Extension, Coagulated Eggs, Edema, Circulation	1000	330	26
		D. rerio	Fish	ELS 24–48 h Movement, Tail Extension	330	100	26
		D. rerio	Fish	ELS 48 h Heart Rate	10	3.3	26
		D. rerio	Fish	ELS 48 h Survival	33	10	26
		O. mykiss	Fish	21 d Reproduction	EC50 = 169–338		27
		L. macrochirus	Fish	21 d Survival	LC50 $\geqslant$ 500		28
		D. rerio	Fish	8w Reproduction	33		28
		P. promelas	Fish	96 h Teratogenesis	EC50 $\geqslant$ 400		28
		X. laevis	Amphibian	96 h FETAX[b]	>4000		29
		P. subcapitata	Algae	72 h Growth, Biomass	EC50 = 244, 118		30
Musk moskene (MM)	Nitro musk	*D. magna*	Invert.	21 d Survival	LC50 $\geqslant$ Sol.		31
		O. mykiss	Fish	21 d Reproduction	EC50 $\geqslant$ Sol.		29
		X. laevis	Amphibian	96 h FETAX	EC50 $\geqslant$ 400		30
Musk xylene (MX)	Nitro musk	*D. magna*	Invert.	21 d Survival	LC50 = 680		32
		D. rerio	Fish	ELS 24–48 h Tail Extension, Coagulated Eggs, Edema, Circulation, Movement	1000	330	27
		D. rerio	Fish	ELS 48 h Heart Rate, Survival	330	10	27
		D. rerio	Fish	14 d Survival	LC50 = 400		33
		X. laevis	Amphibian	96 h FETAX[b]	>400		30
		P. subcapitata	Algae	72 h Growth, Biomass	EC50 $\geqslant$ Sol.[a]		34
		M. aeruginosa	Algae	5 d Cell Count	>10 000		34
Celestolide (ADBI)	Polycyclic musk	*N. spinipes*	Invert.	7 d Developmental Rate, Survival	100		24
		A. tonsa	Invert.	5 d Developmental Rate	EC50 = 160	EC10 = 36	28
		A. tonsa	Invert.	5 d Juvenile Survival	600	240	28
		X. laevis	Amphibian	96 h FETAX	EC50 $\geqslant$ 1000		30
Galaxolide (HHCB)	Polycyclic musk	*D. magna*	Invert.	21 d Development, Reproduction	282 (EC50)		24
		D. magna	Invert.	21 d Growth, Survival	205	11	35
		D. magna	Invert.	21 d Survival	LC50 = 293		36
		A. tonsa	Invert.	5 d Developmental Rate	EC50 = 59	EC10 = 37	26
		A. tonsa	Invert.	5 d Juvenile Survival		300	26
		N. spinipes	Invert.	7 d Developmental Rate, Survival	20		24
		L. cardium	Benthic invert.	96 h Growth	EC50 = 153–563		36
		Capitella sp.	Benthic invert.	119 d Survival, Growth, Development	123 mg kg^{-1}, No effect, 168 mg kg^{-1}		37
		Potamopyrgus antipodarum	Benthic invert.	94 d Adult and Juvenile Survival, Growth, Reproduction	100 Time to 1st reproduction, 10 number of offspring		38
		L. macrochirus	Fish	21 d Growth, Survival	182 LC50 = 452	182	39
		P. promelas	Fish	36 d Hatch, Survival,	>140, 68, 68, 68	>140, 140, 140,	40

(*continued on next page*)

Table 3 (*continued*)

Compound	Category	Species	Trophic level	Endpoint/duration	LOEC (μg L^{-1})	NOEC (μg L^{-1})	References
				Growth, Development		140	
		O. mykiss	Fish	21 d Reproduction	EC50 = 282		36
		D. rerio	Fish	21 d Survival	LC50 = 452		36
		O. latipes	Fish	72 h VTG, ERα[f]	500		41
		X. laevis	Amphibian	96 h FETAX	EC50 ⩾ 100		30
		X. laevis	Amphibian	32 d Survival	LC50 ⩾ 140		30
		P. subcapitata	Algae	72 h Growth, Biomass	466	201	42
		P. subcapitata	Algae	72 h Growth, Biomass	EC50 ⩾ 854, 723		43
Tonalide (AHTN)	Polycyclic musk	*D. magna*	Invert.	21 d Growth, Survival	184–401	89–196	39
		D. magna	Invert.	21 d Development, Reproduction	244 (EC50)		24
		A. tonsa	Invert.	5 d Developmental Rate	EC50 = 26	EC10 = 7.2	26
		A. tonsa	Invert.	5 d Juvenile Survival	160	60	26
		N. spinipes	Invert.	7 d Developmental Rate, Survival	>60		24
		L. cardium	Benthic invert.	96 h Growth	EC50 = 108–708		36
		D. rerio	Fish	ELS 24–48 h Heart Rate	33	10	27
		L. macrochirus	Fish	21 d Growth, Survival	184 LC50 = 314	89	36
		P. promelas	Fish	36 d Hatch, Survival, Growth, Development	>140, 140, 67, 67	>140, 67, 35, 35	40
		O. mykiss	Fish	21 d Reproduction	EC50 = 244		36
		D. rerio	Fish	21 d Survival	LC50 = 314		36
		O. latipes	Fish	72 h VTG, ERα[f]	500		43
		X. laevis	Amphibian	96 h FETAX	EC50 ⩾ 1000		30
		X. laevis	Amphibian	32 d Survival	LC50 = 100		30
		P. subcapitata	Algae	72 h Growth, Biomass	797–835	204–438	42
		P. subcapitata	Algae	72 h Growth, Biomass	EC50 ⩾ 797, 468		43
Benzylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	200, 2600		44
		P. promelas	Fish	7 d Growth	1700		44
Butylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	200, 2600		44
		P. promelas	Fish	7 d Growth	1000		44
		S. trutta	Fish	10 d VTG	134	76	45
Ethylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	9000, 2300		44
		P. promelas	Fish	7 d Growth	17 000		44
Isobutylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	300, 2000		44
		P. promelas	Fish	7 d Growth	3500		44
Isopropylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	4000, 2000		44
		P. promelas	Fish	7 d Growth	9000		44
Methylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	6000, 1500		44
		P. promelas	Fish	7 d Growth	25 000		44
Propylparaben	Preservative	*D. magna*	Invert.	7 d Growth, Reproduction	400, 6000		44
		P. promelas	Fish	7 d Growth	2500		44
		O. latipes	Fish	7 d VTG	99 00[g]		46
Benzophenone-1	UV filter	*P. promelas*	Fish	14 d VTG	4919.4		46
		O. mykiss	Fish	14 d VTG, Growth	4919		47
Benzophenone-2	UV filter	*P. promelas*	Fish	14 d VTG	8782.9		48
		O. mykiss	Fish	14 d VTG, Growth	8783		47
Benzophenone-3	UV filter	*O. mykiss*	Fish	14 d Growth	3900		47
Benzophenone-4	UV filter	*O. mykiss*	Fish	14 d Growth	4897		47
3-benzylidene camphor	UV filter	*Potamopyrgus antipodarum*	Benthic invert.	56 d Reproduction	0.28 mg kg^{-1} sediment		48
		Lumbriculus variegatus	Benthic invert.	28 d Reproduction	6.47 mg kg^{-1} sediment		48
		P. promelas	Fish	14 d VTG, Reproduction, Gonad Histology	434.6, 74, 74		49
		P. promelas	Fish	14, 21 d VTG	435, 74		50, 51
		O. mykiss	Fish	14 d VTG, Growth	453		47
		O. mykiss	Fish	10 d Injection	68 mg kg^{-1}		51
		X. laevis	Amphibian	35 d Metamorphosis	No effect		52
3-(4′-methylbenzy-lidene camphor)	UV filter	*Potamopyrgus antipodarum*	Benthic invert.	56 d Reproduction	1.71 mg kg^{-1} sediment		48
		Lumbriculus variegatus	Benthic invert.	28 d Reproduction	22.3 mg kg^{-1} sediment		48

Table 3 (*continued*)

Compound	Category	Species	Trophic level	Endpoint/duration	LOEC ($\mu g\ L^{-1}$)	NOEC ($\mu g\ L^{-1}$)	References
		O. mykiss	Fish	14 d Growth	415		47
Oxybenzone	UV filter	*O. mykiss*	Fish	14 d VTG	749		54
		O. latipes	Fish	21 d VTG, Hatching	620		54
Ethyl-4-aminobenzoate	UV filter	*P. promelas*	Fish	14 d VTG	4394		49

References: (1) Orvos et al. (2002), (2) Tatarazako et al. (2004), (3) Memmert (2006), (4) Dussault et al. (2008), (5) Ishibashi et al. (2004), (6) Raut and Angus (2010), (7) Fort et al. (2010), (8) Veldhoen et al. (2006), (9) Fraker and Smith (2004), (10) Smith and Burgett (2005), (11) Yang et al. (2008), (12) Wilson et al. (2003), (13) Ciniglia et al. (2005), (14) DeLorenzo and Fleming (2008), (15) Stevens et al. (2009), (16) TCC Consortium (2002), (17) Marchini et al. (1992), (18) Calamari et al. (1982), (19) Kuehn et al. (1989), (20) Smith et al. (1990), (21) Carlson and Kosian (1987), (22) Adema and de Ruiter (1987), (23) Breitholtz et al. (2003), (24) Grutzner (1995b), (25) Wollenberger et al. (2003), (26) Carlsson and Norrgren (2004), (27) Grutzner (1995c), (28) Tas et al. (1997), (29) Chou and Dietrich (1999), (30) Grutzner (1995a), (31) Schramm et al. (1996), (32) Adema and Langerwerf (1985a,b), (33) Sousa and Suprenant (1984), (34) Payne and Hall (1979), (35) Wuthrich (1996a), (36) Gooding et al. (2006), (37) Ramskov et al. (2009), (38) Pedersen et al. (2009), (39) Wuthrich (1996b), (40) Croudace et al. (1997), (41) Yamauchi et al. (2008), (42) Van der Plassche and Balk (1997)), (43) Van Dijk (1997), (44) Dobbins et al. (2009), (45) Bjerregaard et al. (2008), (46) Inui et al. (2003), (47) Kunz et al. (2006c), (48) Schmitt et al. (2008), (49) Fent et al. (2008), (50) Kunz et al. (2006a), (51) Kunz et al. (2006b), (52) Holbech et al. (2002), (53) Kunz et al. (2004), (54) Coronado et al. (2008).

[a] No effects found at concentrations exceeding water solubility.
[b] Frog Embryo Teratogenesis Assay – Xenopus.
[c] Early Life Stage.
[d] Hepatosomatic Index and Gonadosomatic Index.
[e] Vitellogenin.
[f] Estrogen receptor.
[g] Only concentration tested.

short- and long-term exposures than TCS (TCC Consortium, 2002) (Table 2, Table 3). M-TCS toxicity to aquatic organisms (*Daphnia magna* and *Scenedesmus subspicatus*) exposed for short time periods is considerably less than the parent compound TCS (Batscher, 2006a,b). In addition to ionization state affecting bioaccumulation, ionization state is also important when examining toxicity. Orvos et al. (2002) determined un-ionized TCS was slightly more toxic at pH's ranging between 8.17 and 8.21 than ionized TCS at higher pH's. This effect is not observed for TCC as TCC only ionizes at extreme pH's outside environmentally relevant ranges (Young et al., 2008).

In addition to typical acute and chronic studies, a number of studies have investigated effects of TCS exposure on swimming behavior of fish. TCS has induced alterations in swimming performance of *Oncorhynchus mykiss*, *Danio rerio*, and *Oryzias latipes* at concentrations as low as 71 $\mu g\ L^{-1}$ which is considerably greater than other endpoints indicating behavior is not a sensitive endpoint for identifying TCS effects (Orvos et al., 2002; Oliveira et al., 2009; Nassef et al., 2010).

Evidence suggests TCS is weakly estrogenic, likely due to its similarities in structure to the non-steriodal estrogen diethylstilbestrol (Foran et al., 2000; Ishibashi et al., 2004). TCS exposure has been implicated in changes in fin length and sex ratios of medaka (*O. latipes*) (Foran et al., 2000). TCS has also been demonstrated to induce VTG synthesis in male *O. latipes* after 21 d exposure (Ishibashi et al., 2004), and decreased sperm counts and VTG synthesis after 35 d exposure in *Gambusia affinis* (Raut and Angus, 2010). TCS has also been investigated for endocrine effects in *Xenopus laevis* and *Rana catesbeiana* (Veldhoen et al., 2006; Fort et al., 2010) due to similarities in structure to thyroid hormone (TH) (Veldhoen et al., 2006). TCS had no effects on *X. laevis* metamorphosis (Fort et al., 2010) and produced only slight effects in *R. catesbeiana* (Veldhoen et al., 2006) suggesting TCS only minimally affects thyroid hormone (TH) and development in amphibians. To-date, no studies have looked at potential endocrine disruption of other disinfectants with similar structures (i.e. TCC and M-TCS) (Table 3).

Based on toxicity data, algae appear to be the most sensitive trophic group to environmental concentrations of TCS and other disinfectants. However, it is possible that TCS, M-TCS, and TCC could affect benthic invertebrates at environmentally relevant concentrations due to disinfectants potential sorption to sediment although, heretofore, no studies have investigated acute or chronic effects in benthic invertebrates (Orvos et al., 2002). Additionally, TCS has been observed to cause development of antimicrobial strains of resistant bacteria as well as antibiotic resistant bacteria through development of cross-resistance (Braoudaki and Hilton, 2004). The potential environmental impacts of antimicrobial resistance in aquatic ecosystems are low although it could have major implications on human health and aquaculture.

Results of a probabilistic risk assessment indicate minimal effects of TCS on aquatic ecosystems as the 95th percentile of environmental concentrations is below the 5th percentile of sensitive species (Lyndall et al., 2010). Numerous uncertainties remain including effects on benthic organisms and effects of ionization and dissociation on partitioning, toxicity, and bioaccumulation that need to be determined to conduct a comprehensive risk assessment on TCS.

3. Fragrances

Fragrances are perhaps the most widely studied class of PCPs and are believed to be ubiquitous contaminants in the environment (Daughton and Ternes, 1999). The most commonly used fragrances are synthetic musks. Synthetic musks are fragrances used in a wide-range of products including deodorants, soaps, and detergents. Synthetic musks are either nitro musks, which were introduced in the late 1800s, or polycyclic musks, introduced in the 1950s (Daughton and Ternes, 1999). The most commonly used nitro musks are musk xylene (MX) and musk ketone (MK) whereas musk ambrette (MA), musk moskene (MM), and musk tibetene (MT) are used less frequently (Daughton and Ternes, 1999). Nitro musks however, are slowly being phased out due to their environmental persistence and potential toxicity to aquatic species (Daughton and Ternes, 1999). Polycyclic musks are currently used in higher quantities than nitro musks with celestolide (ABDI), galaxolide (HHCB) and toxalide (AHTN) used most commonly and traseloide (ATII), phantolide (AHMI), and cashmeran (DPMI) used less often (Daughton and Ternes, 1999). HHCB and AHTN production alone has been estimated at about 1 million pounds

per year and has thus been placed on the High Production Volume List by the USEPA (Peck, 2006).

Yamigashi et al. (1981, 1983) first identified nitro musks in the environment and conducted the first major monitoring study on MX and MK. MX and MK were found in greater than 80% of samples from river water, WWTP effluent, freshwater fish, and shellfish in Japan. Concentrations were highest in WWTP effluent ranging from 25 to 36 ng L^{-1} and 140–410 ng L^{-1} for MX and MK, respectively (Yamigashi et al., 1981, 1983). For all studies conducted in which fragrance concentrations were reported, MX and MK have been detected in 83–90% of WWTP effluents and approximately 50% of surface waters. Furthermore, Winkler et al. (1998) and Moldovan (2006) identified both nitro musks and polycylic musks in Elbe River (Germany) and Somes River (Romania) water samples ranging between 2 and 10 ng L^{-1} for nitro musks and 2–300 ng L^{-1} for polycyclic musks, with the polycyclic musk HHCB being detected at the highest concentration. Polycyclic musks have been observed more often in surface water (78.3% and 84.6% of samples for AHTN and HHCB, respectively) as well as in greater concentrations than nitro musk compounds worldwide. Kalferlein et al. (1998) and Geyer et al. (1994) published extensive reviews of musks found in the environment, and more specifically in biological compartments. The highest concentrations of polycylclic musks reported to-date has occurred in surface waters in Berlin, Germany that receive substantial input from WWTP at concentrations approaching 10 μg L^{-1} (Table 1) (Heberer et al., 1999).

Nitro and polycyclic musks are water soluble, but high octanol–water coefficients (log Kow = 3.8 for MK and 5.4–5.9 for polycyclic musks) (Schramm et al., 1996; Balk and Ford, 1999) indicate high potential for bioaccumulation in aquatic species (Geyer et al., 1994; Winkler et al., 1998). This potential has been realized by numerous researchers having identified high concentrations of musks in lipids from fresh- and saltwater fish and mollusks (Schramm et al., 1996). Median concentrations of synthetic musks in biota range from approximately 0.1 to 3 mg kg^{-1} of lipid for MK and AHTN, respectively. Dietrich and Hitzfield (2004) compiled bioconcentration (BCF) and bioaccumulation factors (BAF) for synthetic musks and found nitro musks, specifically MX, bioconcentrate and bioaccumulate more than polycyclic musks. MX has been observed to bioconcentrate up to 6700x in common carp (Gatermann et al., 2002), whereas AHTN bioconcentrates much less with BCF values ranging between 597 and 1069 in aquatic species (Dietrich and Chou, 2001; Fromme et al., 2001).

Table 4
Estimated hazard quotients based on the most sensitive endpoint investigated to date and highest observed environmental concentration.

Compound	Trophic group	Toxicity endpoint	Hazard quotient[a]
Triclosan	Invertebrate	EC25 – growth	0.038
	Fish	LOEC – ELS survival	0.032
	Amphibian	LOEC – development	0.130
	Algae	LOEC – growth	19.167
	Plant	LOEC – morphology	0.230
Triclocarban	Invertebrate	LOEC – reproduction	10.962
Musk ketone	Invertebrate	EC10 – development	0.039
	Fish	LOEC – ELS survival	0.039
Musk xylene	Fish	LOEC – ELS survival	0.001
Celestolide	Invertebrate	EC10 – development	0.014
Galxolide	Invertebrate	LOEC – development	0.624
	Fish	LOEC – development	0.183
Tonalide	Invertebrate	EC10 – development	0.942
	Fish	LOEC – ELS survival	0.205
Butylparaben	Fish	LOEC – VTG	0.005

[a] Hazard quotient is calculated by environmental concentration/effects concentration.

Nitro musks have relatively low or no propensity to cause acute toxicity to aquatic taxa studied to-date. Furthermore, only three studies examining MK and MX found acute toxicity to occur at levels below water solubility limits (0.15 mg L^{-1} for MX, 0.46 for MK) (EC, 2003a,b) (Table 2). However, nitro musks are potentially toxic to aquatic organisms over longer time periods with *D. rerio* (zebrafish) early life stage (ELS) studies being most sensitive (Table 3). Additionally, *D. rerio* in general are the most sensitive species studied to-date, whereas amphibians do not appear sensitive to nitro musk exposure (Chou and Dietrich, 1999; Carlsson and Norrgren, 2004). It has been suggested nitro musk transformation products have potential to be highly toxic to aquatic organisms although only minimal data exists (Daughton and Ternes, 1999). Polycyclic musks are more acutely toxic than nitro musks based on published literature. HHCB and AHTN are toxic to aquatic invertebrates at ppb to low ppm levels although they are relatively non-toxic to fish (Table 2), and for longer exposure periods, invertebrates also appear more sensitive to polycyclic musks than fish (Table 3). Similar to nitro musks, polycyclic musks are non-toxic to amphibians. Developmental rates of invertebrates and growth and development for ELS of fish are the most sensitive endpoints studied to date for polycyclic musks. Vitellogenin (VTG) synthesis, indicating potential endocrine effects, was not a sensitive endpoint suggesting musks do not induce estrogenic effects (Dietrich and Chou, 2001).

Based on the highest reported concentrations of synthetic musks in aquatic environments, only AHTN would have the potential to cause adverse effects in wildlife. EC10 values, based on 5 d developmental rates, of the saltwater copepod *Acartia tonsa* are just slightly below the highest environmental concentrations observed (Heberer et al., 1999; Wollenberger et al., 2003) resulting in a hazard quotient of close to 1 (Table 4). However, limited research exists on effects of musks on algae and benthic invertebrates, and therefore, potential risk cannot be accurately determined. Because synthetic musks possess high octanol–water coefficients benthic invertebrates are likely exposed to high concentrations of synthetic musks in sediment and should be tested to evaluate potential toxicity of musks released in WWTP effluent. Only a handful of studies have investigated synthetic musk toxicity to sediment/soil organisms indicating there is potential risk of musk exposure to benthic invertebrates (Balk and Ford, 1999). The polychaete species *Capitella* and snail species *Potamopyrgus antipodarum* were exposed to HHCB for two weeks and 96 d, respectively (Pedersen et al., 2009; Ramskov et al., 2009). Both studies found adult organisms to be insensitive to HHCB although juveniles were much more sensitive for both species. Both studies also examined potential effects for population and both studies concluded there were no population level effects at environmentally relevant concentrations.

Up to eight additional fragrances (acetophenone, camphor, d-limonene, ethyl citrate, indole, isoborneol, isoquinolone, and skatol) have been observed in surface water; however, all fragrances except ethyl citrate have been detected in only a small number of samples (Kolpin et al., 2002; Glassmeyer et al., 2005). Ethyl citrate is a tobacco additive that has frequently been detected in surface water throughout the US (Kolpin et al., 2004; Glassmeyer et al., 2005; among others). Acute and chronic toxicity is not expected to occur with any of these compounds tested individually; however, additional research needs to be conducted.

4. Insect repellants

N,*N*-diethyl-*m*-toluamide (DEET) is the most common active ingredient in insect repellants (Costanzo et al., 2007) and is routinely detected in surface waters throughout the United States (Glassmeyer et al., 2005). DEET was developed in the 1940s and

functions by interfering with insects ability to detect lactic acid on hosts (Davis, 1985). DEET is currently registered for use in 225 products in the US and it is estimated annual usage exceeds 1.8 million kg (USEPA, 1998).

DEET has been detected in WWTP effluent (Glassmeyer et al., 2005; Sui et al., 2010) and surface water (Kolpin et al., 2002; Glassmeyer et al., 2005; Sandstrom et al., 2005; Quednow and Puttmann, 2010) worldwide. DEET is relatively persistent in the aquatic environment, but unlike many other PCPs (i.e. fragrances and UV filters) DEET has a low BCF and is likely not accumulated into aquatic organisms (Costanzo et al., 2007). DEET has been regularly detected in effluent (95% of analyzed samples) and surface water (65% of all analyzed samples) with median concentrations of approximately 0.2 $\mu g\ L^{-1}$ and 55 $ng\ L^{-1}$, respectively. The only other insect repellant detected in WWTP effluent or surface water is 1,4-dichlorobenzene. 1,4-dichlorobenzene has been detected in surface water (40% of surface water screened) receiving significant inputs of WWTP effluent throughout the US at concentrations up to 0.28 $\mu g\ L^{-1}$ (Table 1) (Glassmeyer et al., 2005).

To-date very little data exists pertaining to acute toxicity of DEET to aquatic organisms. Costanzo et al. (2007) summarizes all data published through 2006 and since its publication no additional information has been reported. Data indicates DEET is only slightly toxic to aquatic organisms (Table 2) (Michael and Grant, 1974; Office of Pesticide Programs, 2000). Although DEET is relatively resistant to breakdown and commonly found in surface water, no known studies exist that have examined chronic toxicity of DEET exposure to aquatic organisms. DEET has been observed to inhibit cholinesterase in rats (Chaney et al., 2000), and it is possible similar effects could be observed in fish even though no research has been conducted. Additionally, no studies have been conducted to examine potential endocrine effects on aquatic organisms although studies have been conducted in rats. These studies indicated DEET has no effect on sperm count, morphology, or viability in male rats after 9 week exposure (Lebowitz et al., 1983) thus indicating little potential effects in aquatic species. Based on available information, Costanzo et al. (2007) performed a preliminary risk assessment and concluded DEET is not likely to produce biological effects at environmentally relevant concentrations in aquatic ecosystems; however, due to lack of information on chronic toxicity a definitive assessment could not be made. Similar conclusions are still applicable today as chronic toxicity of DEET to aquatic organisms remains undetermined.

Similar to DEET, a preliminary risk assessment has also been conducted on the moth repellant 1,4-dichlorobenzene. Invertebrates, specifically *D. magna*, appear the most sensitive from short-term exposure (Table 2) whereas fish appear to be most sensitive to long-term exposures (Table 3) (Boutonnet et al., 2004). Based on observed environmental concentrations, it is unlikely acute or chronic effects are occurring to freshwater and marine organisms (Boutonnet et al., 2004). Additionally, there is little potential for bioaccumulation of 1,4-dichlorobenzene (Boutonnet et al., 2004) and to-date there is no indication that it can cause endocrine effects.

5. Preservatives

Parabens (alkyl-*p*-hydroxybenzoates) are antimicrobial preservatives used in cosmetics, toiletries, pharmaceuticals, and food (Daughton and Ternes, 1999). There are currently seven different types of parabens in use (benzyl, butyl, ethyl, isobutyl, isopropyl, methyl, and propyl). In 1987 over 7000 kg of parabens were used in cosmetics and toiletries alone (Soni et al., 2005) and that number has been expected to increase over the last 20 years. Methyl- and propylparaben are the most commonly used in cosmetics and are typically co-applied to increase preservative effects (Peck, 2006). To-date only a handful of studies have examined paraben concentrations in WWTP and surface water. Greatest concentrations of parabens have been identified in surface water with concentrations ranging from 15 to 400 $ng\ L^{-1}$ depending on paraben, whereas effluent had lower concentrations ranging from 50 to 85 $ng\ L^{-1}$ (Table 1) (Benijts et al., 2004; Lee et al., 2005; Gregory and Mark, 2006; Kasprzyk-Hordern et al., 2008; Jonkers et al., 2010).

Of the seven different types of parabens currently in use, benzylparaben appears to be most acutely toxic (Madsen, 2009; Terasaki et al., 2009; Bazin et al., 2010) (Table 2). Methyl- and ethylparaben appear to be least acutely toxic with LC50 values approximately 3× greater than benzylparaben for all trophic groups studied (Table 3) (Bazin et al., 2010). It has previously been reported increasing chain length of parabens' substituents can increase paraben acute toxicity to bacteria (Dymicky and Huhtanen, 1979; Eklund, 1980) and this appears to be true for other trophic groups as well. There is currently a lack of information on the chronic effects of parabens to aquatic organisms with only a single known study examining toxicity in *D. magna* and *Pimephales promelas* (Dobbins et al., 2009). These authors found benzyl- and butylparaben were most toxic to invertebrates and fish whereas methyl- and ethylparaben appeared least toxic. This corresponds directly with results of acute studies, as well as previous studies indicating increased chain length of parabens increases toxicity. In addition to increasing chain length, chlorination also substantially increases toxicity of parabens to both bacteria and *D. magna* (Terasaki et al., 2009).

Based on limited environmental concentration and toxicity data, it appears benzyl-, butyl- and propylparaben could potentially cause adverse effects to aquatic organisms. Dobbins et al. (2009) concluded parabens only pose limited hazard to aquatic organisms; however, parabens, specifically benzyl-, butyl- and propylparaben, can elicit low-level estrogenic responses. *In vitro* studies conducted with fish MCF-7 cell lines and yeast estrogenic screening assays demonstrated parabens can elicit estrogenic responses at low levels (Routledge et al., 1998; Darbre et al., 2002, 2003). Furthermore, Inui et al. (2003) and Bjerregaard et al. (2008) demonstrated parabens can cause VTG synthesis in fish when exposed to low concentrations. Therefore, low level exposure to parabens could potentially cause estrogenic effects at environmentally relevant concentrations. Additional studies have been conducted examining effects of parabens on sexual endpoints including spermatogenesis and serum testosterone in male rats (Oishi, 2002). Both butyl- and propylparaben significantly inhibited spermatogenesis, but did not affect serum testosterone (Oishi, 2002). Golden et al. (2005) reviewed paraben endocrine activity in rats and determined butyl-, isobutyl-, and benzylparaben demonstrate estrogenic activity although their potency is much less than estrogen itself (Golden et al., 2005). These results indicate there are potential affects in aquatic organisms continually exposed to parabens. Preliminary data on environmental concentrations, however, suggest only minimal risk to aquatic organisms as effect concentrations are generally 1000x higher than what has been observed in surface water.

6. UV filters

Growing concern over effects of ultraviolet (UV) radiation in humans has caused an increased usage of UV filters. UV filters are used in sunscreen products and cosmetics to protect from UV radiation and can be either organic (absorb UV radiation, e.g. methylbenzylidene camphor) or inorganic micropigments (reflect UV radiation, e.g. ZnO, TiO_2); however, in this review only organic compounds will be discussed. Typically, three to eight separate UV filters are found in sunscreens and cosmetics and can make

up greater than 10% of products by mass (Schreurs et al., 2002). There are 16 compounds that are currently certified for use as sunscreen agents (SSA's) (Reisch, 2005) and 27 certified UV filters in cosmetics, plastics, among others in the US (Fent et al., 2008). UV filters enter the environment in two ways, either indirectly via WWTP effluent or directly from sloughing off while swimming and other recreational activities. A study in Switzerland estimated the input of four commonly applied UV filters into WWTPs to be as high as 118 g of 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), 49 g of 4-methyl-benzilidine-camphor (4MBC), 69 g of benzophenone-3 (BP3), and 28 g of octocrylene (OC) per 10 000 people per day in high use times (Balmer et al. (2004)). Additionally, Poiger et al. (2004) estimated up to 1263 mg of UV filters are applied per person daily resulting in up to 966 kg of UV filters released directly into a small lake in Switzerland per year. Although UV filters are used at high levels and are likely to enter into aquatic environments, very little is known about their environmental concentrations due to a lack of analytical methods.

Balmer et al. (2004) examined presence of four UV filters (4MBC, BP3, EHMC, and OC) in wastewater effluent, surface water, and fish tissue in Switzerland. WWTP effluent had the greatest concentrations of UV filters with 4MBC being detected at the highest concentrations (2.7 $\mu g\,L^{-1}$) (Balmer et al., 2004) and was also detected at the highest concentrations in surface water (35 $ng\,L^{-1}$) and fish tissue (123 $ng\,g^{-1}$ lipid tissue). Poiger et al. (2004) found similar results in Swiss lakes with BP3 being detected at the highest concentrations (5–125 $ng\,L^{-1}$) (Table 1). Overall, 4MBC has been detected most frequently in WWTP effluent and surface water worldwide (95% and 86% of samples, respectively) whereas OC has been detected much less frequently in both (77% of WWTP effluent samples and 14% of surface water samples). The majority of additional environmental concentration data that exists pertains to bioaccumulation of UV filters in aquatic organisms. UV filters are known to bioaccumulate in fish at levels similar to PCBs and DDT (Daughton and Ternes, 1999) due to their high lipophilicity (log Kow = 3–7) and stability in the environment (Balmer et al., 2004; Poiger et al., 2004). UV filters have been found in lipid tissue in fish at concentrations up to 2 ppm (Nagtegaal et al., 1997). Additionally, UV filters were identified to have bioaccumulation factors of greater than 5000 in fish (21 $\mu g\,kg^{-1}$ in whole fish versus 0.004 $\mu g\,L^{-1}$ in water) (Hany and Nagel, 1995). In the only known laboratory study, 3-benzylidene camphor (3BC) was found to have a bioconcentration factor of 313 in *P. promelas* exposed for 21 d (Kunz et al., 2006a).

A single study indicates UV filters do not appear to be acutely toxic to aquatic organisms (Fent et al., 2009). *D. magna* were observed to be most sensitive over short-term exposures (48 h) to EHMC whereas they were least sensitive to benzophenone-4 (BP4) (Fent et al., 2009) (Table 2). The majority of studies pertaining to UV filters have focused on long-term exposures. Schmitt et al. (2008) investigated effects in benthic invertebrates and observed a significant reduction in *P. antipodarum* reproduction and increased mortality when exposed to 3BC and 4MBC for 56 d and significant decreases in reproduction and increased mortality in *Lumbriculus variegates* when exposed for 28 d to the same compounds (Table 3).

UV filters are well known to bioaccumulate and recent studies have also indicated the potential for estrogenic activity. *In vitro* assays using fish MCF-7 cell lines indicate five UV-A and UV-B sunscreens (BP3, homosalate (HMS), 4MBC, octyl-methoxycinnamate and octyl-dimethyl-PABA) have potential to cause estrogenic effects (Schlumpf et al., 2001; Kunz and Fent, 2006). Additionally, Kunz et al. (2006c) identified 10 UV filters had estrogenic effects using a recombinant yeast assay with rainbow trout ERα. Benzophenone-1 (BP1) was the most potent UV filter with 4′ hydroxybenzophenone (4HB) being the only other compound that demonstrated estrogenicity below 1 $mg\,L^{-1}$ exposure (Kunz et al., 2006c). Aquatic studies using fish (*P. promelas* and *O. mykiss*) indicate numerous UV filters have the potential to cause estrogenic effects and also adversely affect fecundity and reproduction (Table 3). 3BC appears to be the most estrogenic compound inducing VTG in *O. mykiss* and *P. promelas* after 14 d and 21 d exposure, respectively (Kunz et al., 2006a,b; Fent et al., 2008). Other UV filters (BP1 and BP2) also induce VTG in male fathead minnows but at concentrations 10-fold higher than 3BC. Oxybenzone induces VTG production in both *O. mykiss* and *O. latipes* at similar concentrations as 3BC and also significantly decreases fertilized eggs hatchability in *O. latipes* (Coronado et al., 2008). Based on a single study, amphibians do not appear as sensitive as fish as a result of 3BC exposure (Kunz et al., 2004). Recombinant yeast assays using fish hERα also indicate some UV filters also possess antiestrogenic activity (e.g. 4MBC and 3BC), androgenic activity (e.g. BP2 and HMS), and/or antiandrogenic activity (e.g. 4-hydroxy benzophenone [4HB]) (Kunz and Fent, 2006); however, no studies have investigated these effects *in vivo* in aquatic organisms. *In vivo* studies using rats have indicated 4-MBC affected the hypothalamus-pituitary–gonadal system in male rats thus altering gonadal weight and steroid hormone production. 3-BC also affected development of sex organs in male rats after 12 week exposure (Schlumpf et al., 2004). This data substantiates the *in vitro* data and indicates potential risk in aquatic species; however, the extent of risk of UV filters in WWTP effluent and surface water is currently unknown based on the scarcity of environmental concentration data. Additionally, the number of species used to identify toxic effects is minimal and therefore does not allow for a comprehensive risk profile to be developed.

7. Additional compounds

Three additional PCPs have been identified in surface water in the US by United States Geologic Survey researchers. The fixative benzophenone was detected most frequently (67.5% of samples in one study) whereas the flavorant menthol was detected at the highest concentrations (1.3 $\mu g\,L^{-1}$) (Kolpin et al., 2002; Glassmeyer et al., 2005). Benzophenone acute and chronic toxicity has been examined for *Caenorahbditis elegans* and *P. promelas* and both studies indicate benzophenone is relatively non-toxic to aquatic organisms (Marchini et al., 1992; Ura et al., 2002) (Table 2; Table 3). The other compound detected in surface water is methyl salicylate (wintergreen flavoring and liniment) although it has only been detected at low concentrations and in few environmental samples (Glassmeyer et al., 2005).

8. Hazard assessment

A preliminary hazard assessment was conducted for PCPs with sufficient chronic toxicity and environmental concentration data using hazard quotients (Table 4). A hazard quotient is the ratio of the exposure concentration divided by the toxicological benchmark concentration that can give a preliminary assessment of potential adverse effects (Suter, 2007). Hazard quotients exceeding 1 indicate potential effects. Based on published reports of toxicity and environmental concentrations, only triclosan and triclocarban have the potential to cause chronic effects based on hazard quotients greater than 1 (Table 4). Growth of a natural algal assemblage from a stream in Kansas, USA was significantly decreased after 96 h exposure to a concentration of 0.12 $\mu g\,L^{-1}$ TCS (Wilson et al., 2003) which resulted in a hazard quotient of 19. TCC also had a hazard quotient greater than 1 (10.9) for *Americamysis bahia* in response to 28 d exposure to 0.13 $\mu g\,L^{-1}$ (TCC Consortium, 2002). However, both of these hazard quotients could be consid-

ered worst-case scenarios as the environmental concentrations used were the maximum observed (approximately 20× higher than median concentrations (Table 1)) and the most sensitive species and biological endpoint was also considered (Table 3). Therefore, actual hazard is likely much lower than what is predicted here, which coincides with preliminary risk assessments conducted elsewhere (Costanzo et al., 2007; Lyndall et al., 2010).

Insufficient long-term *in vivo* data exists for UV filters and therefore hazard quotients were not capable of being calculated. Additionally, hazard quotients were not calculated for potential endocrine effects due to the dearth of *in vivo* studies on aquatic organisms; however, mammalian studies indicate UV filters are the most likely to cause endocrine effects.

9. Discussion

Based on published data, environmental concentrations and toxicity of PCPs have been largely overlooked in comparison to pharmaceutical compounds. Current published literature for environmental concentrations is fairly substantial for some PCP compounds (ex. TCS, DEET, fragrances) but relatively little is available for others (ex. TCC, UV filters, preservatives). Additional research for surface water concentrations and uptake into biota needs to be conducted in order to develop aquatic risk assessments for PCPs released in WWTP effluent. There are also substantial data gaps in published literature addressing potential toxicity of PCPs to aquatic organisms. The most overlooked trophic group is benthic invertebrates although they are likely exposed to the greatest concentrations of PCPs based on chemical properties of many PCP compounds. Additionally, toxicity of PCPs in algae and vascular plants are also not well represented in PCP literature.

Data developed thus far indicate most PCPs are relatively nontoxic to aquatic organisms at expected environmental concentrations. However, the primary concern for PCPs is their potential to cause estrogenic effects at relatively low concentrations. Preservatives and UV filters are known endocrine active compounds (Routledge et al., 1998; Schlumpf et al., 2001; Darbre et al., 2002, 2003; Kunz and Fent, 2006) with triclosan also suspected to cause endocrine effects (Foran et al., 2000; Ishibashi et al., 2004). Although numerous studies have identified effects in fish, only a handful of studies have examined these effects in non-vertebrate aquatic species (Veldhoen et al., 2006; Fort et al., 2010).

Besides potential for endocrine effects, the other major concern with PCPs is their potential to bioaccumulate in aquatic organisms. UV filters, disinfectants, and fragrances have all been shown to bioaccumulate in biota (Geyer et al., 1994; Winkler et al., 1998; Daughton and Ternes, 1999; Coogan et al., 2007), and can potentially biomagnify in higher trophic levels. To-date, no known studies have examined the potential for biomagnification and potential effects on higher trophic level organisms and subsequent effects on aquatic ecosystems.

As mentioned previously, PCPs have little potential to cause acute or chronic effects in aquatic organisms; however, most studies conducted have investigated compounds individually and not as environmentally relevant effluent mixtures. The more realistic exposure scenario would be through whole effluent testing (WET) to gain information on environmentally relevant mixtures. So far no known studies have investigated PCPs as part of whole effluent on aquatic organisms although it is likely additional effects could be observed using WET testing procedures.

Based on all data published through April 2010 a definitive risk assessment cannot be conducted to determine potential effects of PCP release into aquatic environments. Numerous preliminary risk assessments (TCS, fragrances, DEET, and parabens) have been performed yet no definitive assessment has been made due to incomplete data sets. Specifically the incomplete data include in order of importance are: (1) environmental concentrations of M-TCS, TCC, preservatives, and UV filters, (2) chronic data for DEET and preservatives, (3) endocrine effects of fragrances, (4) bioaccumulation and biomagnification of UV filters, and (5) acute data for M-TCS and UV filters. As these questions continued to be addressed, more definitive aquatic risk assessments can be developed and a better understanding of potential risk of PCP release into aquatic environments will be realized.

10. Conclusions

Personal care products are released into the environment unaltered through normal human usage. Although they can be released at levels greater than many other compounds, including pharmaceuticals, relatively little research has been conducted to identify environmental concentrations and potential toxicity. PCPs are also continually replenished through normal usage in the environment and are thus persistent compounds that warrant acute and chronic studies. Additional investigations into both acute and chronic toxicity need to be conducted in order to understand potential effects and risk of PCP release into surface water. Similar to pharmaceuticals, studies investigating PCP effects on benthic invertebrates is severely lacking. Most studies conducted to-date indicate little short- and long-term toxicity and therefore the primary issues of concern with PCPs are their ability to bioaccumulate to high levels as well as the propensity to cause estrogenic and endocrine effects. This review indicates additional research is needed to understand environmental concentrations, potential toxicity of mixtures, endocrine effects, and potential for bioaccumulation and biomagnification of PCPs in order to accurately identify potential risk of PCP release into the aquatic environment.

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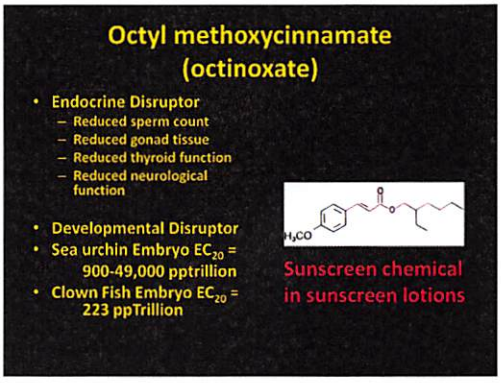
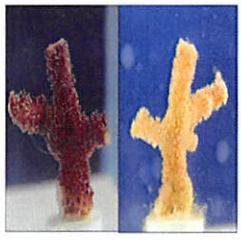
HEL data on file

Sea urchin larva 900 – 49,000 ppt
Clown fish larva 223 ppt



Coral fragment 1 ppb



New Probabilistic Risk Assessment of Ethylhexyl Methoxycinnamate: Comparing the Genotoxic Effects of *Trans*- and *Cis*-EHMC

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ABSTRACT: Ethylhexyl methoxycinnamate (EHMC) is a widely used UV filter present in a large number of personal care products (PCPs). Under normal conditions, EHMC occurs in a mixture of two isomers: *trans*-EHMC and *cis*-EHMC in a ratio of 99:1. When exposed to sunlight, the *trans* isomer is transformed to the less stable *cis* isomer and the efficiency of the UV filter is reduced. To date, the toxicological effects of the *cis*-EHMC isomer remain largely unknown. We developed a completely new method for preparing *cis*-EHMC. An EHMC technical mixture was irradiated using a UV lamp and 98% pure *cis*-EHMC was isolated from the irradiated solution using column chromatography. The genotoxic effects of the isolated *cis*-EHMC isomer and the nonirradiated *trans*-EHMC were subsequently measured using two bioassays (SOS chromotest and UmuC test). In the case of *trans*-EHMC, significant genotoxicity was observed using both bioassays at the highest concentrations (0.5 - 4 mg mL^{-1}). In the case of *cis*-EHMC, significant genotoxicity was only detected using the UmuC test at concentrations of 0.25 - 1 mg mL^{-1}. Based on these results, the NOEC was calculated for both *cis*- and *trans*-EHMC, 0.038 and 0.064 mg mL^{-1}, respectively. Risk assessment of dermal, oral and inhalation exposure to PCPs containing EHMC was carried out for a female population using probabilistic simulation and by using Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE). The risk of *cis*-EHMC was found to be ~1.7 times greater than *trans*-EHMC. In the case of *cis*-EHMC, a hazard index of 1 was exceeded in the 92nd percentile. Based on the observed differences between the isomers, EHMC application in PCPs requires detailed reassessment. Further exploration of the toxicological effects and properties of *cis*-EHMC is needed in order to correctly predict risks posed to humans and the environment. © 2016 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2016.

Keywords: ethylhexyl methoxycinnamate; *trans*/*cis* isomerisation; genotoxicity; probabilistic risk assessment; QIVIVE

INTRODUCTION

Ultraviolet (UV) filters are substances that protect our skin from harmful UV rays. They can be divided into three groups: chemical (organic) UV filters, mineral (inorganic) UV filters and insoluble organic pigments. The most widely used UV filters are organic with 2-ethylhexyl 4-methoxycinnamate (EHMC) being one of the most commonly used and

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most discussed compounds because of its chemical properties. EHMC is used not only to protect human skin from the harmful effects of UV radiation but also as a UV absorber to prevent light-induced product degradation in many personal care products (PCPs) such as lipsticks and lip balms, make-up, perfumes, facial creams, aftershaves, hand creams, face powders and hairsprays (Manová et al. 2013).

Although EHMC protects human skin from harmful UV rays, it has been associated with a number of adverse effects. EHMC is included in the European Union's database of possible endocrine disruptors as a compound with limited knowledge about its health effects and worth further investigation based on accumulating evidence (Petersen et al., 2007). A number of studies have pointed out the potential risks of EHMC in terms of disruption to the hormonal system (Schlumpf et al., 2001,2004,2008a,b; Inui et al., 2003; Ma et al., 2003; Schmutzler et al., 2004; Seidlova-Wuttke et al., 2006a,b; Gotthardt et al., 2007; Klammer et al., 2007; Szwarcfarb et al., 2008; Carbone et al., 2010). Because of its high production volume [>1000 tonnes per year in the EU (Brooke et al., 2008)] and frequent use, EHMC is found in great concentrations in water (Tsui et al., 2014) and has been detected in biota (Janjua et al., 2004, 2008; Schlumpf et al., 2008b). EHMC was also measured in human matrices including; breast milk (Schlumpf et al., 2010); urine (Markogiannaki et al., 2014); and blood (Janjua et al., 2008). In human milk samples UV filters were determined in >85% of samples at concentrations comparable to PCBs, (EHMC was 49% of samples). Because EHMC is not very persistent, and patterns correlated with use of cosmetic, and sunscreen application, its detection unlike typical contaminants tends to be from personal application (Schlumpf et al., 2010). Another effect of concern is its genotoxicity. Although many studies have shown that EHMC is not genotoxic/mutagenic with or without exposure to UV-light (SCC, 1996), the Ames test on the *Salmonella typhimurium* bacterial strain without S9 metabolic activation showed a significant increase in EHMC genotoxicity (Bonin et al., 1982). Given the structure of EHMC, it may metabolize in organisms to form 2-ethylhexanol and 2-ethylhexanoic acid, i.e. developmental toxicants (Horn et al., 2004). Toxicological information about EHMC to date is summarized in Supporting Information Section 1.

EHMC is unstable in light, commercial EHMC is in its initial *trans*-EHMC configuration that may change under UV light to *cis*-EHMC (Chatelain and Gabard, 2001; Miranda et al., 2014). The isomerisation of this compound by light is a relatively fast; the decrease of the sun protection factor (SPF) after 20 min may be more than 30% (Huong et al., 2007). Photoinstability is a key parameter for UV filters effectiveness to protect against UV rays. Because of the much lower ability of *cis*-EHMC to absorb harmful UV light, the protection against harmful effects such as skin damage, premature skin aging and skin cancer is less effective (Huong et al., 2007).

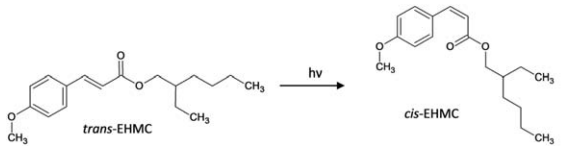

Fig. 1. EHMC photoisomerisation: *trans*-EHMC is transformed into the less stable *cis*-EHMC following UV light irradiation.

All toxicological research conducted to date has been limited to *trans*-EHMC. It is widely known that different isomeric forms can have different effects and toxicological properties in other chemicals (Roberts and Friedlos, 1987; Nishimura et al., 1994; Willett et al., 1998), toxicological information on *cis*-EHMC has to date not been explored.

The aim of this study is to evaluate the genotoxic potential of EHMC and compare the effects of *trans*-EHMC and *cis*-EHMC following irradiation. Because *cis*-EHMC is not commercially available, a designed and validated method of *cis*-EHMC preparation and isolation from a reaction mixture was developed. This study also presents the first exposure risk assessment posed to humans by *cis* and *trans* EHMC isomers found in PCPs. The probabilistic simulation, an innovative tool used in this study, using input variables can yield more realistic results when quantifying human exposure to EHMC.

MATERIALS AND METHODS

Preparation of Samples

EHMC Irradiation

As *cis*-EHMC is not commercially available, it was necessary to develop a method of irradiation by UV light and subsequent separation from the irradiated mixture. *cis*-EHMC is generated under UV light from *trans*-EHMC as shown in Figure 1. *trans*-EHMC (Sigma–Aldrich) dissolved in acetonitrile (0.004 w/w: 99.8%, Sigma–Aldrich) was irradiated using 400 W medium-pressure mercury lamp - through the 2 mm borosilicate glass filter ($\lambda > 280$ nm), manufactured by Teslamp. The solution was continuously mixed using a magnetic stirrer and solution temperature was monitored (temperature ranged from 22 to 25 °C).

The course of irradiation was checked by periodic sampling and GC-FID analysis (Agilent 7890A). A 1 uL sample

Abbreviations

EHMC	ethylhexyl methoxycinnamate
HI	hazard index
NOEC	no observed effect concentration
PCP(s)	personal care products
QIVIVE	*in vitro* to *in vivo* extrapolation
UV	ultraviolet

was injected in spilt mode with an 8:1 ratio at an inlet temperature of 250 °C and a column flow of 22 cm s^{-1}. Separation was performed on a 60m DB-5MS column (0.25 mm, SF 0.25 μm, Agilent Technologies), determination of concentration assumed intensity of *cis*/*trans* isomers to be identical.

The photostationary state in which rate of *cis* to *trans* isomerisation equals rate of reversed reaction and the yield of *cis* isomer is maximal was achieved at the ratio of 46.6: 54.4, *cis*:*trans*. The reaction mixture was evaporated using vacuum evaporator (Heidolph Laborota 4000).

Column Chromatography

Column chromatography was used to isolate *cis*-EHMC from the reaction mixture. The height of the column was 25 cm and the inner column diameter was 5 cm. The column was filled by stationary phase of 150 g silica gel and with the mobile phase DCM:hexane (2:1). The sample (1.6 mL) dissolved in ~3 mL of mobile phase (DCM:hexane, 2:1) and then dripped in a continuous layer onto stationary phase. The mobile phase introduced carefully, so that the stationary phase was not disturbed. Then 350 mL of mobile phase was added to fill the column. A sequential gradient 2:1 (350 mL), 3:1 (250 mL), 5:1 (250 mL), DCM:hexane was used for chromatography.

The 15 mL fractions were collected and analyzed by GC-FID using the parameters noted earlier. The fractions with the greater proportion of *cis* isomer were combined. Because of this method a *cis*-EHMC sample with a purity of >98% was obtained. Confirmation of *cis*-EHMC was done by GC-MS (Hawlett-Packard GCMS 5890/5971). A 1 μL injection in split mode (30:1) with an inlet temperature of 250 °C and the same flow and column as used in FID. Initial oven temperature was 100 °C held for 2 min then temperature ramp 20 °C min^{-1} to 250 °C, holding for 20 min. The mixture was then evaporated by vacuum evaporator (Heidolph Laborota 4000) to ~3 mL and then concentrated under N_2. Remaining solvent residues were then exposed to the low pressure (<133 Pa) for 2 h to evaporate the solvent residues. The *cis*-EHMC was weighed and redissolved in DMSO to 800 mg mL^{-1}. *trans*-EHMC was diluted with DMSO to the same concentration of 800 mg mL^{-1}. Both samples were then tested for genotoxic potential using the SOS chromotest and the UmuC test. This concentration was used to simulate realistic human exposure to EHMC by one application of a PCP containing EHMC.

Genotoxicity Assays

SOS Chromotest

The SOS chromotest was performed without metabolic activation using a genetically modified *E. coli* strain PQ37 (Quillardet et al., 1982; Bartoš et al., 2005). After 2 h of incubation with samples, the activity of β-galactosidase (reporter enzyme for genotoxicity induction along with the DNA repair system) was measured using chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and the activity of alkaline phosphatase (marker of cytotoxicity) was measured using *p*-nitrophenyl phosphate (PNPP) chromogenic substrate. Cytotoxic effect was quantified as a percentage of inhibition of the alkaline phosphatase in comparison with negative control and concentrations causing more than 50% inhibition were excluded from genotoxicity evaluations. Prepared samples of *cis* and *trans* EHMC were diluted to an initial concentration 400 mg mL^{-1} for the SOS chromotest. The eight dilution series 1:1 was prepared in DMSO and 10 μL of samples were used in the test; every concentration was tested triplicate. The standard mutagen 4-nitroquinoline 1-oxide (4-NQO), $c = 0.469$ μg mL^{-1}, was used as a positive control. The activity of β-galactosidase of each concentration was compared with a negative control and statistically evaluated using Dunnett's one-way ANOVA test ($p = 0.05$). The SOS induction factor was then calculated for a range of concentrations (0.02 - 4.00 mg mL^{-1}).

UmuC Test

The UmuC test was performed without metabolic activation using a genetically modified bacterial *Salmonella typhimurium* strain TA 1535 with an incorporated plasmid pSK1002 (Oda et al., 1985). After 4 h of incubation with samples, the activity of β-galactosidase (reporter enzyme for genotoxicity induction along with the DNA repair system) was measured using a chromogenic substrate chlorophenol red-β-galactopyranoside (CPRG). EHMC (5 μL) with a concentration of 400 mg mL^{-1} in DMSO was prepared in eight dilution series 1:1 in DMSO. Each concentration was tested in triplicate. The standard mutagen 4-NQO was used as a positive control (PK1: $c = 0.2345$ μg mL^{-1} and PK2: $c = 0.117$ μg mL^{-1}). None of the tested compounds showed $\Delta D < 0$, the growth factor was > 0.5. The activity of β-galactosidase of each concentration was compared with a negative control and statistically evaluated using Dunnett's one-way ANOVA test ($p = 0.05$).

Risk Assessment

A description of the method of EHMC probabilistic exposure and risk assessment focusing on the female population is included below. Females were focused on as they typically have a greater risk of exposure through PCPs.

Model Background

Chronic Daily Intake. The United States Environmental Protection Agency (US EPA) exposure model was used for chronic daily intake via the dermal, oral and inhalation routes, CDI_{dermal} (Eq. (1)), CDI_{oral} (Eq. (2)), CDI_{inh} (Eq. (3)), respectively (US EPA 1992).

$$CDI_{dermal} = (PI \times f_{perm} \times CA \times SA \times FQ)/BW \quad (1)$$

Where CDI_{dermal} is chronic daily dermal intake [mg kg^{-1} bw day^{-1}], PI is product ingredient–fraction of EHMC in PCP [-], f_{perm} is the UV filter permeation factor through human skin [-], CA is the amount of the applied PCP on the skin [mg cm^{-2}], SA is the area of skin that can be treated [cm^2], FQ is the frequency of applications per day [day^{-1}], BW is body weight [kg].

$$CDI_{oral} = (FI \times CA_{app} \times FQ)/BW \quad (2)$$

Where CDI_{oral} is chronic daily oral intake [mg kg^{-1} bw day^{-1}], FI is fraction ingested from the PCP [-], CA_{app} is the amount of PCP per application [mg], FQ is the frequency of applications per day [day^{-1}], BW is body weight [kg].

$$CDI_{inh} = (PI \times CA_{app} \times IR \times FQ \times ET \times ED)/(AT \times V_{room} \times BW) \quad (3)$$

Where CDI_{inh} is chronic daily inhalation intake [mg kg^{-1} bw day^{-1}], PI is product ingredient–fraction of EHMC in PCP [-], CA_{app} is the amount of PCP per application [mg], RI is inhalation rate [m^3 hr^{-1}], FQ is the frequency of applications per day [day^{-1}], ET is exposure time [hr], BW is body weight [kg], and V is room volume [m^3].

Reference Dose. The reference dose (RfD, Eq. (6)) for both EHMC isomers was determined using QIVIVE approach using suitable dosimetric adjustment factors (DAFs) and *in vitro* no observed effect concentration ($NOEC_{in\ vitro}$) [mg mL^{-1}] (Eqs. (5) and (6)), calculated from our experimental data of the genotoxicity assay UmuC test using the range between statistically significant concentration and no effect concentration.

The dosimetric adjustment factors DAFs (Eq. (4)) were derived as:

$$DAFs = (24 \times V_{female})/(BW \times 4) \quad (4)$$

As the DAFs (Eq. (4)) were used the exposure time extrapolation, a volume inter-species extrapolation [thus the volume of female body obtained from direct physical measurement (Sendroy and Collison, 1966; Marieb and Hoehn 2015)].

$$NOAEL_{in\ vivo} = NOEC_{in\ vitro} \times DAFs \quad (5)$$

In vivo No observed adverse effect level ($NOAEL_{in\ vivo}$) [mg kg^{-1} bw day^{-1}] (Eq. (5)) describes no observed adverse effect for humans, V_{female} [mL] is female body volume and BW [kg] is the body weight of a female.

$$RfD = NOAEL_{in\ vivo}/(UF_H \times UF_S \times UF_A) \quad (6)$$

The composite uncertainty factors used in Eq. (6) were derived as follows:

- an intraspecies UF (UF_H) of 10 was applied to account for human variability in susceptibility;
- a sub-chronic to chronic UF (UF_S) of 10 was applied;
- a UF_A of 3 was applied to account for toxicodynamic differences between *in vitro* cell-based assays and humans according to Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) (Campbell et al., 2015). This value was adopted because a dosimetric adjustment from *in vitro* to *in vivo* has already been incorporated (DAF index) (US EPA, 2006).

Hazard Index. The hazard index (HI) calculation is described in Eq. (7).

$$HI = CDI/RfD \quad (7)$$

When the $HI > 1$, the risk is significant and the systemic effects are assumed to be of concern.

Model Inputs

Product ingredient (PI) was obtained from (Manová et al. 2013), where 116 PCPs were selected on the basis of a product-use questionnaire, categorized into seven groups and analyzed. EHMC was present in 59 of the 116 (51%) tested products. The lognormal distribution was defined for minimum, mean and maximum measured value for the given PCP category. For other categories (perfume, eau de toilette spray, face powder, night cream and hairspray) which were not included by Manová et al. (2013), the normal distribution was used in range of zero and 10%, the maximum authorized concentration (EU, 2009).

Permeation factor (f_{perm}) i.e. the penetrated amount of EHMC containing PCPs through human and pig skin was used in range from 0.004 to 5.7% according to *in vitro* studies using method of static Franz diffusion cells (Treffel and Gabard, 1996; Potard et al., 1999; Benech-Kieffer et al., 2000; Potard et al., 2000; Chatelain et al., 2003; Jimenez et al., 2004; Klinubol et al., 2008; Durand et al., 2009). In these studies, varying amounts ranging from 2 to 8 mg cm^{-2} were applied on the skin. The recommended amount of sunscreen is 2 mg cm^{-2} to ensure safe protection of the declared SPF. It is necessary to note that exposure may be greater because EHMC is contained in many other PCPs (Loretz et al., 2005, 2006). Another uncertainty may be the usage of different receptor fluid containing phosphate buffer saline with albumin, distilled water, gentamicin sulfate, polyethylene glycol 20, etc. in different concentrations that may affect permeability. Likewise the applied amount, formulation, thickness and exposure area of the skin may involve a number of uncertainties and thus differences in outcome.

The PCP amount applied on the skin (CA) [mg cm^{-2}] according to questionnaire studies by Loretz et al. (2005, 2006) and Biesterbos et al. (2013) was used.

TABLE I. Human exposure parameters and inputs, probability distribution types

Parameter	Symbol	Unit	Distribution	Reference
Product ingredient	PI	–	Lognormal/ normal	Manová et al. (2013)
Amount of PCP applied on the skin	CA	mg cm^{-2}	Normal	Biesterbos et al. (2013), Loretz et al. (2005, 2006)
Exposed area of skin	SA	cm^2	Normal/fixed value	US EPA (2011), Ferrario et al. (2000), Bremmer (2006)
PCP application frequency	FQ	day^{-1}	Normal/fixed value	Loretz et al. (2005, 2006), NEGh (2012)
PCP amount per application	CA_{app}	mg	Normal	Biesterbos et al. (2013), Loretz et al. (2005, 2006)
Room volume	Vroom	m^3	Fixed value	Bremmer (2006)
Inhalation rate	IR	$m^3\ hr^{-1}$	Normal	US EPA (2011)
Exposure time	ET	Hr	Fixed value	Bremmer (2006)
Body weight	BW	kg	Normal	US EPA (2011)
Permeation factor	f_{perm}	–	Normal	Benech-Kieffer et al. (2000), Chatelain et al. (2003), Durand et al. (2009), Jimenez et al. (2004), Klinubol et al. (2008), Potard et al. (2000), Potard et al. (1999), Treffel and Gabard (1996)

The used values of the parameters are available in Supporting Information Tables SII, SIII.

The distribution of the mean, 5th and 95th percentile of surface area (SA) of adult females (21 years and older) was obtained from the Exposure Factors Handbook (US EPA, 2011). One half of the head surface area was used for face cream, make-up foundation, face powder, night cream and hairspray application, the hand area was used for hand cream application and the entire body area was used for sunscreen application. The fixed value was used for lip balm and lipstick (Ferrario et al., 2000). In the case of eau de toilette it is assumed that 85% of the product is actually applied to the skin and the value for the exposed body surface was thus set at $8 \times 25\ cm^2 = 200\ cm^2$. The body surface area for perfume application is smaller ($4 \times 25\ cm^2 = 100\ cm^2$) (Bremmer, 2006).

The PCP application frequency (FQ) was obtained from Loretz et al. (2005, 2006), a study which determined the distribution of product usage by consumers for commonly used cosmetic products: lipstick, face cream, make-up foundation, perfume and hairspray. Normal distribution using mean and standard deviation was used for the probabilistic model of risk assessment. The FQ for other PCPs was taken from Existing Default Values and Recommendations for Exposure Assessment (NEGh, 2012).

Amount per application (CA_{app}) using normal distribution (mean, max) was obtained from Biesterbos et al. (2013) and Loretz et al. (2005, 2006).

Room volume (V_{room}) is the volume of the bathroom where the PCPs are applied (Bremmer, 2006).

The mean and 95th percentile was used for inhalation rate (IR) obtained from exposure factors handbook (US EPA, 2011) for free-living normal-weight females (16 years and older).

Exposure time, used in CDI inhalation calculation (ET) was obtained from the Cosmetic Fact Sheet (Bremmer, 2006).

Body weight (BW) was obtained from the Exposure Factors Handbook (US EPA, 2011) as normal distribution using the 5th and 95th percentiles of body weight for females (16 years and older).

Female body volume (V_{female}) was obtained from direct physical measurements in four independent measurements of four population studies (Sendroy and Collison, 1966).

All inputs and parameters are summarized in Table I. All parameters used in the probabilistic simulation are summarized in Supporting Information Tables SII and SIII.

RESULTS

Genotoxicity Assays

Significant genotoxicity ($p = 0.05$) was found for *trans*-EHMC in both SOS chromotest (Table II, Fig. 2) and UmuC test (Table III, Fig. 3) at the greatest concentrations (0.5 - 4 mg mL^{-1}). In the case of *cis*-EHMC, significant genotoxicity ($p = 0.05$) was detected using the UmuC test method at concentrations of 0.25 - 1 mg mL^{-1}.

TABLE II. SOS chromotest results for *trans*-EHMC and *cis*-EHMC obtained by using ONPG chromogenic substrate

SOS Chromotest, *trans*-EHMC			SOS Chromotest, *cis*-EHMC		
c (mg mL^{-1})	IF	SD	*c* (mg mL^{-1})	IF	SD
0.02	0.93	0.08	0.03	0.71	0.10
0.03	0.90	0.20	0.06	1.04	0.07
0.06	1.01	0.14	0.13	0.91	0.06
0.13	1.21	0.14	0.25	0.92	0.11
0.50	**1.36**	0.12	0.50	0.76	0.02
1.00	**1.37**	0.04	1.00	0.90	0.19
2.00	**1.90**	0.12	2.00	0.68	0.10
4.00	**2.53**	0.18	4.00	0.75	0.06

Underlined IF is statistically significant ($p = 0.05$).

EHMC Risk Assessment

NOAEL Calculation

According to experimental data obtained using the UmuC assay, the statistical level of significant genotoxic response and the $NOEC_{in\ vitro}$ was calculated for both isomers, as 0.038 and 0.064 mg mL^{-1}, for *cis* and *trans*, respectively. $NOAEL_{in\ vivo}$ was extrapolated using DAFs for both EHMC isomers, as 194.64 and 331.34 mg kg^{-1} bw day^{-1}, for *cis* and *trans* respectively. The extrapolation includes a number of uncertainties (described in the Discussion) because the data for calculation were measured from the sub-acute screening test. The NOAEL was used predominantly to predict the risk and to include both *cis* and *trans* isomers because they may have different adverse effects.

RfD Calculation

The RfD was calculated from the $NOAEL_{in\ vivo}$ using UFs 300. $RfD_{cis} = 0.649$ mg kg^{-1} bw day^{-1} and $RfD_{trans} = 1.105$ mg kg^{-1} bw day^{-1}.

CDI Calculation

CDI corresponding to each exposure route and to each PCP was calculated using probabilistic Monte Carlo simulation. Chronic daily intake of EHMC is shown in Table IV (the relevant graphs are available in Supporting Information Section 3). Median CDI was 0.1745, 0.0311, and 0.0158 mg kg^{-1} bw day^{-1} for dermal, oral, and inhalation routes, respectively. The route of exposure was found to be in order of importance skin < oral < inhalation. This is not surprising as most PCPs are applied on the skin. Based on usage, the Personal Care Products Council (PCPC) estimated an average of 24 to 87 mg per day in the 95th percentile of lipstick may be ingested assuming 100% of applied material is ingested (Loretz et al., 2005). The inhalation route was found to contribute the least.

Using the calculated data for dermal, oral and inhalation CDI, we modelled predictable hazard risks posed by the cumulative exposure of the different PCPs. According to the different RfD of *cis*- and *trans*-EHMC, the risks posed by *cis*-EHMC exposure were much greater. Median of $sumHI_{cis}$ was 0.3804. In the 92nd percentile the HI = 1 was exceeded and in the 95th percentile $sumHI_{cis}$ was 1.1754 (Table V, Fig. 4). On the other hand, the hazard index of *trans*-EHMC (median of $sumHI_{trans} = 0.2258$) exceeded the threshold HI = 1 in the 98th percentile (Table V, Fig. 4).

The screening probabilistic risk assessment is only able to predict possible hazards and to highlight deficiencies in the exposure evaluation of EHMC containing PCPs and its *cis* and *trans* isomers, which may lead to risks that are not yet sufficiently explored.

DISCUSSION

Genotoxic Response: *cis*- and *trans*-EHMC

The genotoxic effects were measured using two methods: SOS chromotest and UmuC test. Although the assays are

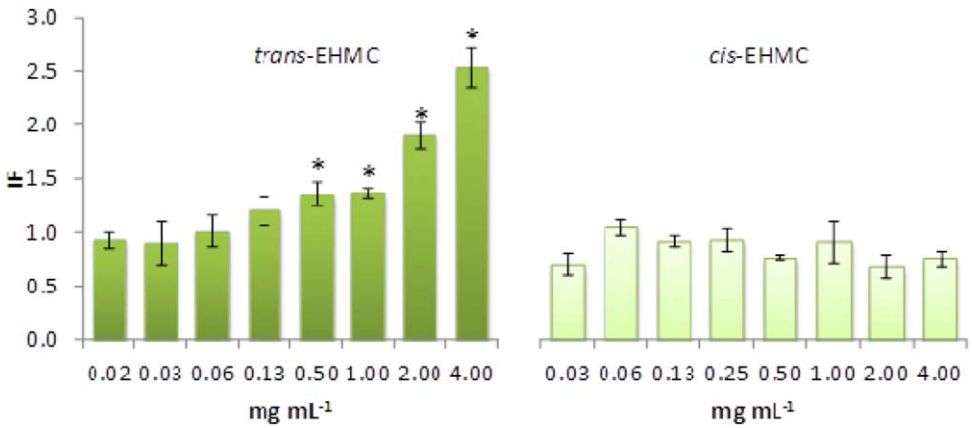

Fig. 2. Genotoxicity bioassay—SOS chromotest: Induction factor (IF) of *trans*/*cis*-EHMC depending on concentration (mg mL^{-1}). Mean values with standard deviations; $n = 3$ for *trans*/*cis*-EHMC. * statistically significant ($p = 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III. The results of the UmuC test of *trans*-EHMC and *cis*-EHMC using CPRG chromogenic substrate

UmuC Test, *trans*-EHMC			UmuC Test, *cis*-EHMC		
c (mg mL^{-1})	IF	SD	c (mg mL^{-1})	IF	SD
0.03	0.95	0.04	0.01	0.83	0.11
0.06	1.00	0.00	0.02	0.87	0.10
0.13	1.08	0.06	0.03	0.89	0.04
0.25	1.07	0.09	0.06	**1.42**	0.05
0.50	**1.24**	0.05	0.13	**1.39**	0.01
1.00	**1.24**	0.06	0.25	**1.40**	0.07
2.00	**1.38**	0.05	0.50	**1.46**	0.03
4.00	**1.25**	0.04	1.00	**1.20**	0.06

Underlined IF is statistically significant ($p = 0.05$).

very similar, different organisms, i.e. *E. coli* and *S. typhimurium*, were used. Different sensitivity responses of the two tests could be caused by the different defence mechanisms of bacterial cells and also by the different transmission of cell membranes of the tested bacterial strains (Van Dyk et al., 1994; Fralick, 1996; Ptitsyn et al., 1997) as well as by tolC mutation (Koronakis, 2003). With respect to our results, we can say that the UmuC test showed a greater sensitivity response to *cis*-EHMC than the SOS chromotest. The higher sensitivity of the UmuC test for selected groups of chemicals was confirmed in a comparative study of three bacterial genotoxicity tests for detecting DNA damage to SOS repair systems (Flegrova et al., 2007).

As a consequence of significant differences in the genotoxic response of *cis* and *trans* isomers (Figs. 2 and 3), it could be assumed that *cis*-EHMC also probably differs in other toxicological effects and properties. Because there is a range of EHMC toxicological effects and because from the findings of this study, a far more detailed evaluation of the toxicological effects of both *cis* and *trans* EHMC is needed (Bonin et al., 1982; Schlumpf et al., 2001, 2004; Inui et al., 2003; Ma et al., 2003; Seidlova et al., 2006b; Carbone et al., 2010; Szwarcfarb et al., 2008; Axelstad et al., 2011).

Risk Assessment

Following the experimental genotoxic effects of *cis*- and *trans*-EHMC, a new approach to probabilistic risk assessment showed significantly greater risks in *cis*-EHMC exposure, ~1.7 times greater than *trans*-EHMC exposure. The RfD, used for probabilistic risk calculation, was determined from $NOEC_{in\ vitro}$ obtained from the genotoxicity UmuC assay. Although RfD is a toxicity value that applies to humans, *in vitro* cell assays-to-human screening extrapolation is required. Ideally, this extrapolation can be accomplished with a chemical-specific physiologically based pharmacokinetic model. In the absence of such a model, dosimetric adjustment factors (DAFs) can be applied (US EPA, 2006).

Probabilistic risk assessment was performed using the most accurate data from questionnaire-based studies though these still leave uncertainties. Parameters CA and FQ obtained from Loretz et al. (2006, 2005) were made in the United States and PCPs consumption habits can differ in comparison with European Union population. Also, the experimental data used for dermal penetration are provided in diverse conditions and pig skin is used instead of human skin in some studies. Moreover, *cis*-EHMC penetration through human skin is unknown and the possible differences in permeation speed and the amount of penetrated *cis*-EHMC in comparison with *trans*-EHMC are crucial (unpublished data, Necasova et al.—manuscript in preparation).

A study by Andersen et al. (2012) that included EHMC, focuses on female exposure to possible endocrine disruptors in consumer products and determines the greatest risk characterisation ratios (RCR) which describe the ratio between exposure and "safe dose level" with regard to the endocrine disrupting effect of the substance. The greater the RCR, the more risk. If the total RCR exceeds 1, the exposure scenario

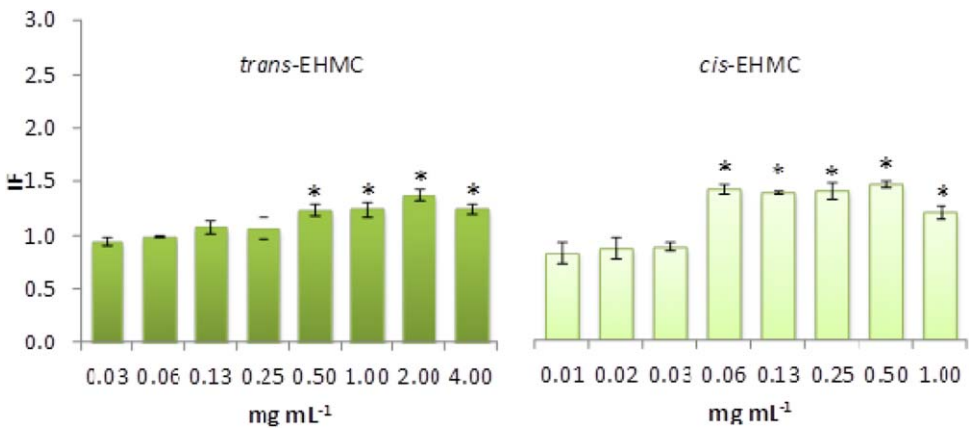

Fig. 3. Genotoxicity bioassay—UmuC test: Induction factor (IF) of *trans*/*cis*-EHMC depending on concentration (mg mL^{-1}). Mean values with standard deviations; $n = 3$ for *trans*/*cis*-EHMC. * statistically significant ($p = 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE IV. EHMC chronic daily intake, CDI (mg kg^{-1} bw day^{-1}), via dermal, oral and inhalation route

Percentile	CDI Dermal Route (mg kg^{-1} bw day^{-1})	CDI Oral Route (mg kg^{-1} bw day^{-1})	CDI Inhalation Route (mg kg^{-1} bw day^{-1})
P5	0.0008	0.0163	0.0055
P15	0.0611	0.0207	0.0084
P25	0.0962	0.0237	0.0105
P35	0.1260	0.0266	0.0125
P45	0.1574	0.0295	0.0146
P55	0.1918	0.0329	0.0171
P65	0.2331	0.0373	0.0201
P75	0.2900	0.0438	0.0240
P85	0.3825	0.0550	0.0306
P95	0.6049	0.0905	0.0460
Median	0.1745	0.0311	0.0158
Mean	0.2235	0.0404	0.0198

Probabilistic risk assessment results using Monte Carlo simulation.

is considered to pose a risk. In comparison to our assessment approach, their study was less detailed. Their risk assessment is based only on a deterministic method and describes only the realistic medium scenario (medium exposure) and the realistic worst case scenario (maximum exposure). In our study, we used a probabilistic approach including more variables in individual parameters for appropriate probabilistic trials (10,000) which adjusts for greater variability in the sample group as well as variability in parameters human individuals and their lifestyle habits. Our approach also included a greater range of EHMC containing products and the *cis*-EHMC.

Manová et al. (2015) produced a human risk assessment of EHMC contained in PCPs, using a probabilistic approach similar to our own study to predict aggregate exposure distributions for EHMC and includes a wider range of PCPs than Andersen et al (2012). The study by Manová et al. (2015) predicts the estrogenic and thyroid-disrupting risk of EHMC using probabilistic simulation. However, it focuses only on dermal exposure, whereas our study also includes oral and inhalation exposure pathways. In comparison with Manová et al. (2015), our improved approach uses selective risk assessment treats *trans*-EHMC and *cis*-EHMC individually. Our study however focuses only on females due to their typical greater exposure to PCPs over longer periods in comparison with men or children (Environmental Working Group, 2004; Markogiannaki et al., 2014). Another reason to choose a female-only study was the additional hazard to offspring that may occur during pregnancy and breast feeding. As EHMC is suspected of having genotoxic effects the exposure of pregnant woman could cause morphologic damages, mutations and malformations in the foetus and young children.

Our study offers a new perspective on EHMC exposure risk assessment in comparison with the afore mentioned studies (Andersen et al., 2012; Manová et al., 2015). We also included sprayable PCPs along with the dermal and oral exposure. Sprayable PCPs include perfumes, and hairspray, and examination of inhalation exposure as an additional exposure pathway was conducted. This is especially of interest in occupational exposure for example hairdressers who may be exposed to high concentrations of hairspray for a long period of time in a small room (Ronda et al., 2009).

The most significant contribution for scientific research and EHMC safety evaluation is the exposure risk comparison of *cis* and *trans* isomers we may be exposed to on a daily

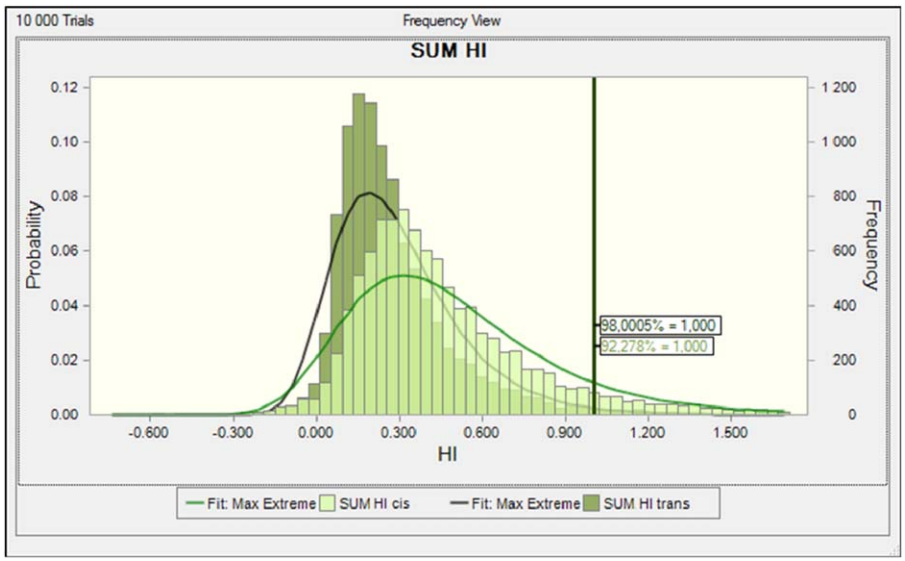

Fig. 4. *trans*-EHMC (dark green) and *cis*-EHMC (light green) hazard index. Sum of all exposure route. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE V. *cis*- and *trans*-EHMC hazard index (HI) via dermal, oral, and inhalation route

	HI Dermal Route		HI Oral Route		HI Inhalation Route		**SUM HI**	
Percentile	*cis*	*trans*	*cis*	*trans*	*cis*	*trans*	***cis***	***trans***
P5	0.0000	0.0000	0.0000	0.0000	0.0088	0.0050	**0.0794**	**0.0460**
P15	0.0387	0.0162	0.0139	0.0071	0.0130	0.0075	**0.1749**	**0.1016**
P25	0.1283	0.0730	0.0297	0.0171	0.0165	0.0097	**0.2368**	**0.1380**
P35	0.2077	0.1234	0.0415	0.0246	0.0200	0.0118	**0.2936**	**0.1718**
P45	0.2859	0.1730	0.0519	0.0311	0.0238	0.0141	**0.3481**	**0.2074**
P55	0.3686	0.2254	0.0618	0.0373	0.0283	0.0169	**0.4122**	**0.2473**
P65	0.4622	0.2848	0.0722	0.0439	0.0337	0.0202	**0.4899**	**0.2922**
P75	0.5775	0.3579	0.0841	0.0514	0.0410	0.0249	**0.5951**	**0.3580**
P85	0.7407	0.4614	0.0998	0.0613	0.0526	0.0322	**0.7587**	**0.4622**
P95	1.0701	0.6703	0.1204	0.0802	0.0799	0.0499	**1.1754**	**0.7233**
median	0.3263	0.1986	0.0569	0.0342	0.0260	0.0154	**0.3804**	**0.2258**
mean	0.3865	0.2368	0.0569	0.0342	0.0329	0.0199	**0.4761**	**0.2878**

Sum of HI for *cis*-EHMC and *trans*-EHMC. Probabilistic risk assessment using the Monte Carlo simulation. Underlined HI (HI > 1), the risk is significant and the systemic effects are assumed to be of concern.

basis. This evaluation of both isomers was based on predicted genotoxic risk in this study. It is important to include the toxicological evaluation of both isomers, and to complete toxicological profile for *cis*-EHMC. Data obtained from our study would be helpful in predicting real risks posed to females according to the different effects of *cis*- and *trans*-EHMC. In further studies the revaluation of EHMC and both its isomers and complex human risk assessment according to all tender and age categories must be performed.

Exposure to EHMC can be great and frequent (Schlumpf et al., 2010). Although EHMC is not a highly persistent compound (half-life of 10 h at 35 °C in human plasma (Thiel, 2008)), in comparison to persistent lipophilic POP chemicals found in breast milk, the EHMC levels were at comparable concentrations to PCBs. The results strongly correlated with a detailed cohort study questionnaire that indicated the large use frequency of PCPs containing EHMC (Schlumpf et al., 2010). Because of the repeated application of cosmetics, an almost continuous uptake of EHMC may be estimated.

Because of the great exposure to EHMC as well as its persistency, a study of EHMC metabolism is needed. Given the structure of EHMC, there is a possibility that its metabolism could lead to the formation of 2-ethylhexanol and 2-ethylhexanoic acid), both of which are developmental toxicants (NTP, 2008). These EHMC metabolites were identified in rat plasma after dosing with EHMC (Mathews et al., 2012). Maternal and developmental NOAEL was found to be 130 mg kg^{-1} day^{-1} in Wistar rats orally exposed to 2-ethylhexanol (Hellwig and Jackh, 1997), which is much less than our determined genotoxicity NOAEL for *cis*-EHMC (194.64 mg kg^{-1} day^{-1}) and *trans*-EHMC (331.34 mg kg^{-1} day^{-1}). Thus the risk exposure of 2-ethylhexanol may be much greater than in the case of *trans*/*cis*-EHMC exposure. Based on these studies, the possible metabolism of EHMC, its effects, toxicokinetic and toxicodynamic may also need to include the metabolites in estimations of the health hazard.

The quantum yield of isomerisation from *trans*-EHMC to *cis*-EHMC due to sun exposure would allow estimates of the rate of transformation. It was found that after 10 min of *trans*-EHMC irradiation in Hept; Diox; AcOEt; THF; ACN; iPro; and Water, the percentage of *cis* to *trans* EHMC was 47.4, 59.0, 57.1, 60.0, 68.1, 66.6, and 16.3%, respectively (Huong et al., 2007). Both the rate of *cis*/*trans* isomerisation and the ratio of the isomers when photostationary state is attained depend mainly on quantum yield of the reaction in either direction, intensity of light and overlap of spectrum of the incident radiation and absorption spectrum of *cis* and *trans* isomers. Photochemical isomerisation is not included in our model, since essential experimental data on photochemical behaviour of EHMC is not currently available. The ability of EHMC to efficiently absorb light and the quantum yield of *trans* to *cis* isomerisation reported to be ~0.2 for 2-naphtyl acrylic acids in homogeneous solution would indicate that rapid formation of *cis*-EHMC can be expected upon exposure to UV-light. Knowledge of quantum yield of isomerisation in matrices that are more relevant to formulations in which EHMC would be needed to refine the risk assessment of EHMC in different exposure scenarios.

CONCLUSION

With due data gaps regarding *cis*-EHMC, this study evaluated the genotoxic effects of EHMC and compared the genotoxic response of the *cis* and *trans* isomers. The genotoxicity of *cis*-EHMC was found to be significantly greater than that of *trans*-EHMC. Furthermore, the results of probabilistic exposure risk assessment revealed that in the case of *cis*-EHMC the HI = 1 value was exceeded in

the 92nd percentile. Because our data indicate major differences between the isomers in genotoxicity and associated risks, it is likely that the toxicokinetics and toxicodynamics of *cis*- and *trans*-EHMC could also differ. Differences in the behaviour, properties and toxicological effects of *trans* and *cis* isomers require further evaluation and reassessment. The new approach to toxicological study and probabilistic risk assessment presented here represent a more detailed approach in the future evaluation of EHMC, its photoproducts and metabolites and its use in personal care products, and may be used in the assessment of other similar compounds.

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In vitro effects of benzophenone-2 and octyl-methoxycinnamate on the production of interferon-gamma and interleukin-10 by murine splenocytes.

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Abstract

Chemical ultraviolet light absorbers (UV-filters) are nowadays widely used in cosmetic and plastic industry. Recent in vitro and in vivo studies have reported that certain chemical UV-filters possess estrogenic activity raising the question of whether these compounds are safe to human health. Work on estrogenic effects of these compounds, however, has focused mostly on reproductive organs, and as the presence of estrogen receptors has been identified in several cells of the immune system, UV screens also may have a great impact on immunity. Thus, we have studied the in vitro effects of two widely used UV-filters--benzophenone-2 (BP-2) and octyl-methoxycinnamate (OMC)--on the production of interferon (IFN)-gamma and interleukin (IL)-10, two cytokines representing Th1- and Th2-type response, respectively, by activated murine splenocytes. Cells were cultured on 48-well plastic plates and stimulated with 12-miristate 13-acetate (PMA) (5 ng/ml) and ionomycin (50 ng/ml) in the presence of different concentrations (10-5-10-8M) of the studied substances or 17beta-estradiol (E2). After 48 hr incubation the supernatants were collected and the levels of IFN-gamma and IL-10 were measured using immunoenzymatic assay. Our results show that BP-2 and OMC at high concentrations (10-5M) shifted the Th1/Th2 balance toward a Th2 response (lower IFN-gamma production and higher IL-10). These effects were comparable to those of E2. Our results clearly show that UV-screens at high doses also may possess immunomodulatory effects some of which resemble those of E2.

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Octyl Methoxycinnamate Modulates Gene Expression and Prevents Cyclobutane Pyrimidine Dimer Formation but not Oxidative DNA Damage in UV-Exposed Human Cell Lines

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Octyl methoxycinnamate (OMC) is one of the most widely used sunscreen ingredients. To analyze biological effects of OMC, an *in vitro* approach was used implying ultraviolet (UV) exposure of two human cell lines, a primary skin fibroblast (GM00498) and a breast cancer (MCF-7) cell lines. End points include cell viability assessment, assay of cyclobutane pyrimidine dimers (CPDs) and oxidated DNA lesions using alkaline elution and lesion-specific enzymes, and gene expression analysis of a panel of 17 DNA damage–responsive genes. We observed that OMC provided protection against CPDs, and the degree of protection correlated with the OMC-mediated reduction in UV dose. No such protection was found with respect to oxidative DNA lesions. Upon UV exposure in the presence of OMC, the gene expression studies showed significant differential changes in some of the genes studied and the expression of p53 protein was also changed. For some genes, the change in expression seemed to be delayed in time by OMC. The experimental approach applied in this study, using a panel of 17 genes in an *in vitro* cellular system together with genotoxicity assays, may be useful in the initial screening of active ingredients in sunscreens.

Key Words: **octyl methoxycinnamate; cyclobutane pyrimidine dimers; oxidative DNA lesions; UV; sunscreens; gene expression.**

Ultraviolet (UV) radiation from sunlight is considered one of the most important environmental factors affecting humans and has been implicated as the main cause for skin cancer (Ananthaswamy *et al*., 1997). It is generally thought that ultraviolet B (UVB) (280–320 nm) and to a lesser extent ultraviolet A (UVA) (320–400 nm) are responsible for the sunlight-induced cancers (Armstrong and Kricker, 2001; Cole *et al*., 1986). The UVA contribution to melanoma skin cancer has been discussed (Mitchell *et al*., 2007). The cellular effects of UV irradiation include DNA damage, cell cycle arrest, immunological depression, apoptosis, and transcriptional changes. UVB irradiation directly causes bulky DNA adducts such as cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (6-4PPs), which are usually repaired by nucleotide excision repair (NER) generally removing bulky DNA adducts (Lehmann, 1995). UV light—in particular UVA but also UVB—mediates oxidative stress indirectly via reactive oxygen species (ROS) (Pelle *et al*., 2003). The UV-generated ROS can induce DNA single-strand breaks (SSBs), DNA-protein cross-links, and oxidized base derivatives, such as 7,8-dihydro-8-oxoguanine (8-oxoG) (Mitra *et al*., 1997). Oxidative DNA damage is preferentially removed by base excision repair (BER) (Helbock *et al*., 1999; Mitra *et al*., 1997).

Sunscreens have become the most popular choice of photoprotection and are recommended in addition to using protective clothing and avoiding the sun (International Agency for Research on Cancer, 2001). Ideally, sunscreens should protect not only against skin cancer but also against effects on the immune system and photoaging of the skin (Elmets and Anderson, 1996; Young and Walker, 2002). There is growing interest in the photostability of sunscreens due to a dramatic increase in their use. The photoinstability of active ingredients in sunscreen has been reported by several studies (Bredholt *et al*., 1998; Serpone *et al*., 2002; Tarras-Wahlberg *et al*., 1999). Upon UV exposure, sunscreens may be degraded to form photoproducts that are potentially toxic. It has been shown that some sunscreens change their spectral performance or may act as photooxidants via generation of free radicals and ROS upon UV exposure (Brezova *et al*., 2005; Gulston and Knowland, 1999). There are some reports of the protective effect of sunscreens against DNA damage formation in cell cultures (Reinhardt *et al*., 2003) and in the skin of volunteers (Al Mahroos *et al*., 2002). The decrease in the efficiency of sunscreens can be caused by different mechanisms: photoisomerization, photodecomposition, and interaction with the formulation or other sunscreen agents (Maier *et al*., 2001). Therefore, knowledge about sunscreen photostability on cellular processes is important.

Octyl methoxycinnamate (OMC) is the most commonly used UVB filter in sunscreens and cosmetics and is listed as a high production volume chemical in the European chemical Substances Information System database (http://ecb.jrc.ec.europa.eu/esis/). Topical application of OMC is well tolerated, with little or negligible skin irritation, allergic contact reaction, and phototoxic effects. However, we have previously reported increased toxicity as a result of breakdown of OMC following UV irradiation (Butt and Christensen, 2000). Such UV-induced molecular breakdown may interfere with cellular processes or induce oxidative damage in human skin. OMC has been shown to degrade into photoproducts when exposed to sunlight, which leads to a decrease in UV absorption efficiency (Butt and Christensen, 2000; Pangnakorn *et al*., 2007). These photoproducts may have a higher toxicity than OMC itself. Other adverse effects of sunscreens have been suggested, including formation of singlet oxygen and various estrogenic effects after *in vitro* and *in vivo* exposure to several UV filters (Allen *et al*., 1996; Schlumpf *et al*., 2001).

To address the need for nonanimal testing of ingredients in cosmetics, we established an *in vitro* cellular system based on two human cell lines, the primary skin fibroblast cell line (GM00498) and the breast cancer cell line (MCF-7). We investigated the effect of OMC with and without UV irradiation on the expression of a panel of 17 genes, selected because of their role in DNA damage response pathways, by quantitative real-time PCR (qRT-PCR) and the expression of p53 protein by Western blotting. The photoprotective capacities of OMC against UV-induced DNA damage, particularly CPDs and oxidative DNA damage, were evaluated by measuring DNA damage using the alkaline elution assay. Investigation of the expression of DNA damage response–related genes, along with genotoxicity assays, may provide sensitive biological end points that could be useful for screening and evaluation of agents to be used in sunscreens.

METHODS

Cell Culture and Chemical Treatment

The human breast carcinoma cell line MCF-7 was obtained from the American Type Culture Collection (ATCC no. HTB22) and was grown in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin (BioWhittaker, Lonza, Switzerland). The human primary skin fibroblast cell line GM00498 was obtained from Coriell Cell Repositories (http://ccr.coriell.org/) and was grown in Quantum medium with L-glutamine (PAA Laboratories GmbH, Austria) and 1% penicillin-streptomycin (BioWhittaker). The two cell lines were incubated at 37°C with 5% CO_2 in air with saturated humidity.

OMC (Eusolex 2292) was purchased from Acros Organics (Geel, Belgium) and dissolved in ethyl alcohol and stored at 4°C. A working solution of OMC was prepared by dissolving the ethyl alcohol dissolved OMC in PBS (without Ca^{2+} and Mg^{2+}).

This experimental design was used to mimic a situation in which an OMC-containing sunscreen lotion is applied to the skin, the individual is exposed to the sun, and then the direct exposure is terminated without removing the remaining sunscreen from the skin. To achieve such a scenario, exponentially growing cells are exposed to UV in the presence or absence of OMC, whereafter cells are grown without UV but still in presence of the UV-irradiated OMC. All cells were pretreated for 1 h with OMC (0, 5, 10, 16, 27, 43, or 60 ppm). Thereafter, half of the samples were irradiated at 4°C with UV (0–2000 s, corresponding to a maximum of 492 J/m^2) at a distance of 28 cm from the light source, whereas the other half was kept in the dark. Thereafter, the PBS/OMC solutions were removed and replaced with culturing medium containing OMC (0–60 ppm), which had been treated with the same UV dose as the corresponding cell sample but without cells. The samples that had been kept in the dark were incubated in medium with OMC (0–60 ppm), which had not been UV treated. OMC was dissolved in PBS and not in medium during its UV exposure to avoid interactions with medium constituents and UV-filtering effects. For cell viability assays, cells were treated as described. Selected UV doses and OMC concentrations were used for downstream analysis. For alkaline elution, the UV dose range was 0–246 J/m^2 (with or without 10 ppm OMC). For gene expression analyses, a single OMC concentration (27 ppm) was used and the UV exposure was 45 J/m^2.

The light source used consisted of five Wolff Helarium B1-01-40W tubes (Germany) mounted inside the lid of a box fitted with an electrical extractor fan for cooling, and the emission spectrum contained wavelengths between 290 and 400 nm (Kinley *et al*., 1997), as measured with a UDT detector (UDT371; United Detector Technology, Hawthorne, CA) and probe 222UV (3.05 mW/cm^2). UV doses in figures are indicated as exposure times (seconds) or as Commission Internationale de l'Eclairage (CIE)-weighted irradiance (J/m^2), i.e., the efficient spectrum of the light source after spectral filtration through OMC at varying concentrations. During UV irradiation, cells were covered with PBS/OMC. The thickness of the PBS/OMC layer was 2.55 cm, selected to obtain a substantial filtration effect and at the same time reducing the toxicity of OMC. The degree of spectral filtration depends on the OMC concentration (Fig. 1, and Supplementary table 1). For example, OMC at 10 ppm reduced the irradiance by ~50% at the level of attached cells, and OMC at 27 ppm reduced the irradiance by ~70% at the level of attached cells.

Genotoxicity Measurement by Alkaline Elution Assay

A semi-automated alkaline elution system was performed as previously described with some modifications (Brunborg *et al*., 1988) but using exogenous enzymes for removing modified bases. Briefly, MCF-7 cells were treated with 10 ppm OMC dissolved in PBS before irradiation with varying doses of UV. Cell samples were then analyzed as described (Brunborg *et al*., 1988) using crude extracts of Fpg and T4 endonuclease V (T4-endo V) enzymes. The Fpg enzyme extract was purified from *Escherichia coli* ER 2566 strain harboring the pFPG230 plasmid as previously described (Boiteux *et al*., 1990). The T4-endo V enzyme extract was purified from *E. coli* AB2480 (uvrA, recA, F'lac IQ1) plus ptac-den V(Apr) strain as previously described (Nakabeppu *et al*., 1982). The activity of the Fpg extract was measured by incubation with a substrate containing ^{3}H-labeled FaPy residues and quantifying the number of ^{3}H-labeled bases released from the substrate as previously described (Olsen *et al*., 2003). The enzymatic activity of the T4-endo V crude extracts was determined using a nicking assay with pure T4-endo V enzyme as a positive control. Various amounts of the extracts were used in initial experiments to establish first-order kinetics of the conversion of specific UV-induced lesions into DNA SSBs (data not shown).

Calibration and calculation of lesion frequencies were carried out using x-rays at the relevant elution pH and assuming an induction of 90×10^{-9}/nucleotide/Gy (~1000 SSB/Gy/diploid cell [$= 3.6 \times 10^{12}$ Da]). The normalized area above curve (NAAC) unit, calculated from elution profiles and used for lesion quantification in our alkaline elution assay (Brunborg *et al*., 1996), was determined at pH 12.25 ± 0.05: 1 NAAC = 22.8 lesions/10^{12} Da. All alkaline elution data (NAAC values) are reported as lesion frequencies (per 10^{12} Da) and presented as the average of five independent experiments.

Determination of Cell Viability

MCF-7 and GM00498 cells were seeded at a density of 5000 cells/100 µl in 96-well culture plates and grown for 24 h. The following day, cells were treated with combinations of OMC (0–60 ppm) and UV (0–2000 s) as described above. After 24-h incubation in medium containing (UV-irradiated) OMC, cell viability

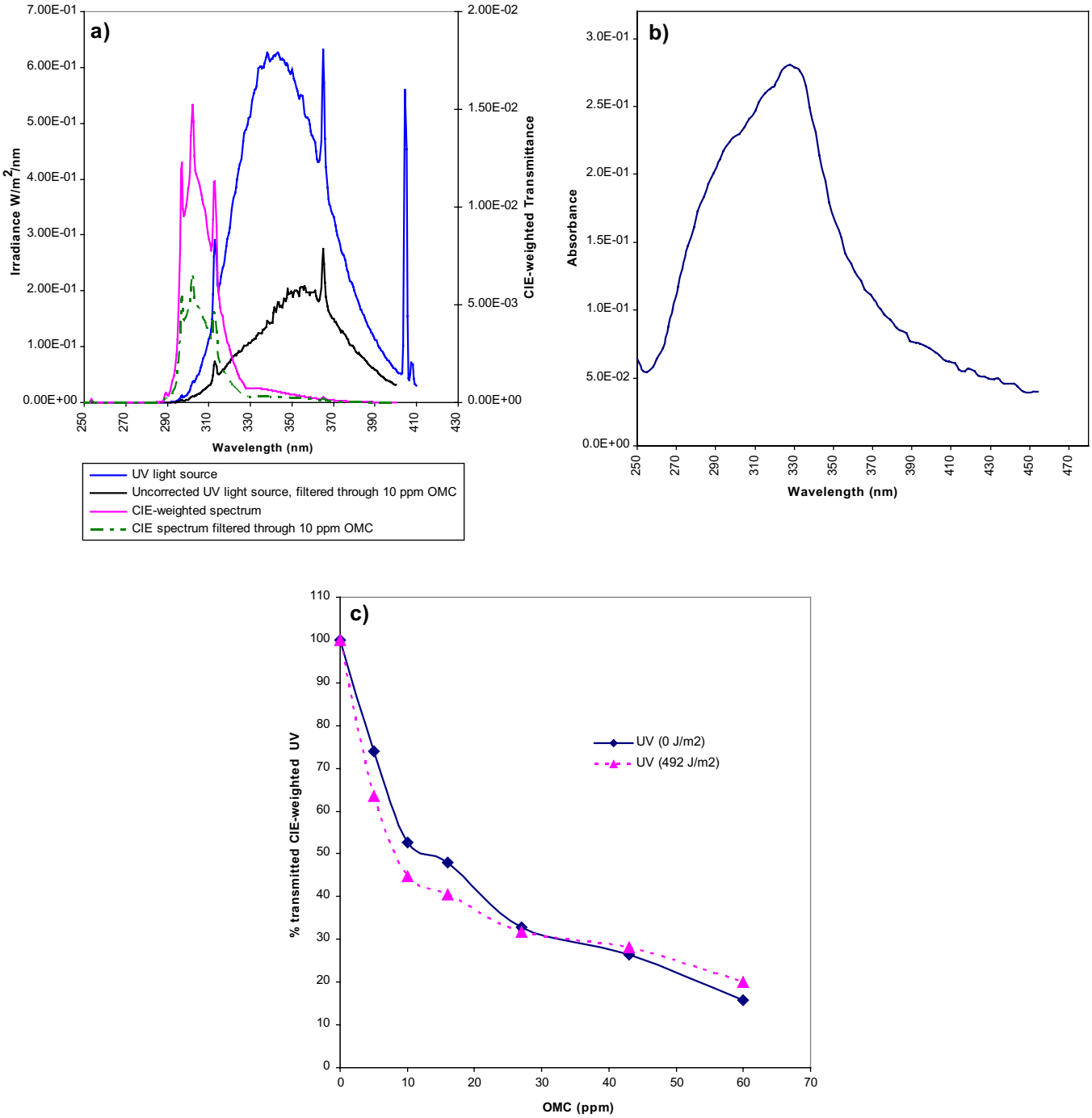

FIG. 1. Light source and transmittance of OMC. (a) Characteristics of the light source, its CIE-weighted spectrum, and the spectrum transmitted through OMC. Spectral irradiance of the UV source (blue line, left axis), spectral irradiance after transmission through OMC (10 ppm, 2.55 cm) (black line, left axis), CIE-weighted spectrum calculated for the light source (purple line, right axis), or after transmission through OMC (10 ppm, 2.55 cm) (broken green line, right axis). The CIE-weighted spectrum was calculated by multiplying the spectral radiation of the lamp with the biological efficiency spectrum for each wavelength. (b) OMC spectral curve, as measured spectrophotometrically in a 27 ppm solution. (c) OMC concentration–dependent reduction in CIE-weighted UV dose (0 or 492 J/m^2 [corresponding to 2000 s]). CIE-weighted light transmittance calculated from measurement of spectra of OMC (0–60 ppm) at a depth of 2.55 cm.

was measured using an 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) according to the manufacturer's instructions. The absorbance was measured at 570 nm by Sunrise plate reader and analyzed with Magellan software, v 1.11 (Tecan, Switzerland). Cell viability was calculated as percentage of absorbance, relative to untreated (no UV and no OMC) control cell cultures; wells containing culturing medium only were used as blanks and were subtracted as background from each sample. The value of untreated control cells was defined as 100% viable, and all data were calculated accordingly. Results are from three independent experiments.

Gene Expression Analysis

RNA preparation. Total RNA was isolated as previously described (Duale *et al*., 2007) using the GenElute kit (Sigma-Aldrich, Inc., Oslo, Norway). RNAs were isolated from three independent experiments. Its quantity and quality were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was determined by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

qRT-PCR analysis. qRT-PCR was used to analyze the transcriptional response of GM00498 and MCF-7 cell lines following the exposure of OMC (27 ppm) alone, co-exposure of OMC (27 ppm) plus UV (45 J/m^2), and UV (45 J/m^2) alone. The reverse transcription reaction and RT-PCR were carried out as previously described (Duale *et al*., 2007). The primer sequences used are listed at RTPrimerDB, real-time PCR primer and probe database (Pattyn *et al*., 2003), or purchased from Qiagen (Germany). The real-time PCR experimental layout of the plate was as follows: For each sample, there were three biological repeats, and for each of those, three technical replicates were run (3 × 3), and 10 samples (i.e., 3 × 3 × 10). We also included three control samples (cDNA from pooled RNA from control samples) in each 96-well plate. This layout allowed simultaneous measurements of all samples in one 96-well plate for each gene, reducing run-to-run variations. All PCR reactions were performed in triplicate, and data are expressed as an average of the triplicates. The average cycle threshold (Ct) measurements for the three independent experiments were used in calculations of relative expression. The endogenous reference genes (house keeping genes [HKGs]) for gene expression studies may depend on the applied treatments; we therefore evaluated the stability of the HKGs (18S rRNA, glyceraldehyde 3-phosphate dehydrogenase [GAPDH], and beta-actin) used in this study by the BestKeeper algorithm (Pfaffl *et al*., 2004). The three HKGs were correlated well with each other (Supplementary table 2 and Supplementary fig. 1). We therefore normalized target genes to the average of all three HKGs in the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The cDNA from pooled RNA from control samples was used as calibrators, and the same cDNA was included in each 96-well plate, allowing control of run-to-run variation. All gene expression data are reported as log2-transformed $2^{-\Delta\Delta Ct}$ values and presented as the average of three independent experiments.

Western Blot Analysis

MCF-7 and GM00498 cells (~2 × 10^6) were treated with OMC (27 ppm) alone, OMC (27 ppm) plus UV (45 J/m^2), or UV alone (45 J/m^2). Samples were washed with ice-cold PBS and incubated for 10 min on ice in cold lysis buffer with a protease inhibitor cocktail (60mM Tris-HCl [pH 6.8], 10% glycerol, 3% SDS, 1mM EDTA, 1mM sodium orthovanadate, 50mM sodium fluoride, 10mM glycerol 2-phosphate disodium salt hydrate [phosphatase inhibitors], and Complete Mini [Roche, Switzerland]). Whole-cell lysates were sonicated on ice. Protein concentration was determined by the Lowry method using Bio-Rad's detergent-compatible protein assay kit according to the manufacture's instruction (Bio-Rad Laboratories, Inc., Hercules, CA). Samples (20 μg per well) were heated in SDS sample buffer for 5 min at 95°C, separated by SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore Inc., Billerica, MA). After the proteins were transferred, the membrane was blocked in 5% blotting-grade nonfat dry milk (Bio-Rad Laboratories, Inc.). The following antibodies were used: anti-p53 (Cell Signaling Technology Inc., Danvers, MA) and anti-GAPDH (Biogenesis, Inc., Hackensack, NJ), used as internal control. Membranes were incubated with the appropriate peroxidase-coupled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and subsequently detected by enhanced chemiluminescence using Amersham's ECL Western Blotting Detection Reagents (GE Healthcare, Norway).

Statistical Analysis

The alkaline elution assay data and the cell viability data were analyzed by a nonparametric Mann-Whitney *U*-test. The gene expression data were analyzed by one-way ANOVA, followed by a post hoc Dunnett's test to allow for multiple comparisons, i.e, comparison of the treatment groups [ΔCt exposed: (Ct exposed target gene − Ct exposed reference gene)] versus common untreated control groups [ΔCt control: (Ct control target gene − Ct control reference gene)], and two-sample *t*-test was used to check for cell type–specific gene expression differences between the two cell lines. All statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL), and $p < 0.05$ was accepted as statistically significant.

RESULTS

Photoabsorption and Stability of OMC

The spectral irradiance of the light source in Figure 1 peaks at 350 nm but with a significant irradiance also in the UVB region (< 320 nm). The CIE-weighted spectrum for erythema induction was used to calculate the total efficiency of the light source with respect to CPD formation since the CIE-weighted spectrum is similar to the spectral efficiency for CPDs (Young *et al*., 1998). The resulting convolution curve is obtained by multiplying—for each wavelength—the irradiance of the light source with the CIE-weighted efficiency. The spectral absorbance of OMC dissolved in PBS was measured spectrophotometrically (Fig. 1b) and was used to calculate the CIE-weighted spectral irradiance without or with OMC (10 ppm) (Fig. 1a); the integrated radiant exposure at 10 ppm OMC was reduced to 52%. Also included in Figure 1a is the irradiance of the light source filtered through 10 ppm OMC (i.e., with no CIE correction). This curve is more relevant for oxidative lesions, which—unlike CPDs—are induced at highest efficiency in the UVA region. The integrated radiant exposure of the light source was reduced by OMC (10 ppm and 2.55 cm depth) to 32%, illustrating that OMC has some absorption also in the UVA region.

The photostability of OMC was investigated (Fig. 1c). OMC (0–60 ppm) dissolved in PBS was exposed for up to 2000 s (corresponding to 492 J/m^2) at the depth (2.55 cm) used during cell exposure. The transmitted CIE-weighted spectral UV irradiance is shown as a function of OMC concentration; there was no major change during the exposure.

Photoprotective Efficacy of OMC Measured by Alkaline Elution Assay

The semi-automated alkaline elution assay, in combination with Fpg or T4-endo V enzymes, was used to measure 8-oxoG and CPDs. The T4-endo V enzyme recognizes CPD, which is a representative lesion after UV exposure, whereas 6-4PPs—which are also formed—are not recognized by the enzyme. The main mutagenic lesion recognized by Fpg is 8-oxoG, but the enzyme has also affinity to other modified purines, such as ring-opened dG and dA.

Figure 2 shows UV dose–dependent increase in both CPDs and Fpg-sensitive lesions. OMC-protected cells had significantly lower levels of CPDs ($p < 0.01$) after exposure to UV (3 or 6 J/m^2) compared to exposure without OMC. At higher UV exposure times (13 or 128 J/m^2), the CPD levels for OMC-protected cells were also lower than those for unprotected cells;

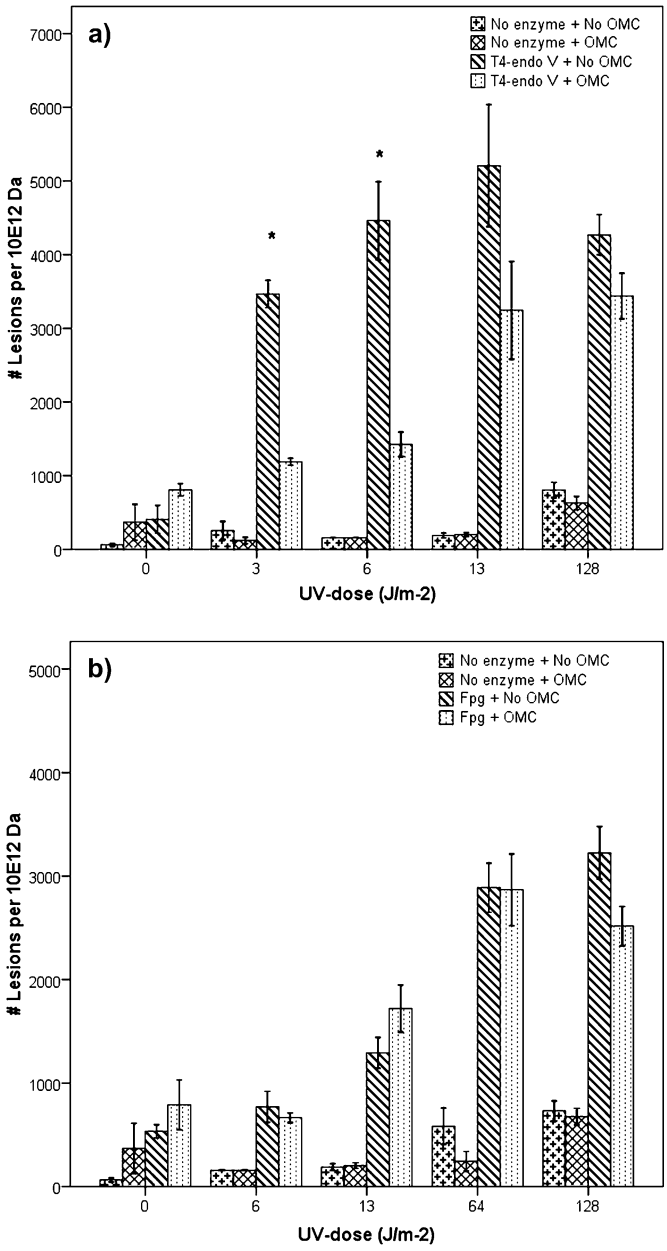

FIG. 2. DNA lesions measured with the alkaline elution assay in cellular DNA after irradiation with UV, unfiltered or filtered through OMC. Exposure of MCF-7 cell line with UV (0–128 J/m^2). (a) The number of CPDs (with T4-endoV enzyme). Asterisk represents significant reduction in net amount of induced CPDs ($p < 0.01$) in the presence of OMC (10 ppm, 2.55 cm) at low UV exposure times (3 and 6 J/m^2). (b) The number of oxidated DNA lesions (with Fpg enzyme). No significant reduction in net amount of Fpg-sensitive DNA lesions in the presence of OMC at all UV exposure times. Bars represent means ± SE of five independent experiments.

however, the observed OMC protection was not statistically significant (Fig. 2a). Figure 2b shows that, within the UV dose range used (0–128 J/m^2), OMC provided no significant protection in the induction of the Fpg-sensitive lesions. There was no net increase in DNA SSBs (i.e., using no enzymes) at the lowest UV doses but a slight increase at 64 and 128 J/m^2 of UV exposure (Fig. 2). We observed a very high response using T4-endo V at low UV doses approaching saturation (i.e., exceeding the dynamic range of the alkaline elution system) at 13 J/m^2. Therefore, all high dose exposures were not included, and the 128 J/m^2 dose illustrates that no further increase was found with this dose.

In Table 1, these data are compared with the reduction in UV dose provided by OMC, calculated both with and without CIE correction. For CPDs, there were substantial relative reductions in lesion frequencies in the presence of OMC. For Fpg-sensitive sites, on the other hand, no such change was observed with OMC filtration. The results indicate that OMC-mediated UV light filtration reduced the formation of CPDs but not Fpg-sensitive sites. OMC by itself did not induce any significant genotoxic effects as measured by the alkaline elution assay.

Cell Viability Measurements

The MTT assay was used to measure cell viability of GM00498 and MCF-7 cells after UV exposure and in the presence or absence of OMC. Figure 3a shows that, for both cell types, there was around 10–25% reduction in cell viability from OMC-alone treatments at the two highest concentrations (43 and 60 ppm). Figures 3b and c show UV exposure–dependent reduction in cell viability. This reduction was highest at the lowest OMC concentrations after UV exposure time at and above 500 s (Figs. 3b and c and Supplementary table 2). This trend was the same for both cell types. In microscopic examination of propidium iodide (PI)/Hoechst-stained cells, changes in cell viability followed similar trends as with the MTT assay (data not shown). Based on these results, for the gene expression studies, we chose a low cytotoxic OMC (27 ppm) concentration, which resulted to ~12% reduction in cell viability and when co-exposed with UV (500 s, corresponding to 45 J/m^2 with 27 ppm OMC) resulted to ~25% reduction in cell viability.

Gene Expression

We were interested in identifying genes that are significantly differentially expressed among cells protected by OMC during UV irradiation compared to those exposed to UV alone and OMC alone in the dark. For this purpose, qRT-PCR was used to evaluate the transcriptional responses of a panel of 17 DNA damage response–related genes. These transcripts consist of five NER pathway–related genes, three BER pathway–related genes, five antioxidant-related enzymes, two immediate-early response genes, and GADD45A and TP53. By visual inspection of Figures 4a–d, we observe that there were clear differences between the gene expression profiles of the cells co-exposed with OMC and UV or UV-alone on the one hand and those exposed to OMC in the dark on the other hand. Overall, there were no significant differences in gene expression patterns between the GM00498 and the MCF-7 cell lines, and the observed gene expression changes therefore did

TABLE 1
CPDs and Fpg-Sensitive Sites Induced by UV Light, With and Without OMC Filtration

UV exposure time (s)	UV dose (J/m^2)[a]	OMC-filtered UV dose (J/m^2)[a]	Mean lesions/10^{12} Da		Relative (%) lesions with OMC[b]
			No OMC	OMC	
CPDs					
25	6	3	4064	1451	36
50	12	6	4934	1370	28
100	25	13	6034	2628	44
1000	246	128	4852	3581	74
Fpg-sensitive sites related to UVB irradiance					
50	12	6	771	665	86
100	25	13	1292	1389	108
500	123	64	2919	2834	97
1000	246	128	2764	2503	91

Notes. UV dose–dependent CPD formation was observed, and there was a substantial relative reduction in lesion frequency with OMC (Column 6). (At the highest dose, there was less change due to saturation of alkaline elution lesion detection.) Fpg-sensitive sites were clearly induced in a dose-dependent manner, but no substantial relative change in their frequency with OMC was found (Column 6). The calculated UV dose was significantly reduced by OMC, irrespective of CIE correction (52% UV light remains after filtration of the CIE-corrected spectrum through OMC (10 ppm), and 32% UV light remains after filtration of the uncorrected spectrum through OMC).

[a]CIE-corrected UV dose.

[b]Relative (%) lesions remaining, with OMC [(Column 5)/Column 4 × 100]. OMC filtration: 10 ppm, 2.55 cm depth.

not suggest cell type–specific differences. Furthermore, exposure of cells with OMC alone had no major effects on the transcription of several genes. It should be noted, however, that some genes showed downregulation upon the exposure to OMC alone (Figs. 4a–d and Supplementary table 3).

Sunscreens (OMC) protect the cells from the UV-induced DNA lesions. Therefore, we expected that the expression of NER pathway genes would not be significantly affected in the OMC-protected cells. Figure 4a shows the expression pattern of the five NER pathway genes studied: *DDB1*, *DDB2*, *ERCC1*, *XPA*, and *XPC*. We observed a time-dependent induction of the messenger RNA (mRNA) levels of these genes (some of them were expressed at moderate levels and others at low levels; however, their transcription pattern was similar) in cells co-exposed to OMC and UV, with maximum induction at 24-h postirradiation (Fig. 4a). With UV alone, the mRNA level of these genes was increased. The induction was maximum at 3-h postirradiation, thereafter slowly declining to control levels at 24 h (Fig. 4a). The expression pattern of these genes was in somehow similar, even though some of them showed a moderate or a low expression level. Downregulation of xeroderma pigmentosum, complementation group A (XPA), a key enzyme in the NER pathway, was observed in both cells with OMC-alone treatment.

We observed a time-dependent upregulation of the mRNA level also of the BER pathway gene *APEX1* following exposure to OMC plus UV or to UV alone (Fig. 4b). Two other BER pathway genes, *MPG* and *OGG1*, were also somewhat upregulated upon OMC plus UV at 24-h postirradiation, but their induction was not statistically significant. Downregulation of OGG1 was observed with OMC-alone treatment.

The gene expression patterns of the five antioxidant-related genes: *CAT*, *SOD1*, *SOD2*, *MT1A*, and *MT1B*, which protect cells against oxidative stress, are shown in Figure 4c. We observed time-dependent upregulation of the mRNA levels of these genes in both cell lines following UV-alone or OMC plus UV exposure; the net increase was less pronounced in the latter case (Fig. 4c). Some of these genes showed downregulation upon the treatment to OMC alone (Fig. 4c).

We also conducted gene expression analysis for some other selected DNA damage response–related genes: *FOS*, *JUN*, *GADD45A*, and *TP53* (Fig. 4d). Time-dependent induction of the immediate-early genes, *JUN* and *FOS*, was observed following OMC plus UV exposure, with maximum upregulation at 24 h. Exposure to UV alone had an opposite effect, with mRNA levels increasing to maximum levels at 3-h postirradiation and then declining at longer times (Fig. 4d). In addition, the transcriptional level of *GADD45A* gene, which is involved in DNA damage response, cell cycle arrest, and apoptosis, was significantly induced both by UV alone and by OMC plus UV, although the temporal changes were somewhat different (Fig. 4d). The mRNA level of *TP53* gene was not affected in either cell lines following the exposure.

The Involvement of p53 Protein in the UV-Induced DNA Damage Signaling Pathways

The tumor suppressor gene p53 is an important transcription factor activated by DNA damage. Some of the genes affected by UV alone or OMC plus UV are known p53 target genes. Using Western blot, we investigated the p53 protein expression levels following UV alone or OMC plus UV (Figs. 5a and b). The level

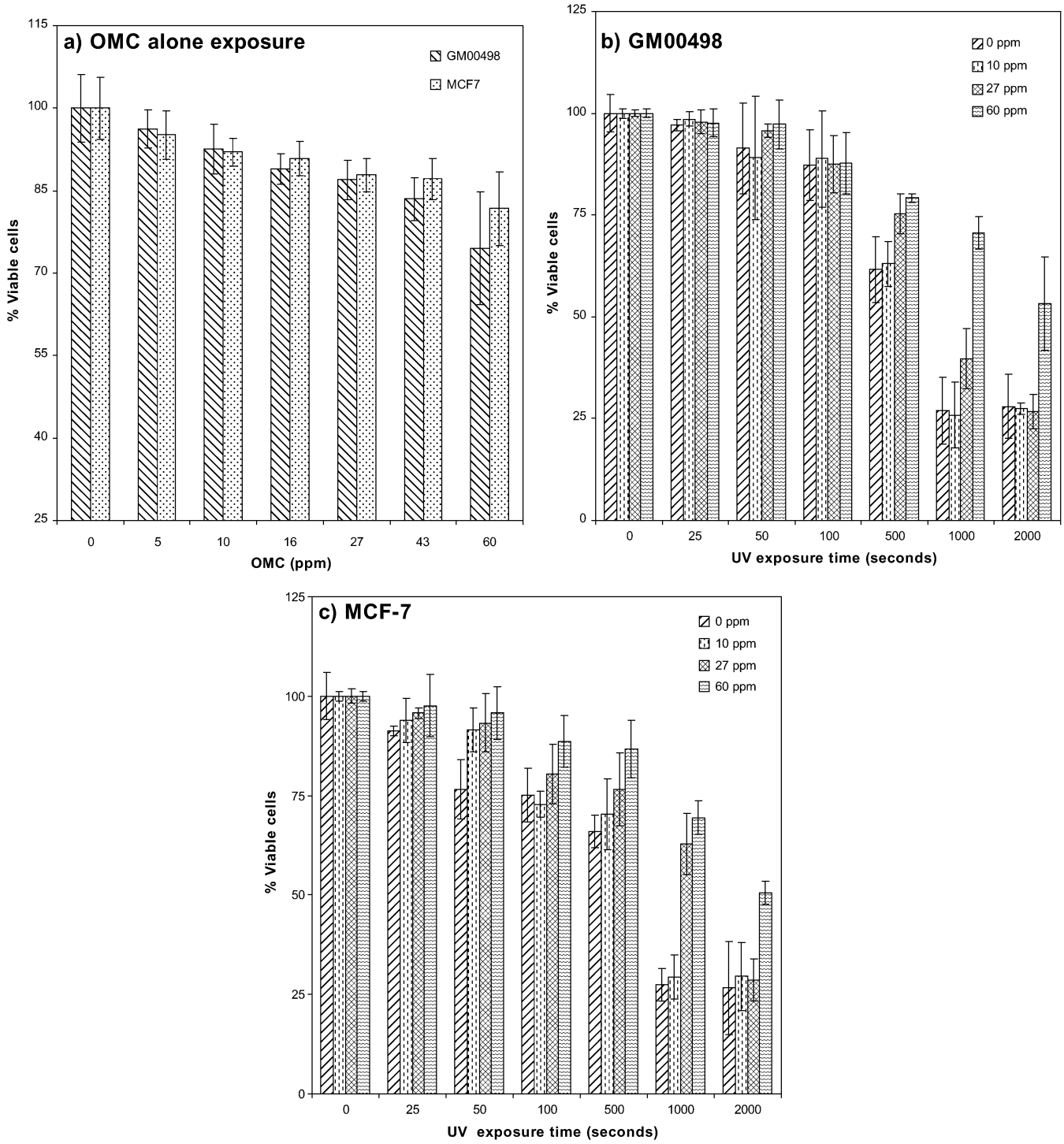

FIG. 3. Cell viability measurements. Cell viability measured with the MTT assay. (a) GM00498 and MCF-7 cells exposed to OMC (0–60 ppm), with cell viabilities expressed as percentage of viable cells relative to untreated controls. (b) GM00498 cells exposed to various doses of UV in the presence of OMC (0–60 ppm), and cell viabilities were expressed as percentage of viable cells relative to UV unirradiated cells. (c) MCF-7 cells exposed to various doses of UV in the presence of OMC (0–60 ppm), and cell viabilities were expressed as percentage of viable cells relative to UV unirradiated cells. Bars represent means ± SE.

of p53 protein was markedly increased by UV exposure; this increase was lower after OMC plus UV. However, the p53 expression level after OMC plus UV exposure was higher than in control cells and cells exposed to OMC alone (Fig. 5). The expression of GAPDH protein levels was not affected upon the exposures.

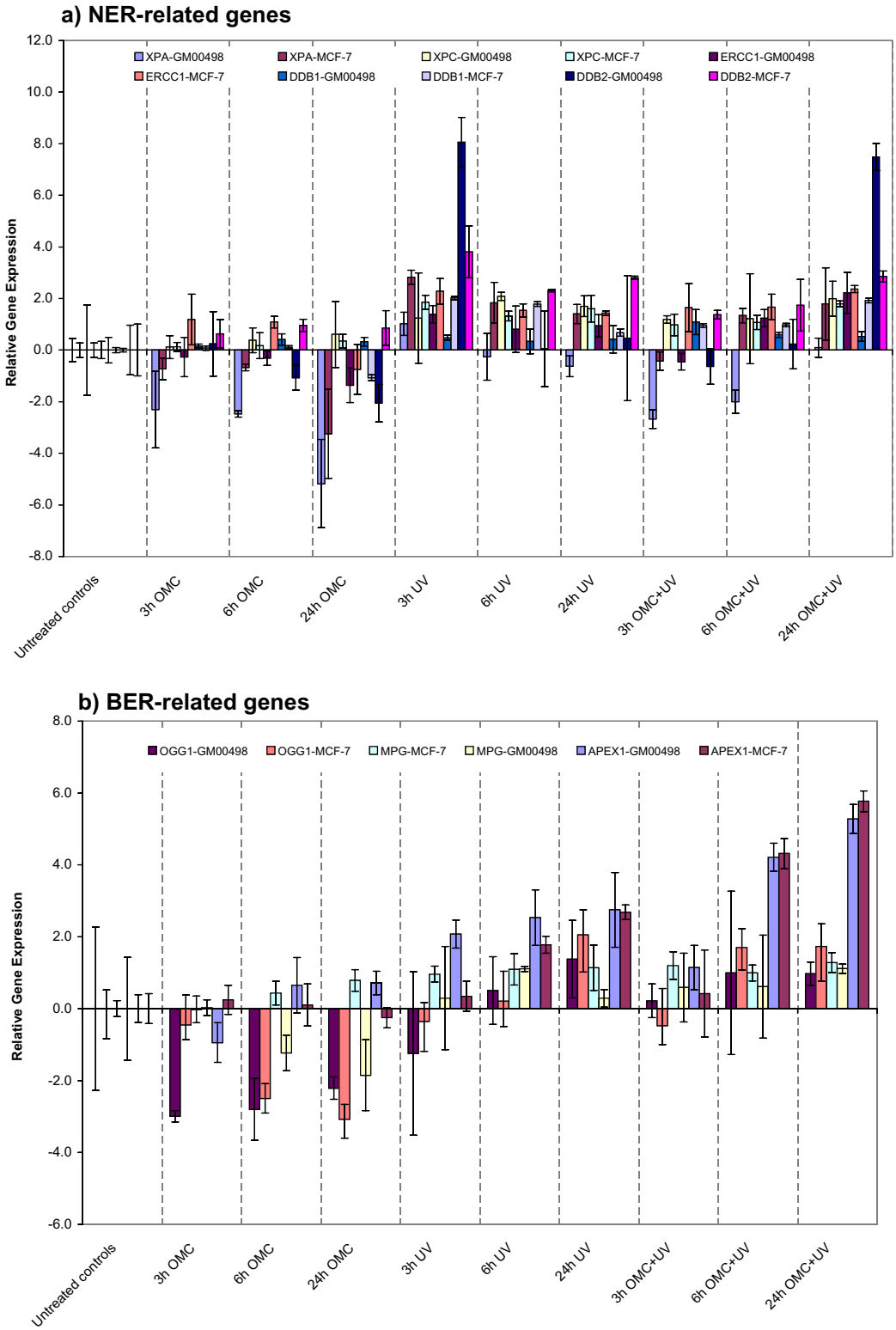

FIG. 4. Time-dependent transcriptional response. The mRNA level of GM00498 and MCF-7 cells exposed to UV (45 J/m^2) alone, OMC (27 ppm) plus UV (45 J/m^2), or OMC (27 ppm) in the dark were analyzed by qRT-PCR. (a) The mRNA level of five NER pathway–related genes. (b) The mRNA level of three BER pathway genes. (c) The mRNA level of five antioxidant-related genes. (d) The mRNA level of two immediate-early response genes (*FOS* and *JUN*) and two DNA damage response–related genes (*TP53* and *GADD45A*). The relative mRNA expression level data are presented as log2-transformed $2^{-\Delta\Delta Ct}$ values. Bars represent means ± SE of three independent experiments.

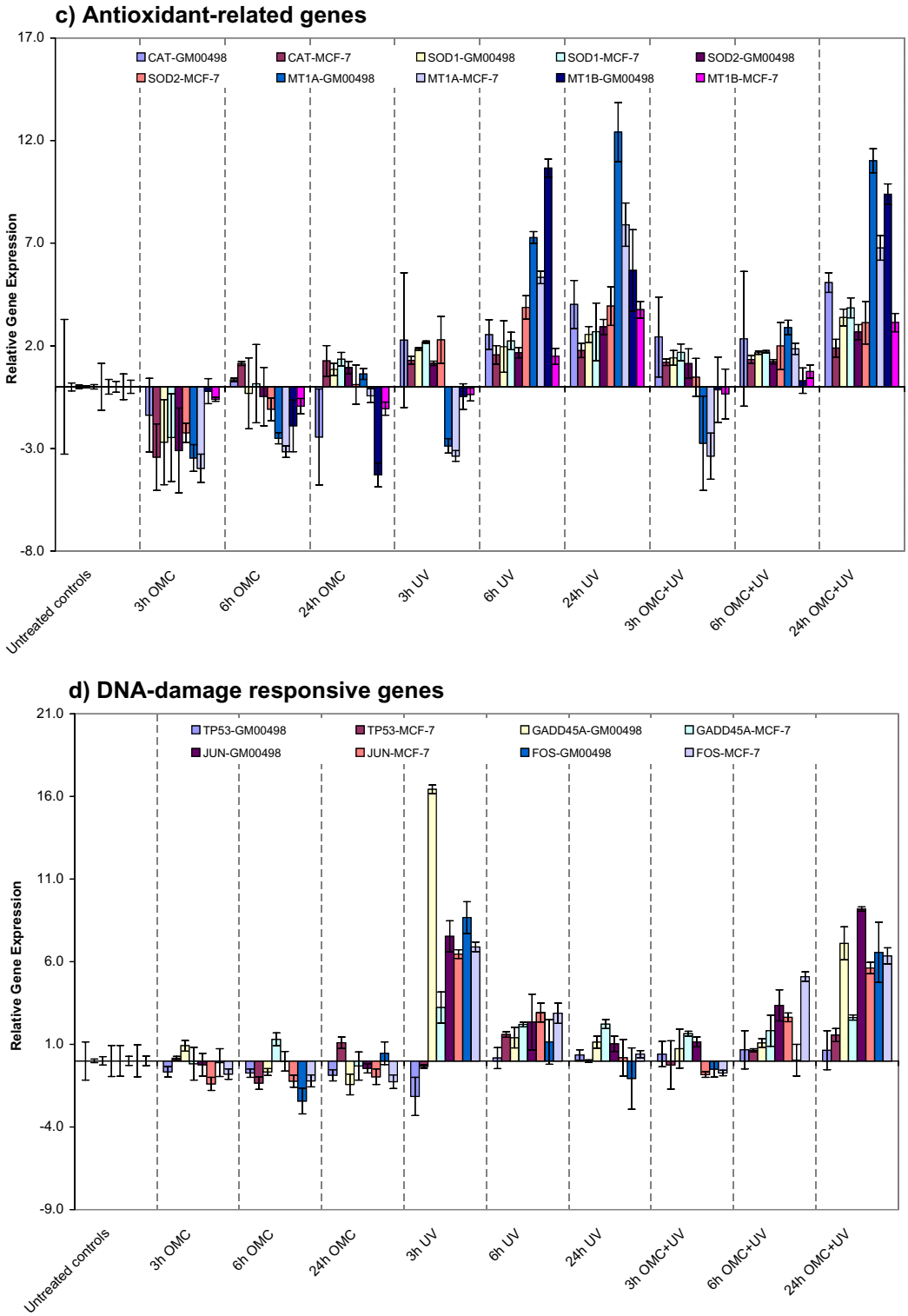

Fig. 4. continued

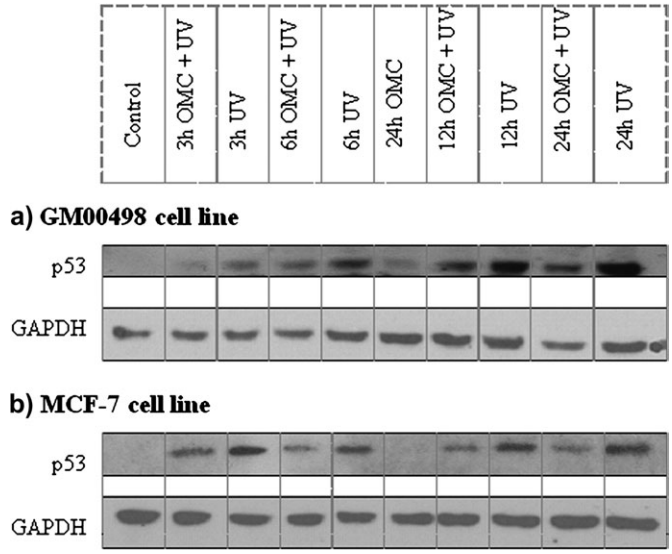

FIG. 5. p53 protein expression analysis. Time-dependent induction of the p53 protein following the exposure of GM00498 or MCF-7 cells with OMC (27 ppm), UV (45 J/m^2) alone, or OMC (27 ppm) plus UV (45 J/m^2), as analyzed with Western blot. (a) GM00498 cells. (b) MCF-7 cells. GAPDH protein was used as internal control.

DISCUSSION

In this study, we evaluated photoprotection of OMC as well as the effects on UV-induced gene expression of this common sunscreen. With 10 ppm OMC in a buffer solution at a depth of 2.55 cm, we observed 52% reduction in the CIE-weighted dose (Fig. 1). This amount of OMC (25.5 μg/cm^2) is comparable to the concentration found in the epidermis of human skin (5–25 μg/cm^2) at normal use of OMC-containing sunscreen of protection factor 15 (Jiang *et al*., 1999). Furthermore, the UV doses used were in a range relevant to human solar light exposure.

The alkaline elution assay allows the sensitive measurement of DNA SSBs. To evaluate the photoprotection efficiency of OMC against UV-induced DNA damage, this assay was modified (Epe *et al*., 1993; Kielbassa *et al*., 1997) to include the T4-endo V enzyme, which has specific activity against UV-induced CPDs, or the Fpg enzyme, which recognizes oxidative purines, particularly 8-oxoG. With these modifications, it was possible to measure these lesions in a dose-dependent manner.

OMC significantly protected against UV-induced CDP formation (Fig. 2a). OMC is marketed as a UVB filter and is not claimed to provide protection against lesions induced by UVA. OMC absorbs also in the UVA region, and one would expect a reduced level of UVA-induced oxidative lesions. No such protection was observed (Fig. 2 and Table 1). Mouse lymphoma LY-R showed similar trends in DNA lesion-specific protection of OMC (data not shown).

Sunscreens are used as a major defense against solar UV, and one would anticipate major reductions in UV-induced transcriptional changes in DNA damage response genes when the cells are protected with OMC. A main finding from the gene expression analysis was the OMC-mediated characteristic delay of some genes known to be involved in DNA damage response. This information should be considered as relevant in systematic screening of sunscreen ingredients. We observed modulation of several genes that participate in DNA damage response pathways upon UV irradiation of OMC-protected cells (Fig. 4). There were clear differences in gene expression profiles between cells co-exposed with OMC plus UV compared to OMC-treated cells in the dark. Cells exposed with OMC plus UV showed similar expression patterns as the UV alone–treated cells. For some but not all genes studied, there was an apparent UV dose reductive effect of OMC at short times after exposure; furthermore, the change in expression of some genes seemed to be delayed in time (Fig. 4). Both cell lines responded similarly to the exposures, although further optimization would be expected to lead to more clearly distinguishable effects.

There are studies suggesting that the use of sunscreens can reduce DNA damage and p53 protein expression following UV exposure (Berne *et al*., 1998; Marrot *et al*., 2002). On the basis of these studies, we expected reduction in the p53 protein expression levels following UV irradiation in the OMC-protected cells. A slight induction of p53 protein expression was found in OMC-protected cells (Fig. 5). It is possible that the observed p53 protein induction in OMC-protected cells is a response to UV-induced oxidative damage rather than response to CPDs. OMC in the dark had no effect on p53 protein expression (Fig. 5). A similar accumulation of p53 protein in cells protected with photounstable sunscreen formulation has been reported (Fourtanier *et al*., 2006). Furthermore, p53 expression in MCF-7 cells was observed following UVB but not UVA (Wang *et al*., 1998).

One consequence of p53 induction is cell cycle arrest, giving the cells sufficient time to repair the damage in its genome. In our study, we observed an upregulation of the *GADD45A* gene both in OMC-protected and in UV alone–exposed cells (Fig. 4). It has been reported that *GADD45A* is induced by a wide spectrum of DNA-damaging agents, including UV (Brown, 2003; Hollander *et al*., 1993). It is also worth noting that the *GADD45A* gene is one of several known p53 target genes involved in a variety of DNA damage response–related pathways (Wang *et al*., 1999).

The decision whether the cells should undergo apoptosis or arrest in the cell cycle for DNA damage repair seems to be determined by the UV dose. We identified several important DNA repair genes related to different DNA repair pathways to be induced in response to OMC plus UV (Fig. 4). The product of *DDB2* gene, which was clearly induced in our study (Fig. 4), forms—together with *DDB1* gene—the UV-damaged DNA-binding protein complex. This complex is involved in several processes including repair, transcription, and cell cycle regulation and is essential for the initial recognition of CPDs

during global genomic repair (Hwang *et al*., 1998; Tang *et al*., 2000). We found a downregulation of XPA by OMC alone, and the biological relevance of this change is unclear; however, moderate changes in the expression of XPA has limited effects on NER efficiency (Muotri *et al*., 2002).

Concerning BER pathway–related genes, we observed induction of *APEX* mRNA level following OMC plus UV exposure (Fig. 4). *APEX1* is responsible for cleavage of apurinic/apyrimidinic (AP) sites via its 5′-endonuclease activity, and it also plays a role in p53 activation and redox-dependent activation of AP-1 (c-Jun/c-Fos) (Evans *et al*., 2000). The expression level of the highly relevant BER gene *OGG1* was significantly downregulated by OMC alone. OGG1 gene is responsible for the excision of 8-oxoG and may be regulated at a posttranslational level and has been reported to be acetylated upon oxidative stress (Bhakat *et al*., 2006).

A notable observation in this study is a change in the temporal pattern of gene expression from OMC, suggesting a possible interference with normal progression of cell division. This change was found for the FOS and JUN genes (Fig. 4). UV belongs to the extracellular influences that activate the immediate-early mitogen-regulated genes, such as c-fos and c-jun (Holbrook and Fornace, 1991), and these genes may promote cell growth after the DNA lesions have been repaired. UV irradiation has been reported to upregulate the expression of *FOS* and *JUN* genes, via phosphorylation of JNK and p38, leading to the activation of AP-1 and NFκB and increasing damage tolerance (Xia *et al*., 1995).

Cells are also protected against oxidative stress by an interacting network of antioxidant enzymes, such as superoxide dismutases, catalases, metallothioneines, and various peroxidases (Packer and Valacchi, 2002; Vayalil *et al*., 2003). We observed time-dependent upregulation of the mRNA level of these genes following the exposure of OMC plus UV or UV alone (Fig. 4). Antioxidant genes are considered to be constitutively expressed; however, their mRNA levels can be regulated by various environmental stresses. The expression patterns of these genes did not follow the same temporal trend as was observed for the DNA damage response genes (*FOS*, *JUN*, *GADD45*, and *DDB2*) following OMC plus UV and UV-alone exposure.

In conclusion, our data support the notion that OMC provides protection against CPDs in cellular DNA and the degree of protection correlates with the reduced transmission of UV light in OMC. With regard to oxidative DNA lesions, protection did not seem to be provided. The gene expression results suggest that the overall cellular response to DNA damage was significantly altered by OMC, and the effects were in general similar in the two cell types used. Among the panel of 17 selected genes studied, several are considered as general biomarkers of genotoxic response. The systematic approach applied in this study—with appropriate standardization—may have a potential use in the initial screening and evaluation of sunscreen active agents.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Cell cycle delay, mitochondrial stress and uptake of hydrophobic cations induced by sunscreens in cultured human cells.

Xu C1, Parsons PG.

Abstract
Reports of systemic absorption of sunscreens prompted a study of the effects of emulsions of 3 commonly used sunscreens on cultured human cells; vegetable oil and paraffin oil were used as controls. Ethylhexyl p-methoxycinnamate (EHMC), octyl p-dimethylaminobenzoate (PABA) and oxybenzone (OB) inhibited cell growth and DNA synthesis and retarded cycle progression from G1 in the dose range 25-100 micrograms/mL. An extended period of exposure (up to 24 h) was required for maximum uptake of sunscreens and for inhibition of cell growth. Melano-cytes and fibroblasts tended to be more resistant than tumor cell lines (melanoma, cervical carcinoma). Sunscreens had no major effects on the transcription of certain genes, as judged by the activity of reporter constructs driven by the p53, c-fos and metal response (sheep metallothionein Ia promoter) elements and transfected into a human melanoma cell line (MM96L). The activity of the cytomegalovirus promoter was also not affected. A cell line (CI80-13S) with mitochondrial dysfunction was significantly more sensitive to growth inhibition by EHMC and PABA than the other cell lines tested. Treatment of MM96L with the mitochondrial inhibitor ethidium bromide sensitized the cells to killing by cotreatment with sunscreens, in association with increased cellular uptake of ethidium bromide. These results established conditions for studying the action of sunscreens on cultured human cells. Further studies are required to determine whether the mitochondrial stress and changes in drug uptake associated with sunscreens in the above cell lines are relevant to their action in vivo.

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The effect of UV-filters on the viability of neuroblastoma (SH-SY5Y) cell line

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ABSTRACT

Topical application of cosmetic products, containing ultraviolet filters (UV filters) are recommended as a protection against sunburns and in order to reduce the risk of skin cancer. However, some UV filters can be absorbed through skin and by consuming contaminated food. Among the chemical UV filters, benzophenone-3 (BP-3), 3-(4-methylbenzylidene)camphor (4-MBC) and 2-ethylhexyl-4-methoxycinnamate (OMC) are absorbed through the skin to the greatest extent. So far, these lipophilic compounds were demonstrated to influence the gonadal and thyroid hormone function, but their effect on central nervous system cells has not been investigated, yet.

In the present study, we investigated the effect of some UV filters on cell viability and caspase-3 activity in SH-SY5Y cells. It has been found that benzophenone-2 (BP-2), BP-3, 4-methylbenzophenone (4-MBP) and OMC present in the culture medium for 72 h in high concentration (10^{-5} and 10^{-4} M) and 4-MBC only 10^{-4} M produced a significant cytotoxic effect, as determined both by the MTT reduction test and LDH release assay. In contrast to necrotic changes, all tested UV filters increased caspase-3 activity in much lower concentrations (from 10^{-8} to 10^{-7} M). Proapoptotic properties of the test compounds were positively verified by Hoechst staining.

The obtained results indicated that UV filters adversely affected the viability of nerve cells, most likely by enhancing the process of apoptosis. The most potent effect was exerted by BP-3 and 4-MBC and at concentrations that may be reached *in vivo*. Since human exposure to UV filters is significant these compound should be taken into consideration as one of the possible factors involved in pathogenesis of neurodegenerative diseases.

1. Introduction

Ultraviolet (UV) chemical filters are used in sun protection products and other cosmetics: perfumes, lipsticks, body lotions, shampoos, conditioners and hair sprays. They are also added to the production of food packaging to protect from the damaging effects of sunlight. Because UV light, by induction of DNA damage and inhibition of skin immune system activity, increases the threat of skin cancers, including melanoma, therefore, the appropriate protection is required. However, some compounds used as UV filters can be absorbed through skin and exert systemic effects. The most common group of chemical filters contains benzophenones derivatives: benzophenone-3 (BP-3), benzophenone-2 (BP-2), and 4-methylbenzophenone (4-MBP). They are often used in cosmetic products in combination with two or more sunscreens such as: 3-(4-methylbenzylidene) camphor (4-MBC), and 2-ethylhexyl-4-methoxycinnamate (OMC). In cosmetic products, sunscreens are in a concentration of up to 10% (Schreurs et al., 2002). They are highly lipophilic and can therefore bioaccumulate in humans and in the environment (Fent et al., 2010).

A wide range of *in vitro* and *in vivo* studies have identified sunscreens as an endocrine-disrupting chemicals (EDCs). The most examined so far action of UV filters is related to their effect on the estrogen receptors (ER). *In vitro* studies showed estrogenic activity of some UV filters, binding to estrogen receptors α (ERα) and estrogen receptor β (ERβ) and influence on the ER levels (Schlumpf et al., 2001). However, though there are individual differences in the interaction with ERα and ERβ, they preferentially bind to ERβ.

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In many of ecotoxicological studies, 4-MBC and OMC decreased the level of vitellogenin, which is a phenotypic endpoint for the estrogenic activity in fish. In *in vivo* acute models, estrogenic activity of BP-3, 4-MBC and OMC has been confirmed by demonstrating an increase in uterine weight in immature female rats (Schlumpf et al., 2001, 2004a,b) and oophorectomized rats (Klammer et al., 2005). In addition to estrogenic activity, these compounds *in vitro* exhibit also antiandrogenic and antiprogestagenic action, but such activity *in vivo* was confirmed only for OMC. Another adverse effect of 4-MBC, BP-3 and OMC is related to the interaction with the hypothalamic–pituitary–thyroid axis (HPT) and as a result they can cause thyroid dysfunction (Schmutzler et al., 2007).

Experimental *in vivo* studies showed that BP-3, 4-MBC and OMC could rapidly pass through the skin to systemic circulation. They can be detected in plasma 1–2 h following application (Janjua et al., 2008). In the same experimental study, concentrations of UV filters in male plasma and urine were higher than in female samples, which indicated a gender-related differences in metabolism, distribution and in the accumulation of these compounds (Janjua et al., 2004). Importantly, dermal application is the main route of human exposure of these compounds, thus they enter the systemic circulation without being metabolized by the liver, posing a potential risk to all human tissues. The exposure of human body is significant and still growing. For example, in the U.S. population, the presence of benzophenone-3 and its metabolites was revealed in 98% of urine samples (Calafat et al., 2008). Human exposure to UV filters can occur via dermal absorption, but also through food chain, for example, by consumption of contaminated fish (Weisbrod et al., 2007; Fent et al., 2010). The presence of benzophenone derivatives was also shown in the adipose tissue, breast milk (Ye et al., 2008; Schlumpf et al., 2010) and semen (Schlumpf et al., 2008; Leon et al., 2010) where they can cause adverse effects. Breastfed babies are exposed to the action of UV filters, which are present in 85% samples of human milk (Schlumpf et al., 2010). BP-3 can also penetrate from blood to placenta and its high concentration in mothers' urine were associated with increased birth weight in boys and decreased birth weight in girls (Wolff et al., 2008).

The effects of sunscreens on the central nervous system (CNS) have not be studied, yet. Sunscreens as lipophilic compounds are likely to pass through the blood–brain barrier and, therefore, toxic effects on the central nervous system cells cannot be ruled out. Moreover, other compounds of similar structure and belonging, as UV filters, to the group of endocrine-disrupting chemicals are known to alter neuronal transmission, synaptic plasticity and induce apoptotic or neurotoxic changes (Kajta and Wojtowicz, 2013; Frye et al., 2012). In the present study, we investigated the effect of some UV filters on cell viability (LDH release and MTT reduction) and apoptotic process (caspase-3 activity and Hoechst staining) in human neuroblastoma cell line (SH-SY5Y cells). Considering human exposure to UV filters and their blood concentration, we have selected the following compounds: benzophenone derivatives: BP-3, BP-2, 4-MBP and other substances: 4-MBC and OMC. SH-SY5Y cell line represents a well-established experimental model to study neurotoxic liability of chemical compounds *in vitro*.

2. Materials and methods

2.1. Chemicals

Benzophenone-3 (2-hydroxy-4-methoxybenzophenone), benzophenone-2 (2,2′,4,4′tetrahydroxybenzophenone), 4-methylbenzophenone, 2-ethylhexyl-4-methoxycinnamate, 3-(4-methylbenzylidene) camphor were obtained from Sigma–Aldrich Ltd.

3-[4,5-Dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), methyl sulfoxide (DMSO, dimethyl sulfoxide) were obtained from Sigma–Aldrich Ltd.

2.2. Cell cultures

The SH-SY5Y neuroblastoma cell line was obtained from the American Type Culture Corporation (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Germany), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma–Aldrich Ltd.), and kept in humidified atmosphere of 5% CO_2/95% O_2 at 37 °C.

2.3. Treatment of cells

One day before the experiment, cells were seeded in 96-well plates in the medium with a reduced amount of serum (1% FBS). For Hoechst staining, cells were cultured on Millicell EZ slide (Millipore). The SH-SY5Y cells were treated with benzophenone-2, benzophenone-3, 4-methylbenzophenone, 3-(4-methylbenzylidene) camphor, 2-ethylhexyl-4-methoxycinnamate at concentrations from 10^{-8} to 10^{-4} M for 24 or 72 h. All investigated chemical compounds (BP-2, BP-3, 4-MBP, OMC, 4-MBC) were dissolved in the smallest possible volume of ethanol and then diluted with water and were added to culture medium in the volume of 10 μl. The control cultures were supplemented with the same amount of an appropriate vehicle in which the final concentration of ethanol was 0.8% for BP-2; 1.5% for 4-MBC and OMC; 1.9% for BP-3 and 2.3% for 4-MBP. To evaluate the effect of solvent on cell viability, the culture without solvent was also added to each assay. As a positive control of cell death and apoptosis respectively 1% Triton and 1 μM staurosporine were used.

2.4. Measurement of lactate dehydrogenase (LDH) release

Toxicity of chemical substances was measured by release of lactate dehydrogenase (LDH) into the culture media at 24 h or 72 h post treatment. LDH activity was determined in medium using a colorimetric method (Cytotoxicity Detection Kit, Roche Diagnostic GmbH, Germany), according to which in the reaction of pyruvic acid with 2,4-dinitrophenylhydrazine amount of colored hydrazone was formed, which is proportional to the LDH activity in the sample. It could be quantified by measuring the absorbance at 490 nm. The results were expressed as a percentage of control cells cultured only with the solvent (mixtures of water and ethanol). In this assay Triton, a compound that damages all cells was used as a positive control.

2.5. MTT reduction assay

Cell viability was measured at 24 h or 72 h post treatment, by determining the cellular reducing capacity. It was estimated from the extent of MTT reduction to the insoluble intracellular formazan, which depends on the activity of intracellular dehydrogenases and is independent of changes in the integrity of the plasma membrane. Briefly, culture medium was replaced and SH-SY5Y cells were incubated with MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide for 3 h at 37 °C. MTT was prepared in PBS and added at a final concentration of 0.15 mg/ml. Then, the crystals of formazan were dissolved in DMSO and the absorbance of each sample was measured at 570 nm in a Multiscan plate reader (Labsystem, USA). The results were expressed as a percentage of control cells incubated only with solvent.

2.6. Caspase-3 activity assay

Activity of caspase-3 was measured 24 h after treatment with UV filters, using fluorometric Caspase-3 assay kit (Sigma–Aldrich, St. Louis, MO, USA), according to which the amount of fluorochrome 7-amino-4-methyl coumarin (AMC) is released from the substrate Ac-DEVD-AMC (caspase-3 fluorogenic substrate) upon cleavage by enzymes, like caspase-3. A yellow-green fluorescence produced by free AMC is proportional to the caspase-3 activity present in the sample. Cell lysates were incubated with Ac-DEVD-AMC (50 μM) for 60 min at 30 °C both in the absence and in the presence of a specific caspase-3 inhibitor (Ac-DEVD-CHO; 50 μM) and the fluorescence was measured with a fluorescence plate reader at 360 nm excitation and 460 nm emission wavelengths. After subtracting non-specific fluorescence, caspase-3 activity was calculated from standard curve and presented as pmol of AMC/min/ml.

2.7. Hoechst staining

In order to visually assess apoptotic changes in neurons, such as nuclear condensation and cell shrinkage, the cells were stained with Hoechst dye (Hoechst 33342, Molecular Probes, Eugene, Oregon, USA) as described previously by Leskiewicz et al. (2008). Twenty four hours after treatment with UV filters (at a concentration of 10^{-7} and 10^{-8} M) or staurosporine, cells were fixed for 30 min with 4% paraformaldehyde and exposed to Hoechst 33342 (0.8 μg/ml) for 30 min. Cultures were washed 3-times with 10 mM PBS and dried. Nuclear condensation and cell body shrinkage were evaluated under a Leica fluorescence microscope. Cells with bright condensed or fragmented nuclei after Hoechst's staining were considered to be apoptotic. The number of cells with apoptotic morphology per one hundred cells was counted in four fields per one slide from three independent experiments. The results were shown as a percentage of apoptotic cells compared with total number of cells.

2.8. Statistical analysis

The data obtained in three independent experiments, each performed in 4–5 wells, were presented as the percentage of control ± SEM. The significance of differences between the means was evaluated by the Duncan's test following a one-way analysis of variance (ANOVA).

3. Results

The cells were exposed to 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M solution of UV filters for 24 or 72 h. Cytotoxic effect of benzophenone derivatives, OMC and 4-MBC was measured by the MTT reduction test and LDH release assay. After 24-h exposure to the test compounds in the culture medium, only BP-3 and 4-MBC, at the highest concentrations tested, showed a significant cytotoxic effect (Table 1). BP-3 at 10^{-4} M concentration increased by 35% and 4-MBC by 360% release of cytoplasmic enzyme lactate dehydrogenase to culture medium. The viability of cells, analyzed in the MTT reduction test, was decreased by about 15% by 10^{-4} M BP-3 and by about 75% by 10^{-4} M 4-MBC. Other compounds (BP-2, 4-MBP, OMC) did not change significantly both parameters studied (data not shown).

Exposure of SH-SY5Y cells to UV filters for 72 h produced cytotoxic effect and the results obtained with the MTT reduction test and LDH release assay were similar (Fig. 1). Benzophenone-2 at 10^{-5} M concentration decreased viability of neuroblastoma cells by about 15% and at 10^{-4} M by 25%, while lower concentrations of this compound had no effect (Fig. 1A). Similarly, LDH release was also increased only by two highest tested concentrations, 10^{-5} M by 18% and 10^{-4} M by about 50% (Fig. 1B). Other benzophenone derivatives have also given statistically significant results. Benzophenone-3 decreased viability of cells by about 15% at 10^{-5} M and by 25% at 10^{-4} M concentration (Fig. 1C). At the same concentrations, this compound also increased LDH activity by 19% and 44% for 10^{-5} M and 10^{-4} M concentrations, respectively (Fig. 1D). Similar cytotoxic effect was observed after 4-MBP and OMC. 4-Methylbenzophenone in a concentration of 10^{-5} M decreased by about 13% and in a concentration of 10^{-4} M by 17% MTT reduction and in the same concentrations enhanced by 20% and 50% LDH release (Fig. 1E and F). OMC decreased viability of cells by about 18% and 20% and increased LDH release by 20 and 50%, respectively, at 10^{-5} M and 10^{-4} M concentrations (Fig. 1G and H). 3-(4-Methylbenzylidene) camphor showed a cytotoxic effect only at the highest concentration tested. At 10^{-4} M, 4-MBC decreased MTT reduction by about 50% and increased LDH activity by 120% (Fig. 1I and J).

Table 1
The effect of benzophenone-3 and 3-(4-methylbenzylidene) camphor on LDH release and MTT reduction in SH-SY5Y cells. The neuroblastoma cells were treated with a mixture of water and ethanol (vehicle), benzophenone-3 (BP-3) or 3-(4-methylbenzylidene) camphor (4-MBC) at concentration from 10^{-8} M to 10^{-4} M for 24 h. The results are shown as a percentage of control cells incubated with mixtures of water and ethanol. Statistical significance of the effects was evaluated by Dunett's test following a one-way analysis of variance (ANOVA). $^{*}p < 0.05$ *vs.* control cultures.

Treatment	LDH	MTT
–	88.59 ± 3.9*	78.79 ± 3.3*
Vehicle	100 ± 4.6	100 ± 4.4
BP-3 10-8 M	101.44 ± 7.2	91.3 ± 2.4
BP-3 10-7 M	102.85 ± 5.5	92.3 ± 8.8
BP-3 10-6 M	97.39 ± 2.4	91.1 ± 4.4
BP-3 10-5 M	108.60 ± 7.6	88.3 ± 2.95
BP-3 10-4 M	135.14 ± 8.7***	83.2 ± 5.2*
Triton	336.37 ± 5.9***	24.1 ± 2.2***
–	91.59 ± 3.9	89.79 ± 3.3
Vehicle	100 ± 7.7	100 ± 4.7
4-MBC 10-8 M	105.1 ± 6.5	112.6 ± 7.7
4-MBC 10-7 M	99.2 ± 8.8	109.8 ± 6.3
4-MBC 10-6 M	104.1 ± 4.6	112.3 ± 5.6
4-MBC 10-5 M	103.3 ± 4.8	108.1 ± 3.0
4-MBC 10-4 M	360.1 ± 4.3***	26.2 ± 1.7***
Triton	402.1 ± 4.4***	23.8 ± 2.7***

Next, the effect of these compound on caspase-3 activity was determined. The activity of caspase-3, the main executive enzyme in programmed cell death, was measured using fluorometric Caspase-3 assay kit. It was found that the test UV filters enhanced the activity of this enzyme in a concentration-dependent manner and at concentrations lower by three orders of magnitude than those that evoked cytotoxic effect (Fig. 2). From our group of sunscreens, BP-3 and 4-MBC were the most potent chemical compounds, which increased activity of caspase-3 already in a concentration of 10^{-8} M (Fig. 2B and E). BP-2, 4-MBP and OMC affected caspase-3 activity in concentrations from 10^{-7} M to 10^{-4} M (Fig. 2A, C and D).

Since the increase in caspase-3 activity does not always lead to cell death, therefore, to demonstrate the presence of apoptosis Hoechst staining was performed. As in the case of caspase-3, cells were cultured with UV filters for 24 h and this assay was done for the two lowest concentrations of the test compounds, *i.e.*, 10^{-7} M and 10^{-8} M, thus the concentrations that may be reached in *in vivo* conditions.

As with caspase-3 activity, BP-2, BP-3, 4-MBC and OMC at 10^{-7} M concentration significantly increased the number of cells with fragmented nuclei (Figs. 3 and 4). Moreover, these compounds already at a concentration of 10^{-7} M intensified chromatin condensation. Only 4-MBP, which raised the caspase-3 activity did

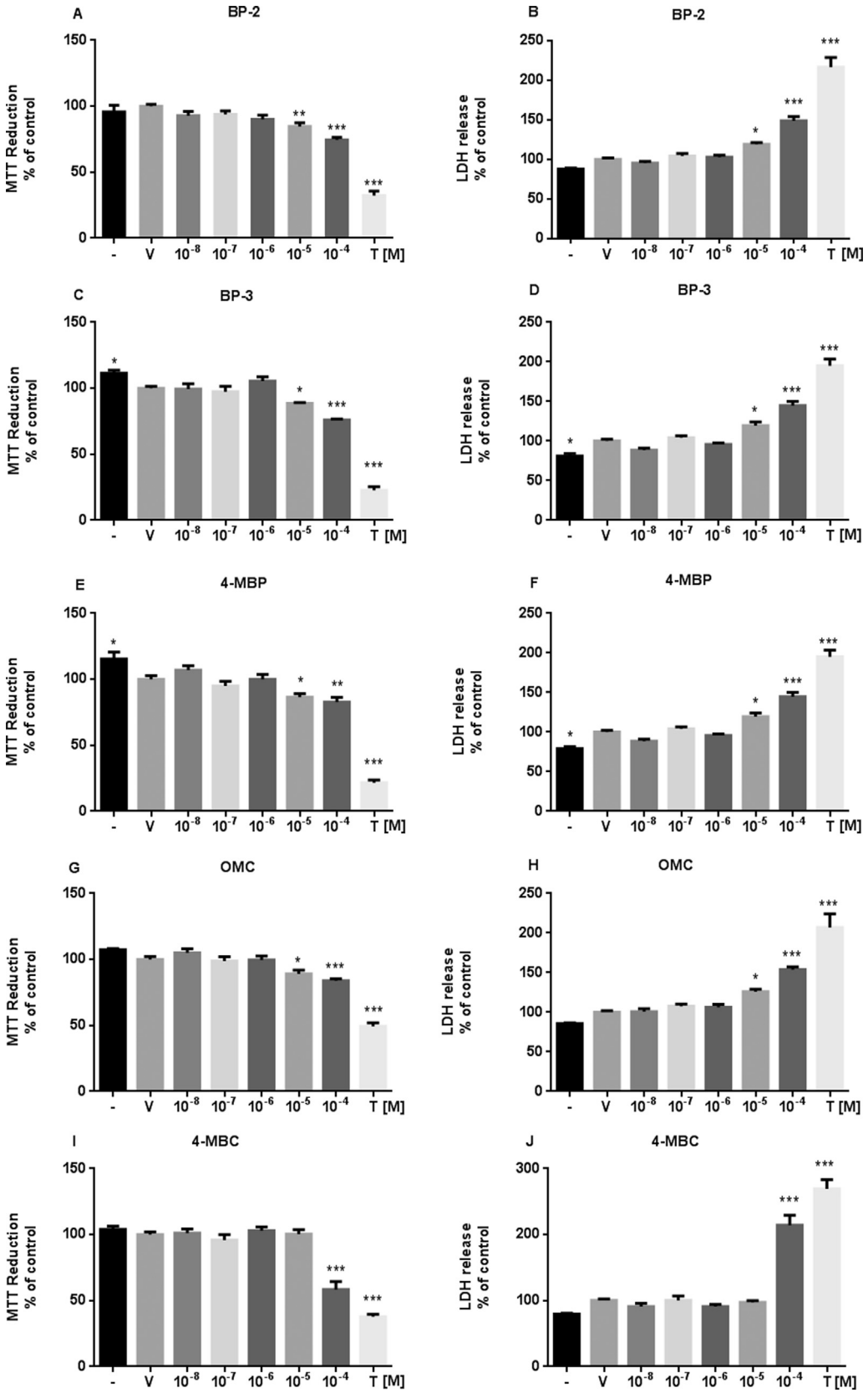

Fig. 1. The effect of benzophenone-2 (BP-2; A and B), benzophenone-3 (BP-3; C and D), 4-methylbenzophenone (4-MBP; E and F), 2-ethylhexyl-4-methoxycinnamate (OMC; G and H), and 3-(4-methylbenzylidene) camphor (4-MBC; I and J) on MTT reduction and LDH release in SH-SY5Y cells. The results are shown as a percentage of control cells incubated with mixtures of water and ethanol (V—vehicle). Statistical significance of the effects was evaluated by Dunett's test following a one-way analysis of variance (ANOVA). $^{*}p < 0.05$ *vs.* control cultures.

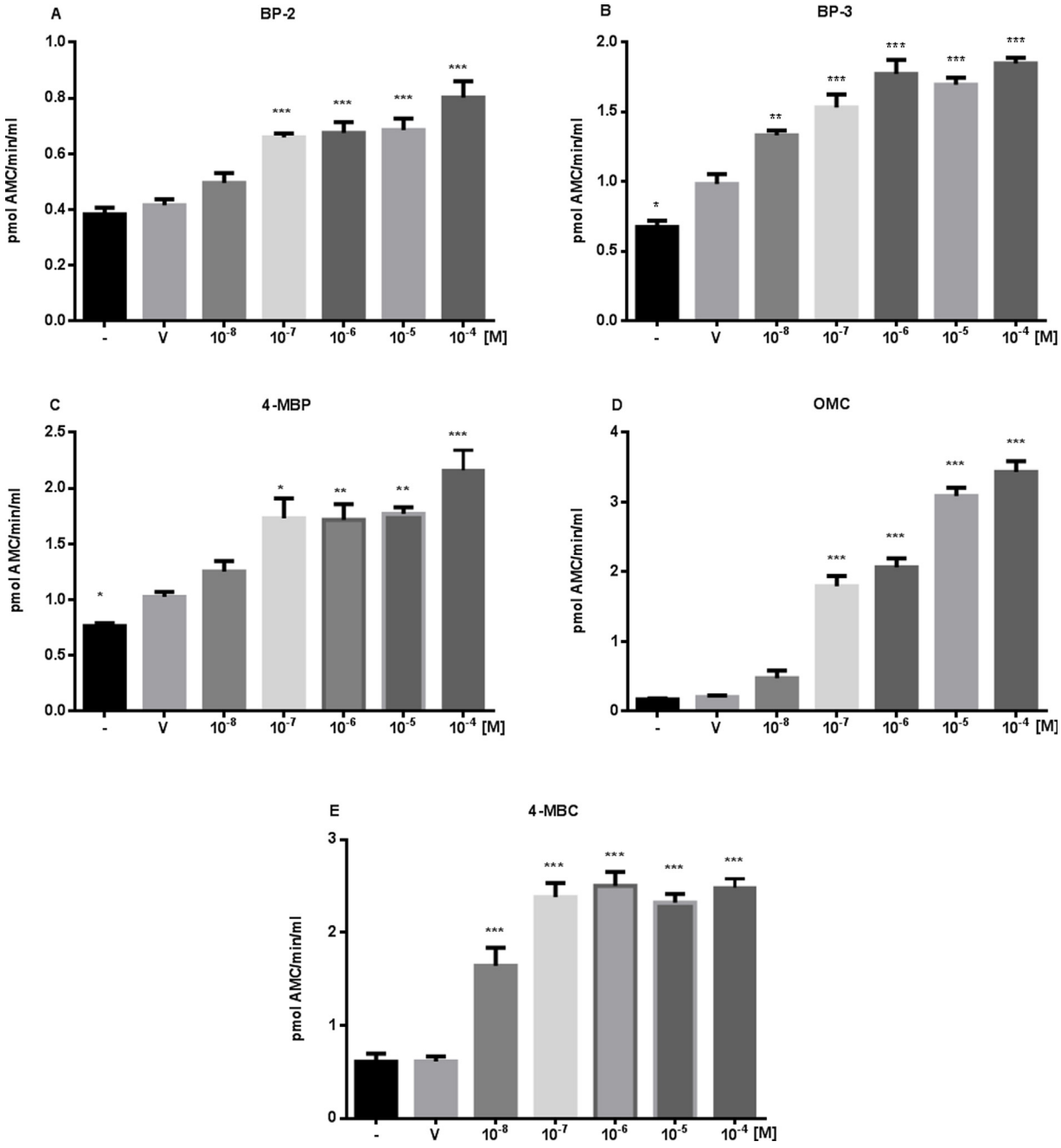

Fig. 2. The effect of benzophenone-2 (BP-2; A), benzophenone-3 (BP-3; B), 4-methylbenzophenone (4-MBP; C), 2-ethylhexyl-4-methoxycinnamate (OMC; D) and 3-(4-methylbenzylidene) camphor (4-MBC; E), V-vehicle on caspase-3 activity in SH-SY5Y cells. The results are shown as a caspase-3 activity in pmol of AMC released per minute per ml of cells lysate. Statistical significance of the effects was evaluated by Dunett's test following a one-way analysis of variance (ANOVA). $^{*}p < 0.05$ *vs.* control cultures.

not increase the number of apoptotic cells. However, in the case of this compound, the final concentration of ethanol in culture medium was 2.3% and this vehicle alone significantly intensified chromatin condensation and under these conditions 4-MBP did not enhance the effects of ethanol.

Due to difficulties in dissolving BP-3 and 4-MBP, it was necessary to use a relatively large volume of ethanol, so as a result in the case of BP-3 cells were cultured in the presence of 1.9% ethanol and for 4-MBP culture medium contained 2.3% of ethanol. Such amount of alcohol induced cell damage leading to the increased LDH release and lowered MTT reduction, while the ethanol content up to 1.5% (in the case of other compounds) did not significantly affect these markers (Table 1,Fig. 1). Similarly, the presence of 1.9% or 2.3% ethanol in culture medium significantly increased caspase-3 activity and the number of apoptotic cells (Figs. 2 and 4).

4. Discussion

The present *in vitro* study demonstrated a significant influence of UV filters on SH-SY5Y neuroblastoma cells. Cytotoxic effect of these compounds was time-dependent, because after 24 hours only BP-3 and 4-MBC and only in a very high concentration (10^{-4} M) attenuated MTT reduction and increased LDH release,

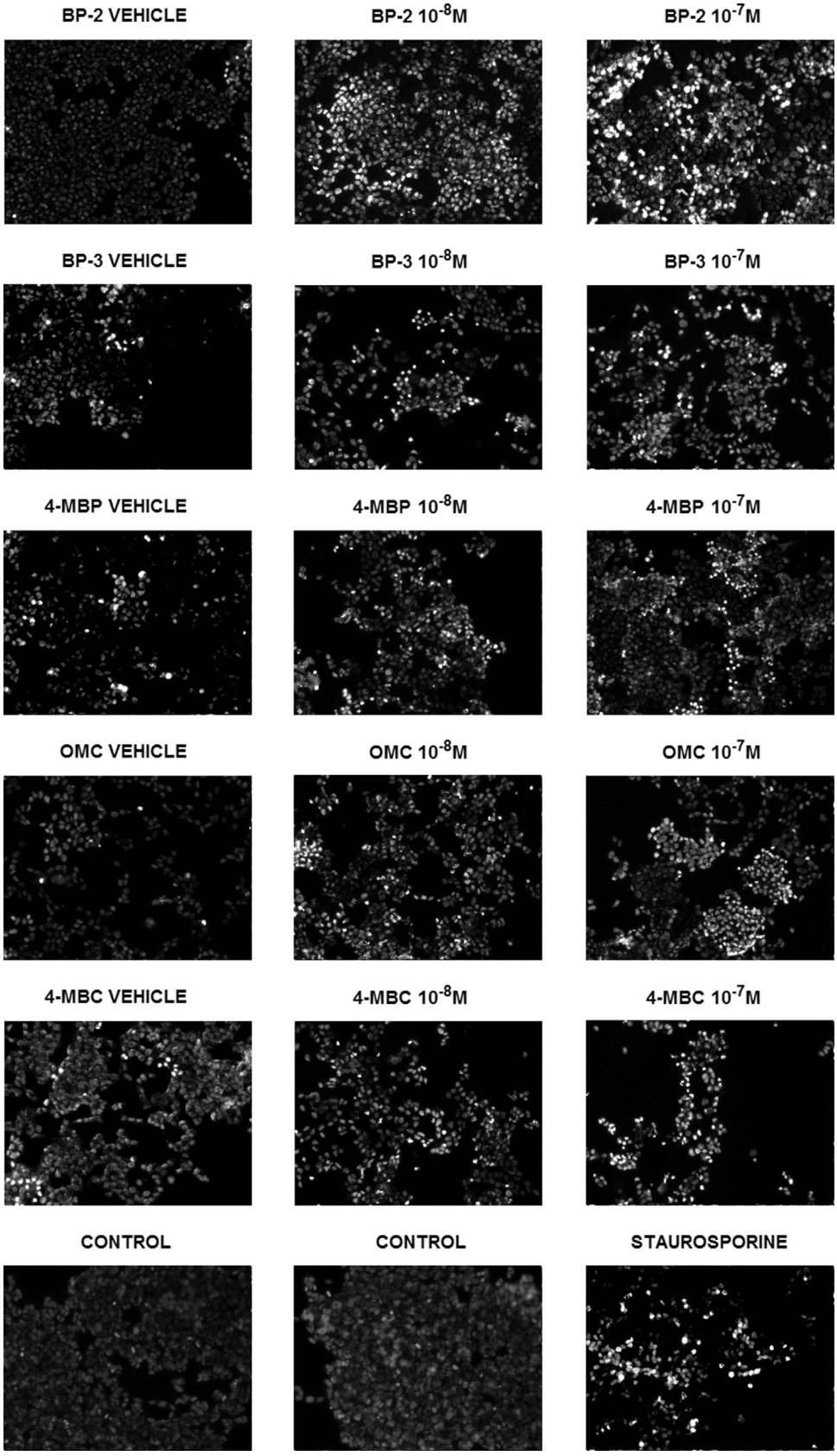

Fig. 3. Photomicrographs of the effect of benzophenone-2 (BP-2), benzophenone-3 (BP-3), 4-methylbenzophenone (4-MBP), 2-ethylhexyl-4-methoxycinnamate (OMC), and 3-(4-methylbenzylidene) camphor (4-MBC) on the number of cells with apoptotic morphology after staining with Hoechst 33342. Cells were cultured for 24 h with: medium alone (–), medium with appropriate vehicle (V) and UV filter in a concentration of 10^{-8} or 10^{-7} M or staurosporine.

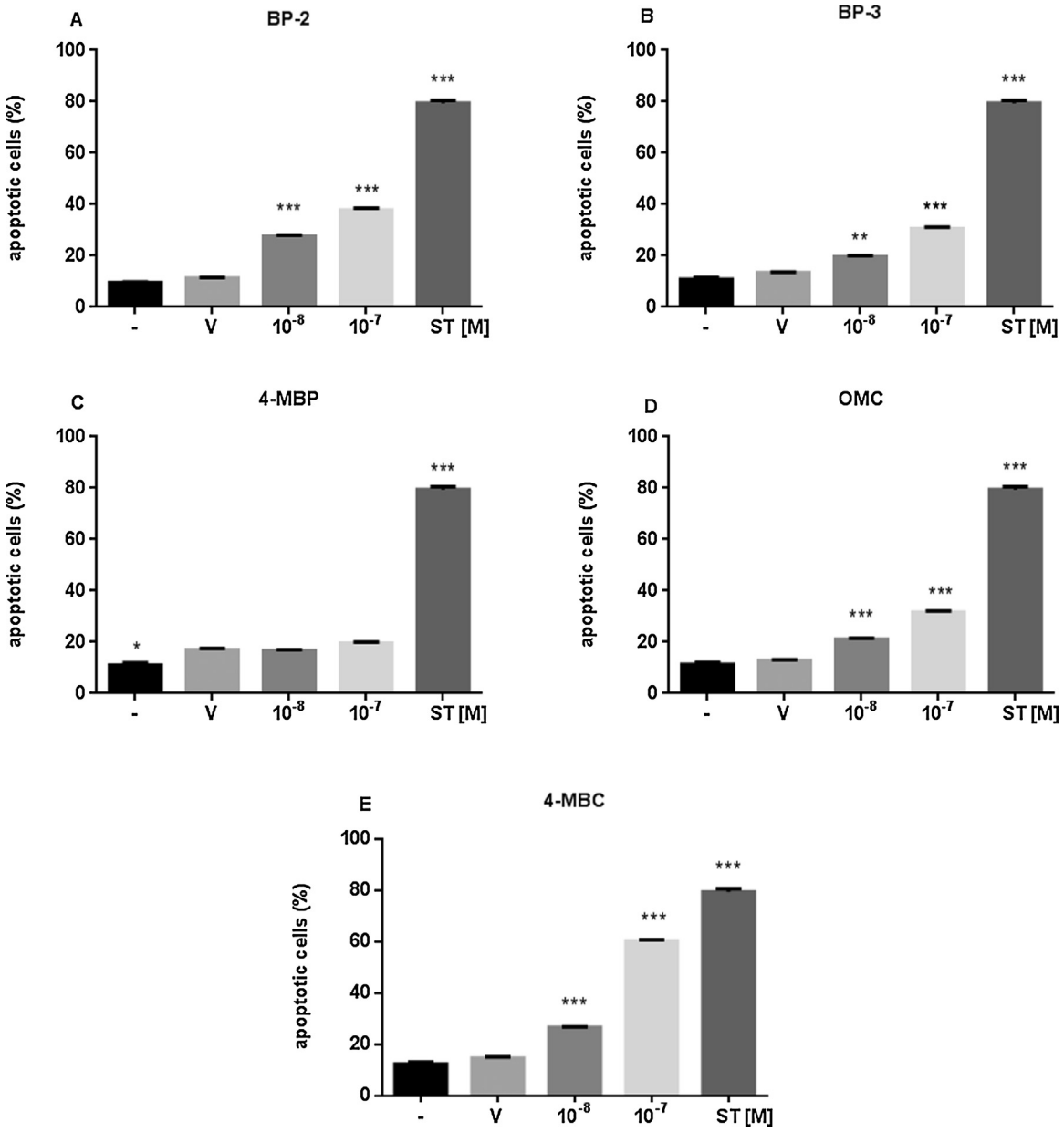

Fig. 4. The effect of benzophenone-2 (BP-2; A), benzophenone-3 (BP-3; B), 4-methylbenzophenone (4-MBP; C), 2-ethylhexyl-4-methoxycinnamate (OMC; D), and 3-(4-methylbenzylidene) camphor (4-MBC; E) on the number of cells with apoptotic morphology after staining with Hoechst 33342. Cells were cultured for 24 h with: medium alone (−), medium with appropriate vehicle (V) and UV filter in a concentration of 10^{-8} or 10^{-7} M or staurosporine (ST). The results are shown as a percentage of apoptotic nuclei compared with the total number of cells. Statistical significance was evaluated by Dunett's test following a one-way analysis of variance (ANOVA). $^{*}p < 0.05$ *vs.* cultures with appropriate vehicle.

while other test compounds had no effect on these parameters. When exposure of neuroblastoma cell line to UV filters was extended to 72 h statistically significant results for BP-3, BP-2, 4-MBP and OMC were observed in concentrations from 10^{-5} M to 10^{-4} M in both tests and only 4-MBC caused cell damages in the same concentration (10^{-4} M) as during 24 h exposure in culture medium. The fact that the test compounds at the same concentrations affected not only MTT reduction but also LDH release indicated the induction of necrotic cell death. MTT reduction itself, reflecting the activity of mitochondrial enzymes, may be caused by a decrease in metabolism and thus cell viability but also may be due by changes in cell proliferation. However, concomitant increase in LDH activity in the culture media showed a damage of cellular membrane and in consequence necrotic cell death. The increased necrosis was evoked after high concentrations of the test compounds, most probably higher than their putative concentration in the brain after dermal exposure to UV filters.

In contrast to necrotic changes, activity of the main apoptotic enzyme, caspase-3, was increased in cell cultured with low concentrations of the tested compounds. All tested UV filters increased caspase-3 activity in a concentration about three orders of magnitude lower than those that evoked necrosis, so at concentrations that they may reach *in vivo*. However, caspase-3 activation is not a conclusive evidence that these compounds indeed induce apoptotic cell death since some studies indicated that activation of this enzyme was involved not only in apoptosis but also in migration, proliferation and differentiation processes of brain cells (Garnier et al., 2004). However, visualization of chromatin condensation or nucleus fragmentation by Hoechst's

staining after culturing cells with UV filters indicated that BP-2, BP-3, 4-MBC and OMC in fact induced the process of apoptosis. On the other hand, Hoechst's staining did not confirm the proapoptotic activity of 4-MBP, but this may result from the adverse action of the ethanol used to dissolve this compound.

The results of the present study suggest that the test UV filters may be *in vivo* the cause of induction or exacerbation of apoptotic process in brain cells, but rather they should not induce the process of necrosis. On the other hand, it should also be take into account that in the current studies, the cells were exposed to test compounds for up to 72 h, while people may be exposed for a lifetime.

Data on concentrations of UV filters in the body are still scarce and on the central nervous system function are lacking at all. Most of the studied adverse effect of sunscreens are estimated after their oral exposure. However, the major route of UV filters absorption to human body is via dermal application, therefore, they enter the circulatory system without being metabolized in the liver. Thus, there is a high risk that UV filters reach all tissue of the body, as was observed in rats after dermal exposure to 3-(4-methylbenzylidene) camphor (Søeborg et al., 2006).

Among the chemical UV filters, BP-3, 3-benzylidene camphor (3-BC), 4-MBC and OMC, tested in humans, are absorbed through the skin to the greatest extent, while BP-3 concentration in human serum is about 10 fold higher than those of 4-MBC and OMC (Krause et al., 2012). It has been demonstrated that after dermal application of a formulation containing 5% BP-3, the maximum concentration of this compound in plasma was 200–300 μg/l, and after 24 h about 80–200 μg/l (Tarazona et al., 2013). Similarly, Janjua et al. (2008) found that after a single application of the BP-3, its concentration in the serum in men was about 250 ng/ml. Thus, blood levels of BP-3 (10^{-6}–10^{-7} M) or 4-MBC and OMC (10^{-7}–10^{-8} M) are in a concentration range which in the present study significantly increased caspase-3 activity and evoked nuclear changes.

So far, little is known about the level of UV filters in the tissues. BP-3 as a lipophilic compound accumulates in organs, especially in the liver, kidneys, spleen and testes, and its concentrations in these organs in rats is about 10-fold higher than in serum (Okereke et al., 1994). BP-3 levels in the brain have not been determined but due to its lipophilic properties and the fact that this compound is transferred to the fetus and milk, BP-3 should, to a considerable degree, pass through the blood–brain barrier. BP-3 and OMC were detected in lipid fraction of human milk at appropriately 117 ng/g of lipids and 35.5 ng/g of lipids (Schlumpf et al., 2010). So far, the demonstration that this compound affected the expression of ERα androgen receptor (AR) and aromatase in the brain of fish (Blüthgen et al., 2012) is the only evidence of the central action of BP-3. In contrast to the BP-3, 3-BC was demonstrated to be present in the rat brain following topical application and 4-MBC was shown to affect gene expression in the brain (Faass et al., 2009; Søeborg et al., 2006).

The obtained results suggest that UV filters may adversely affect the structure and function of nerve cells and in consequence be one of the factors involved in the pathogenesis of neurodegenerative disorders. Current data indicate that apoptotic process plays a key role in pathogenesis of neurodegenerative disorders, such as Parkinson's disease or Alzheimer's diseases (Uttara et al., 2009). It is worth mentioning that in neurodegenerative diseases apoptotic changes in brain cells appear many years before clinical symptoms of these diseases, so the exposure to even weak toxins, which do not cause significant changes in the CSN in a short time, in a long term can interfere with any of the major mechanisms of neuroprotection and in turn gradually initiate or potentiate apoptotic process. Because exposure to UV filters is increasing, these compounds should also be taken into consideration as one of the possible factors involved in nerve cells damage. Thus, studies on their effect on the CNS function and in particular, the exact determination of their effect on nerve cell apoptosis, are really needed.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Sequential assessment via daphnia and zebrafish for systematic toxicity screening of heterogeneous substances☆

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ABSTRACT

Environment and organisms are persistently exposed by a mixture of various substances. However, the current evaluation method is mostly based on an individual substance's toxicity. A systematic toxicity evaluation of heterogeneous substances needs to be established. To demonstrate toxicity assessment of mixture, we chose a group of three typical ingredients in cosmetic sunscreen products that frequently enters ecosystems: benzophenone-3 (BP-3), ethylhexyl methoxycinnamate (EHMC), and titanium dioxide nanoparticle (TiO_2 NP). We first determined a range of nominal toxic concentration of each ingredient or substance using Daphnia magna, and then for the subsequent organismal level phenotypic assessment, chose the wild-type zebrafish embryos. Any phenotype change, such as body deformation, led to further examinations on the specific organs of transgenic zebrafish embryos. Based on the systematic toxicity assessments of the heterogeneous substances, we offer a sequential environmental toxicity assessment protocol that starts off by utilizing Daphnia magna to determine a nominal concentration range of each substance and finishes by utilizing the zebrafish embryos to detect defects on the embryos caused by the heterogeneous substances. The protocol showed additive toxic effects of the mixtures. We propose a sequential environmental toxicity assessment protocol for the systematic toxicity screening of heterogeneous substances from Daphnia magna to zebrafish embryo in-vivo models.

1. Introduction

Contemporary ecological evaluation protocols have a critical problem that can inaccurately assess toxic effects of mixtures, and we may misunderstand their true toxicology. The misunderstanding can underestimate the mixtures' toxic effects, which can pose a threat to public health. Most evaluation methods are based on an individual substance's toxicity rather than on a whole mixture of various substances (Abdelraheem et al., 2015; Backhaus and Faust, 2012; Buckley and Farraj, 2015; Herzog et al., 2013; Marcone et al., 2012; Nabeshi et al., 2011; Nazarenko et al., 2012; Rider et al., 2013). Pesticides for agriculture (Bussy et al., 2015; Jang et al., 2014b; Seiber et al., 2014), industrial products for consumers (Álvarez et al., 2009; El-Didamony et al., 2013; Mastella et al., 2014; Misik et al., 2014), and by-products from the automobile and electronic industries (Feng et al., 2015; Snow et al., 2014) are introduced into ecosystems in mixture forms. Besides toxic effects caused by single substances, there are toxic effects caused by a culmination of heterogeneous substances that eventually affect people. In order to truly portray the toxic effects of substances, toxicity evaluation targeting heterogeneous substances is a next step after understanding toxicology of a substance.

Sunscreen is widely used all across the world (Kaiser et al., 2012b). Sunscreen is a cosmetic mixture of tens of various substances and its popularity in use for skin protection against UV-light from the sun spontaneously results in the introduction of the mixture into the environment. In this regard, we chose several substances from sunscreen as the subject of this study (Fent et al.,

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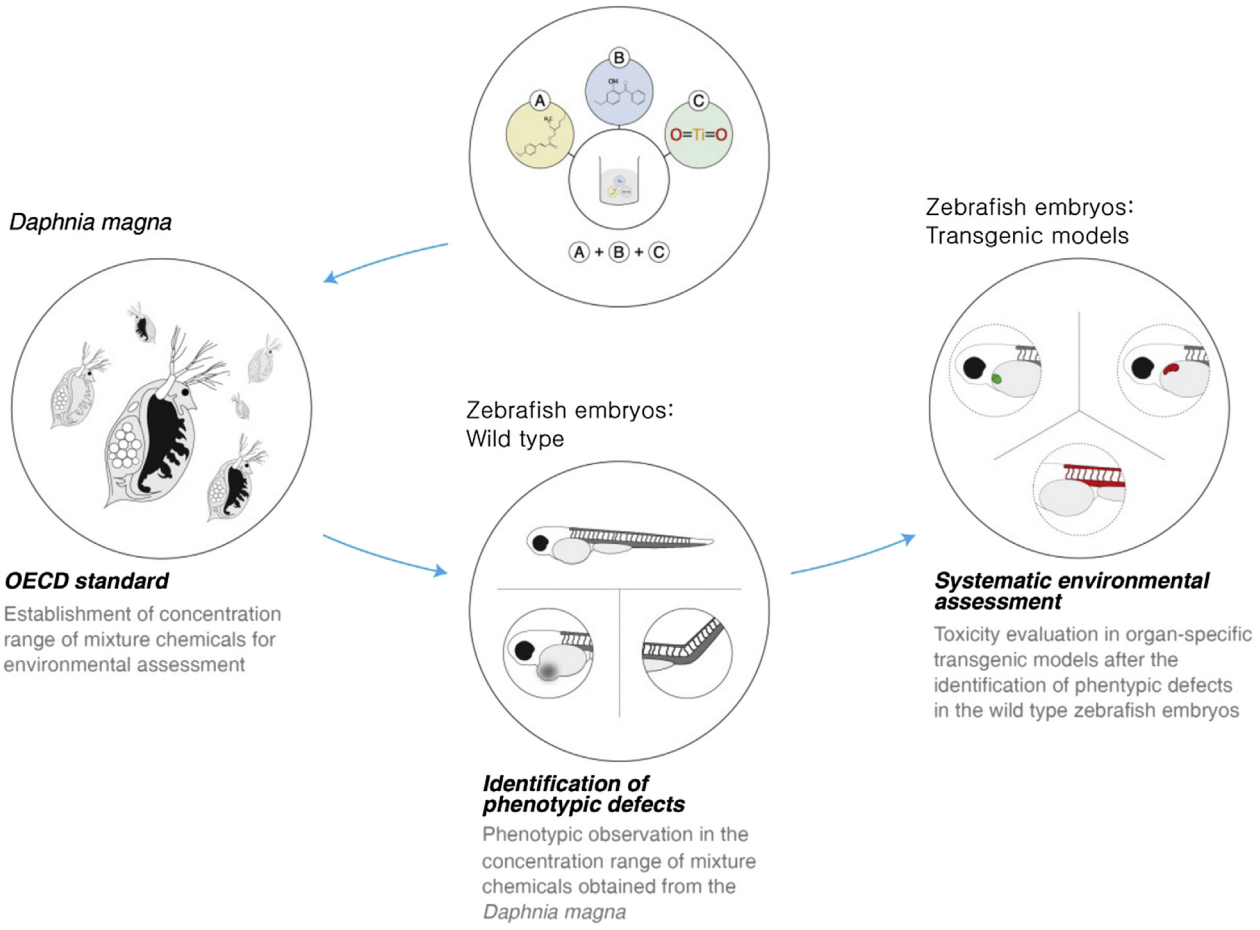

Scheme 1. Sequential environmental toxicity assessment of heterogeneous substances based on the Daphnia magna, wild-type zebrafish, and transgenic model embryos.

2010; Liao and Kannan, 2014; Rodil et al., 2008).

We refer to the substances of interest from sunscreen as UV-filter substances from this point on. The compositions of the substance mixtures used for this research were chosen based on currently used substances in commercial products. Substances composing sunscreen products are largely divided into two groups (Jain and Jain, 2010): one group emphasizes instantaneous and powerful UV protection that is heavily based on chemicals (Gago-Ferrero et al., 2012; Ignasiak et al., 2015; Jain and Jain, 2010; Kim and Choi, 2014; Roussel et al., 2015), while the other group advocates a slower rate of UV protection application through the use of sunscreens that utilize nanoparticles (Bayat et al., 2015; Cho et al., 2013; Moloney et al., 2002; Morabito et al., 2011; Popov et al., 2010; Samberg et al., 2010; Schroeder and Krutmann, 2010). The former can be seen as the more effective group, but the very chemicals that provide instant skin protection do not assure non-toxic effects to the human body, and are thus recommended in small amounts. In contrast, the sunscreens that utilize nanoparticles, although they do not apply instantaneous UV protection, are generally considered to be safer on the human body than their counterparts.

27 sunscreens are listed in the Cosmetics Directive, and 43 chemicals are listed as UV-filters in ingredients used in cosmetics in the EU (Wahie et al., 2007). In U.S. Food and Drug Administration (FDA)-approved sunscreen substances, the concentration of a specific UV-filter varies in between 0.5 and 25% (Sambandan and Ratner, 2011). It is estimated that these UV filters have been constantly produced more than 10,000 tons annually for the global market and entered aquatic environment via either direct pathways such as human recreational activities or indirect pathways such as wastewater (Ramos et al., 2015). To evaluate the combined toxicities of mixtures with various combination of UV-filters rather than a single UV-filter, two different types of UV-filters, ethylhexyl methoxycinnamate (EHMC) (UVA protection) (Christen et al., 2011; Kaiser et al., 2012a; Sieratowicz et al., 2011) and Benzophenone-3 (BP-3) (UVB protection) (Kim and Choi, 2014; Kim et al., 2014; Sieratowicz et al., 2011), were chosen based on their toxicities to aquatic organisms. To better understand the interaction between toxic- and non-toxic substances in a mixture, titanium dioxide nanoparticles (TiO_2 NPs) was also chosen in this study, due to the reports that there was no noticeable toxicity of TiO_2 NPs (George et al., 2014; Marcone et al., 2012; Pavagadhi et al., 2014; Strigul et al., 2009; Zhang et al., 2012; Zhu et al., 2010) to aquatic organisms such as Vibrio fischeri, Daphnia magna, and zebrafish embryo (approximately up to 10 mg/L).

Among the three substances we chose to combine into a single mixture for our research, BP-3 and EHMC, have already been individually evaluated regarding their toxicities. The third substance, TiO_2 NPs, is a physical UV-filter substance. These three substances were combined into various single mixtures that each had different substance compositions. The various mixtures were subject to toxicity assessment.

For the *in-vivo* models, we chose Daphnia magna and zebrafish to carry out the sequential environmental toxicity assessment (Scheme 1). Daphnia magna is a recommended standard *in-vivo* model by the Organization for Economic Co-operation and

Table 1
Nominal test concentrations and heterogeneous substance combinations of the 3 different UV-filters, Ethylhexyl methoxycinnamate (EHMC), benzonphenone-3 (BP-3), and titanium dioxide (TiO_2) NPs, in Daphnia magna and zebrafish embryos.

Tested 3 different UV-filters				
Toxicity	EHMC (μg/ml)	BP-3 (μg/ml)	TiO_2 (μg/ml)	Reference
EC_{10} for Daphnia magna	0.1	1	–	(Sieratowicz et al., 2011; Strigul et al., 2009; Zhu et al., 2010)
EC_{50} for Daphnia magna	0.5	2	–	
Nominal test concentration of UV filters for Daphnia magna and zebrafish embryo				
EHMC (μg/ml)	0.1, 0.5, 1.0, 2.0, 4.0			
BP-3 (μg/ml)	1.0, 2.0, 4.0, 8.0, 10.0			
TiO_2 (μg/ml)	1.0, 2.0, 4.0, 8.0, 10.0			

Mixture combination of UV filters for daphnia magna and zebrafish embryo				
	EHMC (μg/ml)	BP-3 (μg/ml)	TiO_2 (μg/ml)	Reference
Mix. 1	0.1	1.0	1.0	(Sambandan and Ratner, 2011)
Mix. 2	0.5	1.0	1.0	
Mix. 3	0.1	2.0	2.0	
Mix. 4	0.5	2.0	2.0	
Mix. 5	0.1	4.0	4.0	
Mix. 6	0.5	4.0	4.0	

'-' = Not calculated.

Development (OECD) because it shows sensitive reactions when exposed to substances. (OECD) Our nominal concentrations were established based on the 50% effective concentrations (EC_{50}) obtained from toxicity assessments with Daphnia magna. We introduce Zebrafish in-vivo model to comply with the OECD's policy regarding animal model use for ecological evaluation. It is an advantageous model to evade the strict policy of OECD—which does not allow animal experiments for ecological evaluations—while fulfilling the need for in-vivo assessment. A zebrafish embryo until 7 days post fertilization is not considered to be an animal. Zebrafish embryo is an excellent model, because in-vivo model is an essential part of toxicology of substances to understand what really goes on in biological system. These nominal concentrations were used as guidelines to treat the wild-type zebrafish embryos, whose viability and morphology were then assessed. To observe the toxic effects on the internal organs of zebrafish embryos, the heart and liver of the transgenic models were used and monitored, and data obtained was then compared to other results reported in published literature.

2. Material and methods

2.1. Daphnia magna

Daphtoxkit F™ involving Daphnia magna ephippia was purchased from MicroBioTests Inc., Gent, Belgium. Daphnia magna ephippia was cultured for 72 h in ISO standard freshwater of Daphtoxkit F™. Hatched Daphnia magna neonates were transferred into a 500-ml glass beaker filled with 400-ml filtered tap water from a water recirculating system (20–30 daphnia per beaker) and continuously subcultured under a long photoperiod (16L: 8D, 16 h of light followed by 8 h of darkness) at 22 ± 1 °C water temperature in a climate incubator at Korea Institute of Science and Technology Europe (KIST Europe), Germany. The Daphnia magna were fed 1×10^5 cells/ml *Spirulina* once daily and culture water was also exchanged once daily (≥80%). The Daphnia magna whose neonates were collected and used for acute toxicity test in this study had been bred in captivity for over 15 generations in the KIST Europe laboratory.

2.2. Zebrafish models

This study used Tg(cmlc2:EGFP), Tg(fli1:mCherry), and Tg(lfabp:DsRed) lines, which express enhanced green fluorescent proteins, mCherry fluorescent proteins, and red fluorescent proteins on the surfaces of cardiac tissue, blood vessels, and liver tissue respectively, and wild-type (standard AB strain) zebrafish. Each zebrafish line was kept at 28 °C under a daily cycle of 14 h of light exposure and 10 h of dark conditions (Westerfield, 2007). The zebrafish embryos were gathered from the natural mating of their parents.

2.3. UV-filter substances

Ethylhexyl methoxycinnamate (EHMC, CAS 5466-77-3), benzophenone-3 (BP-3, CAS 131-57-7), and liquid type TiO_2 NP (<100 nm size, CAS 13463-67-7) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of EHMC and BP-3 UV-filter substances were dissolved in methanol (Sigma-Aldrich, purity > 99%) at a concentration of 1 mg/ml, and were stored at −20 °C until used for the toxicity tests. The aqueous solution of TiO_2 NPs was diluted in distilled water at a concentration of 1 mg/ml and stored at room temperature for all toxicity tests.

2.4. Test concentrations

Combinations of the chemicals and the nanoparticle in mixtures were prepared the stock solutions of BP-3, EHMC, which were dissolved in methanol, and TiO_2 NPs which was diluted in distilled water. Daphnia and zebrafish eggs were immediately exposed to the mixtures to prevent precipitation and aggregation of chemicals and the nanomaterial, before beginning to the test for mixture toxicity.

Nominal test concentrations of each UV-filter substance and their collaboration mixtures are shown in Table 1. The test concentrations of the three different UV-filter substances and their mixture combinations were set at a range of toxicological properties based on the lowest observed effective concentration (i.e., EC_{10}) and the 50% effective concentration (i.e., EC_{50}) for Daphnia magna (Sieratowicz et al., 2011). The concentrations of TiO_2 NPs in mixtures of UV-filters were kept the same as those of BP-3 as several studies examining the toxicological effects of TiO_2 NP on Daphnia magna reported that the value of EC_{50} for TiO_2 was higher than 100 mg/L after 48 h of exposure (Strigul et al., 2009; Zhu et al., 2010). The composition ratio between the three different UV-filter substances when they were in a heterogeneous substance state were influenced by the mixture composition of

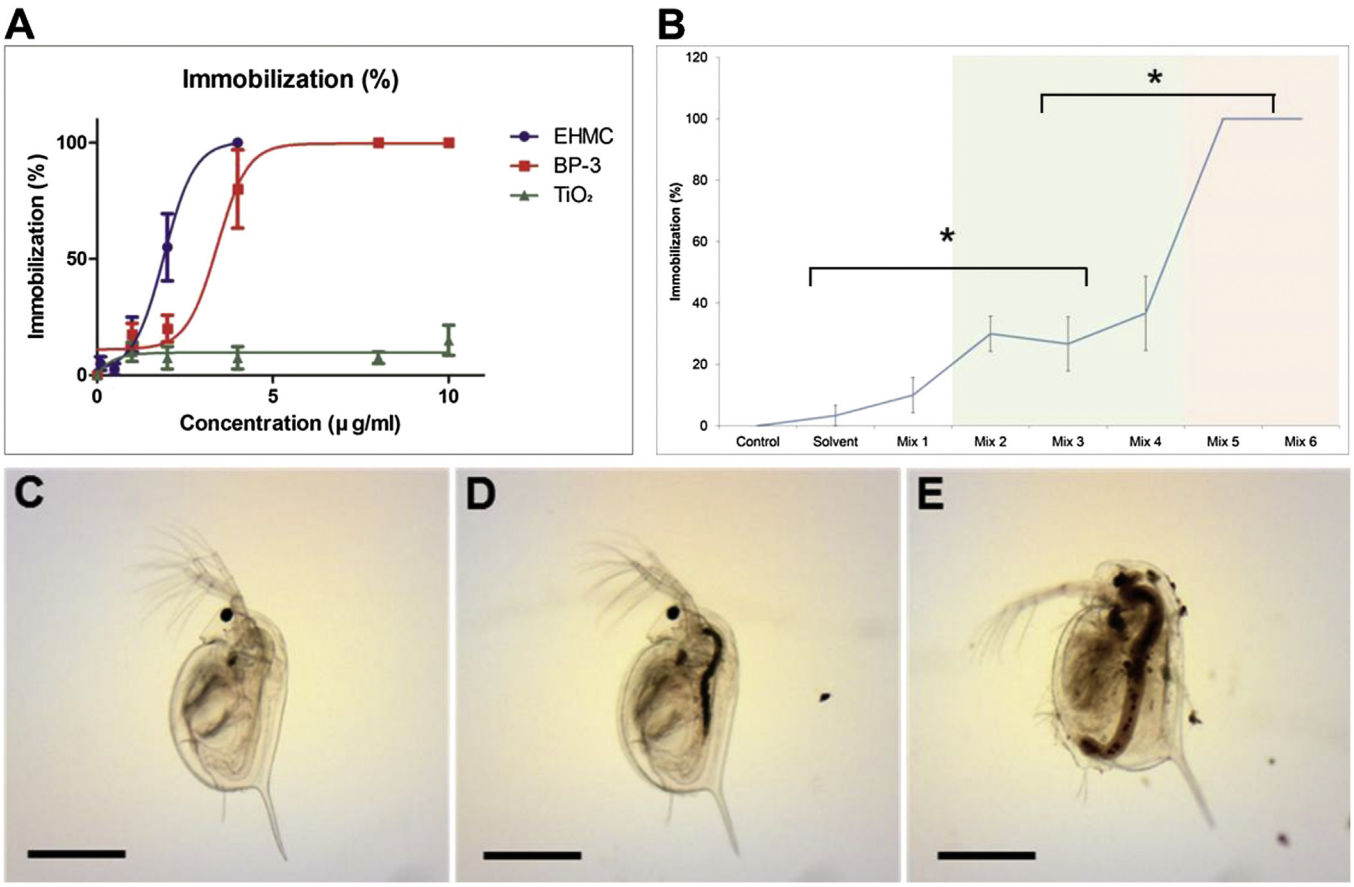

Fig. 1. Comparison of results from individual UV-filter substance treatments and heterogeneous substance treatments on Daphnia magna. (A) Fitted sigmoidal dose-response curves for the tested each UV-filter substance, ethylhexyl methoxycinnamate (EHMC), benzonphenone-3 (BP-3), or titanium dioxide (TiO_2) NP, (B) Toxic effects between three heterogeneous UV-filter mixtures. Values of means ± SEM (n = 3–4). Values of 50% effective concentration (EC_{50}) for EHMC and BP-3 on the immobilized Daphnia magna were 2.17 ± 0.26 and 3.03 ± 0.38, respectively. * denotes significant differences between all groups ($P < 0.05$). (C) Phenotypic observation of control group in Daphnia magna. Phenotypic observation of (D) live Daphnia magna and (E) the dead Daphnia magna exposed to mixture 5 and mixture 6. All scale bars = 500 μm.

current substances used in sunscreen products as approved by the Food and Drug Administration (FDA) (Sambandan and Ratner, 2011).

2.5. Toxicity test of UV-filters and their mixtures on daphnia magna and zebrafish embryo

Both the Daphnia magna acute toxicity test and the zebrafish embryo developmental toxicity test were conducted in 6 well cell culture plates (Cellstar®, greiner bio-one, Germany) filled with 10 ml per test solution. Tap water was filtered with Millipore® 0.22 μm GSWP filter (Merck KGaA, Germany) and sterilized at 120 °C for 2 h, and functioned as either the dilution medium, or the control medium. The amount of the methanol was set to a maximum of 0.001% in the media for all toxicity tests. The methanol was used as the solvent control medium.

2.6. Daphnia magna acute toxicity test

The acute toxicity tests were conducted for 48 h with younger than 8 h old Daphnia magna neonates and were performed with 4 replicates per exposure group (10 Daphnia magna per replicate) in compliance with the OECD Test Guideline 202 (OECD, 2004). The immobilization (mortality + immobility) at each exposure group was recorded by calculating the 50% effective concentration (EC_{50}) for the three different UV-filters. Acute toxicities between the concentrations of components in the substance mixtures of UV-filters were also assessed by Daphnia magna immobilization.

2.7. Zebrafish embryo developmental toxicity test

Developmental toxicity tests using embryos at 6 h post fertilization (hpf) were performed with 2 or 3 replicates per exposure group and 10–30 fertilized eggs per replicate, as indicated in the OECD Test Guideline 212 (OECD, 1998). To examine the toxicities of each UV-filter and a mixture of a combination of the UV-filters on zebrafish embryos, the wild type zebrafish (i.e., AB strain) embryos were exposed to each UV-filter or a mixture for 6 days. The test solutions were refreshed once every 3 days. During an experimental period, unhatched eggs, dead larva, and abnormal larva were recorded to calculate biological response index [BSI = (unhatched egg involving dead eggs + total dead larva + survival abnormal larva)/(initial) × 100]. Embryos were assessed for embryotoxic (i.e., LC_{50}) and developmental toxic effects (i.e., BSI) for 6 days after exposure to each UV-filter and the mixtures. To investigate the organ-specific toxicity, transgenic zebrafish embryos (i.e., Tg strain) were exposed to each UV-filter or the mixtures. The test solutions were exchanged every 2 days during an exposure period of 96 h. ZEISS Stemi 2000, LEICA MZFLIII, ZEISS Imager Z1 and ZEISS axioskop, LEICA S6D, and LEICA DMI6000B were used to observe the transgenic zebrafish embryos at approximately 48, 72, and 96 hpf.

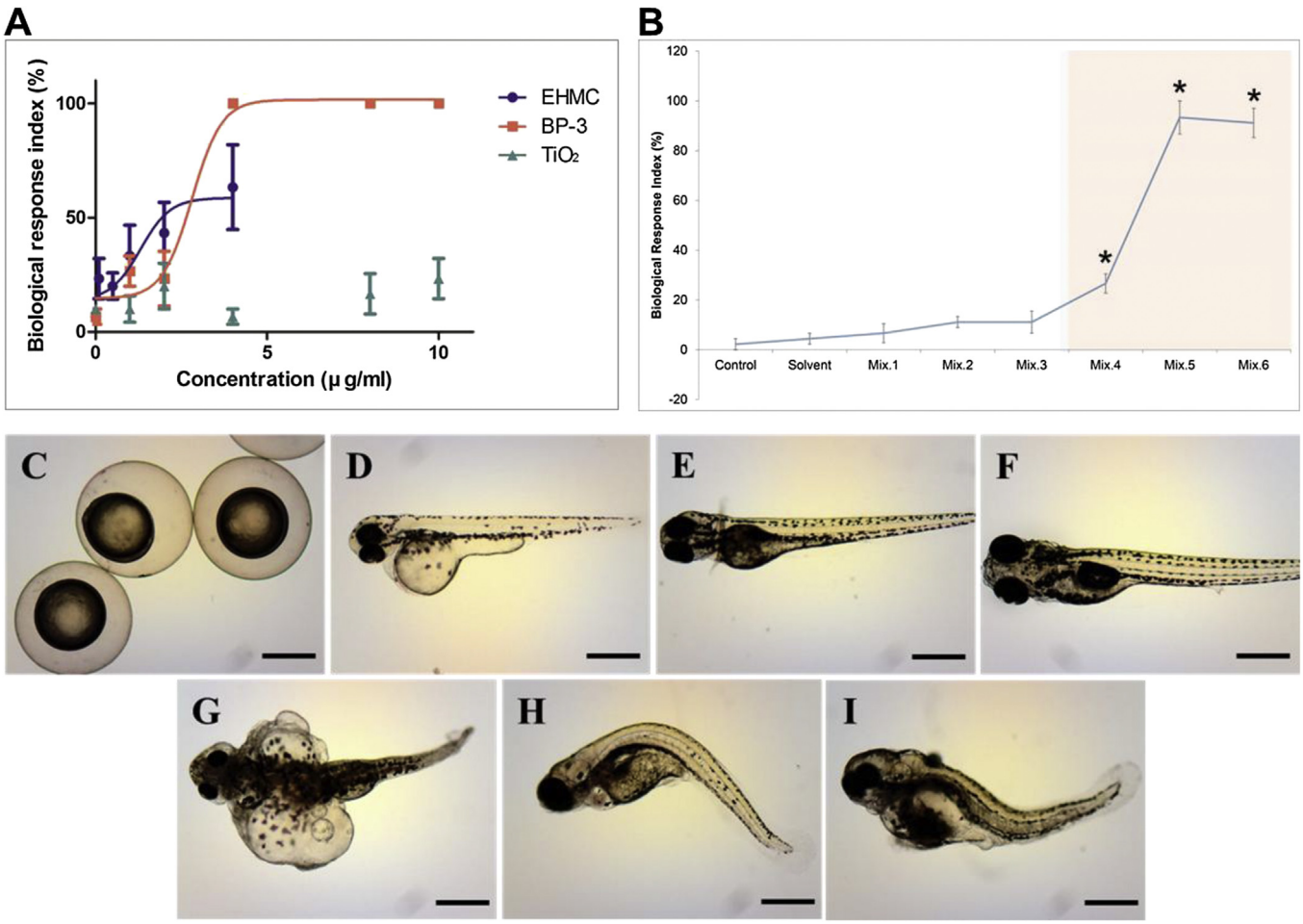

Fig. 2. Comparison of results from individual UV-filter substance treatments and heterogeneous substance treatments on wild-type zebrafish. (A) Fitted sigmoidal dose-response curves for ethylhexyl methoxycinnamate (EHMC), benzonphenone-3 (BP-3), and titanium dioxide (TiO_2) NP, (B) Toxic effects of the different concentrations of components in mixture of three UV-filters. Values of means ± SEM (n = 3). Values of 50% effective concentration (EC_{50}) for BP-3 on biological response index of zebrafish embryo were 2.41 ± 17.20. * denotes significant differences between all groups ($P < 0.05$). (C) Normal embryos at 6 hpf, (D) 48 hpf, (E) 72–96 hpf, and (F) larva at 4 days after hatching in the control groups. (G) Abnormal larva with pericardial edema, (G, H) bent spine. All scale bars = 500 μm.

2.8. Examination of zebrafish heart functionality

The control group for heartbeat count was the WT zebrafish. We measured the heartbeat counts at 48 hpf and 72 hpf for the control group and experimental groups of zebrafish. We selected 5 zebrafishes from each group to count heartbeat within 15 s.

2.9. Statistical analysis

Values of EC_{50} for both Daphnia magna and zebrafish embryo exposed to three different UV-filters were plotted on a fitted sigmoidal does-response curve, and were calculated by a four parameter logistic equation (SigmaPlot version 12.5, Systat Software, Inc., San Jose, CA, USA). Comparison between the combined effects of the mixtures was carried out using a *post hoc* Student-Newman-Keuls (SNK) test in the one-way ANOVA (SigmaPlot version 12.5, Systat Software, Inc., San Jose, CA, USA). All data are shown with the mean and ±standard error of mean (SEM). Statistical significance was set at $P < 0.05$.

3. Results

3.1. UV-filter substance analysis on daphnia magna

The range of nominal concentrations of each UV-filter substance, BP-3, EHMC, or TiO_2 NP, was established for toxicity assessment based on the results from previously published reports (Sambandan and Ratner, 2011; Sieratowicz et al., 2011; Strigul et al., 2009; Zhu et al., 2010). Each UV-filter substance was then applied to each group of Daphnia magna within the established range of nominal concentrations.

Both the BP-3 treated and the EHMC treated Daphnia magna exhibited immobilization in response to each UV-filter substance with EC_{50} values of 2.17 μg/ml and 3.03 μg/ml, respectively (Table 1; Fig. 1A). Higher concentrations of these UV-filter chemicals led to significant defects (Table S1; Fig. 1A; Fig. S1). However, no sign of defects was observed from the TiO_2 NP treated Daphnia magna. This is consistent with previous research reports (Pavagadhi et al., 2014) (Table S1; Fig. 1A; Fig. S1). These results were utilized in the subsequent experiment that made heterogeneous substances from combining the three UV-filter substances (Fent et al., 2010; Sieratowicz et al., 2011; Zhu et al., 2010).

The six different combinatorial mixtures, with compositions that are listed in Table 1, were then applied to the Daphnia magna. When the concentrations of the EHMC and BP-3 changed, the probabilities of immobilization probability varied correspondingly in dose dependent manners (Fig. 1B). In addition, TiO_2 NP in heterogeneous substances had no effect on establishing defects in Daphnia magna even though they were present as part of a heterogeneous substnace. The defect incidence rate did not change

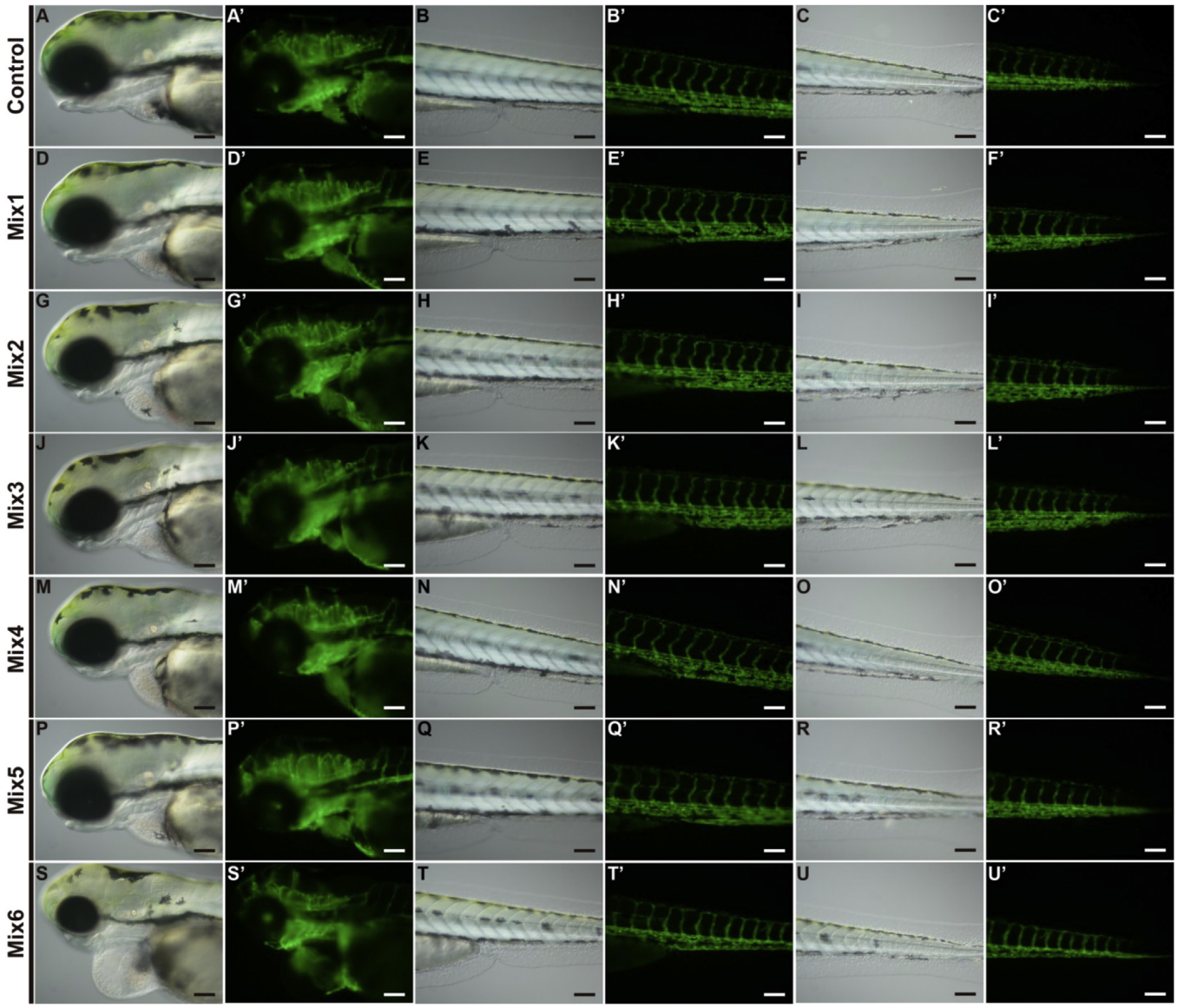

Fig. 3. Optical observation of Tg(flk1:EGFP) zebrafish embryo phenotype at 72 hpf upon treatment with UV-filter mixtures. UV-filter mixtures, with the compositions in Table 1, were added to the zebrafish embryos at 6 hpf, and observed at 72 hpf. The zebrafish embryos treated with mixture 6 showed cardiac edema phenotypes. Other groups did not exhibit defects in the zebrafish embryos. The scale bar represents 100 μm.

through all observed concentration ranges of TiO_2 NP in the mixtures, which correspond with the results observed in Daphnia magna treated with TiO_2 NP alone (Fig. 1B–E).

Defects on the Daphnia magna's physical structure were observed in dose dependent manner. However, in Fig. 1B, although it was possible to observe two patterns regarding mixture 3 and mixture 4, and mixture 2 and mixture 4, it was impossible to comprehensively interpret the data from the groups with 2–6 mixtures treatment. Based on the results above, it was confirmed that the defects in Daphnia magna were observed in the groups with EHMC and BP-3 in a dose dependent manner.

The patterns of defect transformation between the samples containing mixture 3 and mixture 4 and between the samples containing mixture 2 and mixture 4 were recognized to be dose dependent (Fig. 1B), yet these results do not assist in comprehensive evaluating the EHMC and BP-3 possible synergistic effect. Results from mixtures 2, 3, 4 imply that the UV-filter substance mixtures do in fact affect Daphnia magna, but it was hard to attribute the results from a synergistic reaction that arose directly from the UV-filters in the heterogeneous substnace. Instead, the results indicate that it was the Daphnia magna's characteristic quick response to foreign substances which made it hard to track the synergic reaction we were hoping to observe. From this standpoint, Daphnia magna is not an appropriate model to observe the course of pathogenesis.

3.2. UV-filter substance analysis on zebrafish

To successfully address and observe the course of pathogenesis caused by UV-filter substance mixtures, the zebrafish embryo was selected as an appropriate *in-vivo* model for the study. The UV-filter substances concentrations, determined based on the experiment with Daphnia magna, were applied to the zebrafish embryos. The new animal model has offered a number of advantageous features to this study.

Hatching, defects, and mortality of the embryos were observed upon treatment with the UV-filters. As with Daphnia magna,

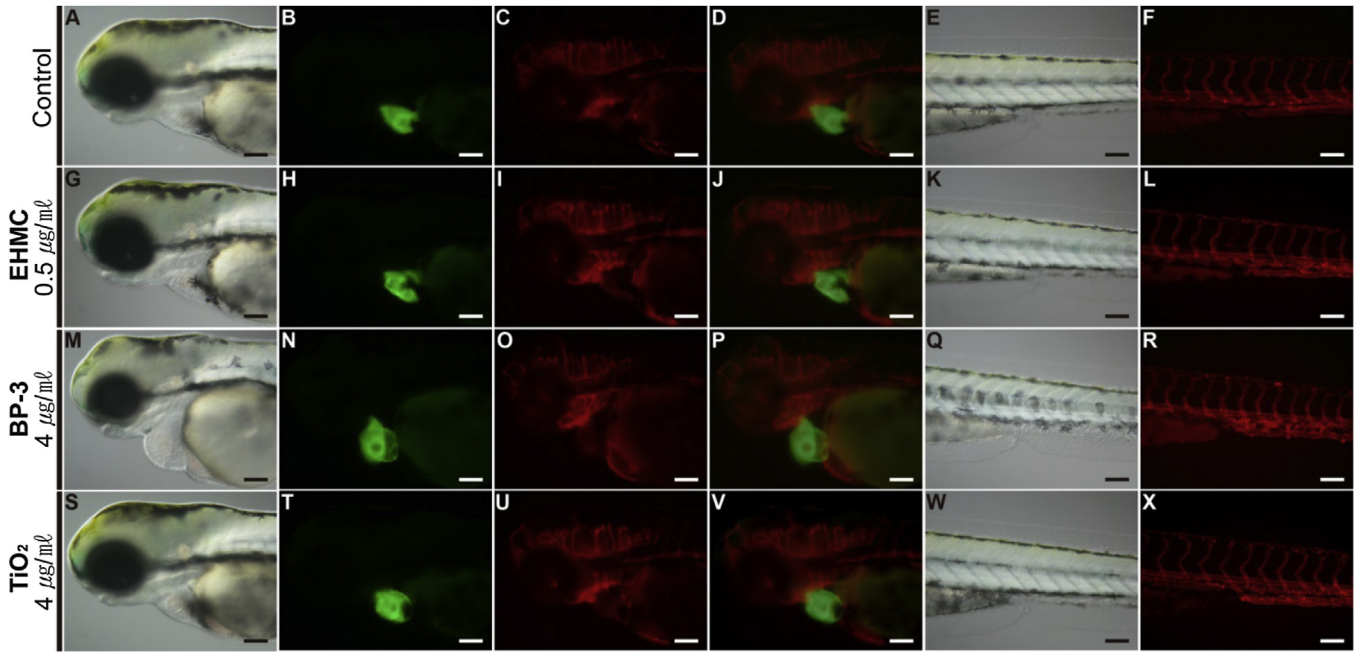

Fig. 4. Observation of phenotypes via Tg(cmlc:EGFP) X Tg(fli1:mCherry) zebrafish embryos upon treatment with each UV-filter substances. To examine the potential effects of the UV-filter substances on the development of the zebrafish heart and blood vessel, transgenic zebrafish modified to express eGFP and mCherry fluorescent protein in the heart and the blood vessel, respectively are treated and observed in 72 hpf. Minimal differences in the shape of the heart and vessel are observed among with the control (A–F), transgenic zebrafish embryos treated with EHMC (G–L) and TiO_2 (S–X). On the other hand, those treated with BP-3 (M–R) show a significantly different morphology in heart phenotype. Scale bar represents 100 μm.

treatment conditions were with single compounds and with trinary mixtures (Tables S2 and S3; Fig. 2A, B). The experimental group treated with BP-3 had an EC_{50} value of 2.41 μg/ml whereas the experimental groups treated with the EHMC or TiO_2 NP did not show any EC_{50} value. As expected, the BP-3 treated group exhibited defects in hatching, showed deformation, and possessed a higher mortality (Table S2; Fig. 2A).

Based on the results from the toxicity assessment on Daphnia magna regarding single UV-filters, zebrafish embryos were subject to the same treatment conditions. Fortunately, unlike the Daphnia magna results, we were able to deduce a clear interpretation for the zebrafish models. When comparing Figs. 1B and 2B, defects can be seen in Daphnia magna groups treated with the low concentration mixtures 2 and 3, but the same defects cannot be detected in the zebrafish models treated with the same mixtures. This indicates that the zebrafish is relatively less sensitive to the heterogeneous substances, and consequently, is a better model for toxicological assessments regarding heterogeneous substances.

Groups treated with the mixtures 4 through 6 had defects, while TiO_2 NPs did not elicit any defect, which was identical to the outcome from the test with Daphnia magna (Table S3; Fig. 2B–I). EHMC and BP-3 are shown to influence the zebrafish models in the same dose dependent manner, as can be seen in the comparison analyses between mixture 3 and mixture 4, and between mixture 3 and mixture 5.

3.3. UV-filter substance analysis on blood vessel transgenic zebrafish embryos

In order to gain a comprehensive understanding of complex effects of mixture 3 through 6, further assessments were planned and carried out to observe heart edema and bent tail morphology for phenotypic analysis using wild-type zebrafish embryos. Any observed defects from the wild-type embryos warranted additional analysis on alterations in vasculatures, heart, and liver of transgenic zebrafish embryos.

Blood flow is heavily influenced by the repercussions and action mechanisms of infiltrated toxic agents, thus we have used transgenic zebrafish embryos which expresses Green Fluorescent Proteins (GFP) on the vasculature surfaces. The scrutinized transgenic models are shown in Fig. 3.

Because blood circulation is the primary method by which toxins are transported throughout the body, the blood vessel transgenic models were analyzed to understand the effects of the mixtures. Mixtures 4, 5 did not yield toxin effects, such as bent tail or cardiac edema, which were seen in the wild-type models, and even mixture 6 yielded nothing but a cardiac edema phenotype (Fig. 3S, S′). Nevertheless, based on the observed hatching rate at 96 hpf (Table S4), there were defects caused by the toxic mixtures.

3.4. UV-filter substance analysis on heart transgenic zebrafish embryos

To assist with the interpretation of the effects from the mixtures, each UV-filter was introduced to ultimate transgenic models which were offsprings from breeding a heart transgenic model which expresses GFP at the heart, with a blood vessel transgenic model which expresses mCherry fluorescent protein in blood vessels. These offspring embryos were treated with various concentrations of non-mixed singular UV-filters (Table S5; Fig. 4). The experimental group treated with 4 μg/ml of BP-3 exhibited cardiac edema whereas the groups treated with EHMC and TiO_2 NP did not show any change in phenotype (Fig. 4M–P). Similar results were recorded for the groups at 48 hpf, 72 hpf, and 96 hpf (Figs. S2 and S3; Fig. 4).

Functional assessment was carried among the groups with no observed phenotypic change (Jang et al., 2014a). Those with cardiac edema were subjected to heartbeat counting at 48 hpf, 72 hpf, and 96 hpf (Movie S1 – S4). The number of heartbeats was counted within 15 s at the aforementioned three stages, and showed the BP-

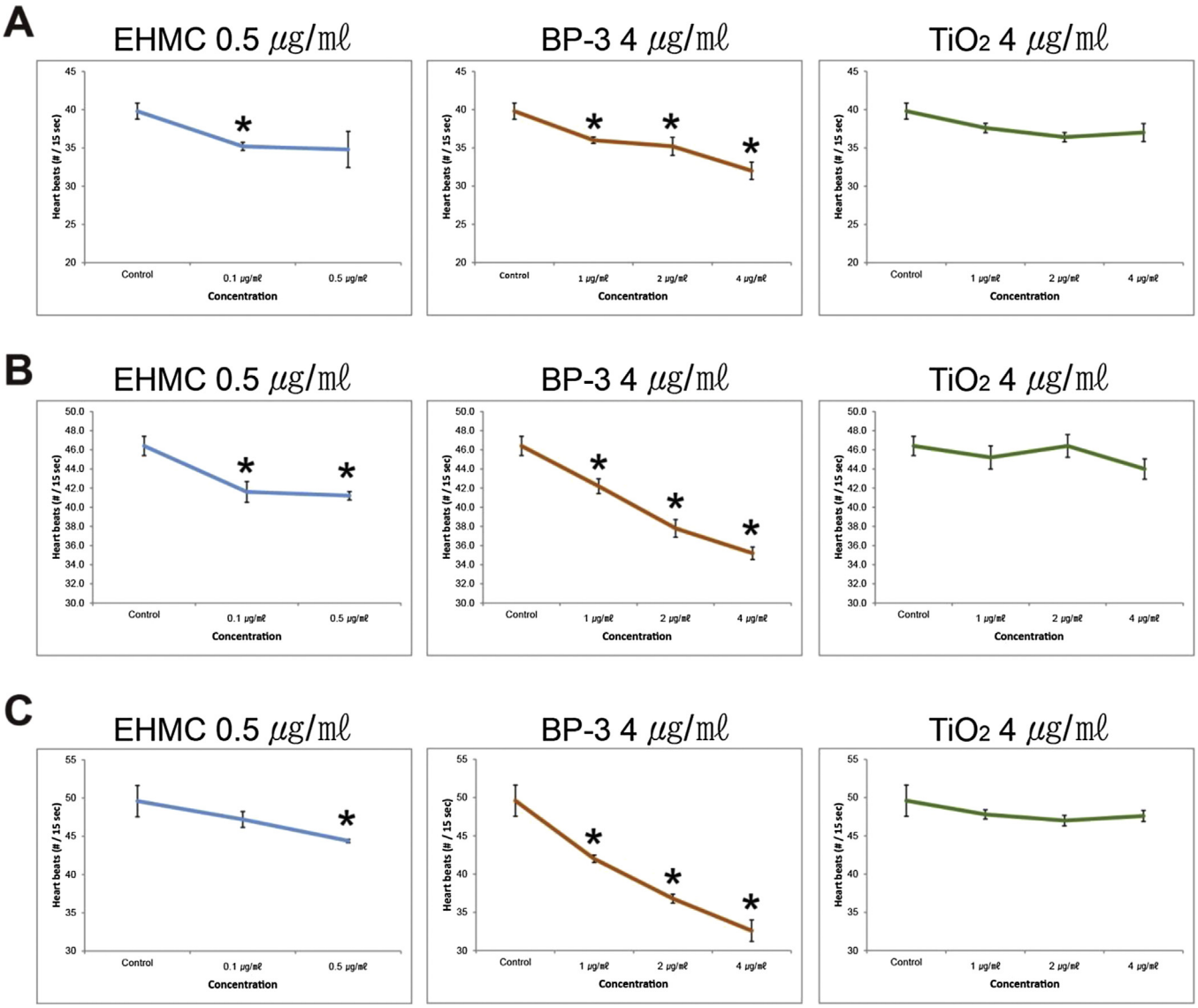

Fig. 5. Assessment of heart functionality by heart rate. To examine the potential effects of the UV-filter substances on the development of zebrafish heart, the heart rate of the zebrafish embryos was recorded upon treatment. (A) At 48 hpf, (B) 72 hpf, and (C) 96 hpf, treatments with the EHMC and BP-3 chemicals induced drops in the heart rates; in the case of the BP-3 treated group, the higher the treatment concentration, the larger the drop. Results from the three separate experiments are presented as heartbeat numbers compared to the control (no treatment). Means ± SEM (n = 5). *p < 0.01 from the control group.

3 experimental group to have deteriorated cardiac contractility in a dose dependent manner (Table S6; Fig. 5). The EHMC experimental group also showed a similar result in cardiac contractility, but TiO_2 NPs still did not show any defects in the functional assessment test (Table S6; Fig. 5A–C). These results support the dose dependent phenotypic changes found in the wild-type zebrafish embryo experimental groups with EHMC.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.06.001.

3.5. UV-filter substance analysis on liver transgenic zebrafish embryos

To see the effects of the UV-filters on hepatotoxicity, the DsRed expressing liver transgenic zebrafish embryo was utilized (Table S7; Fig. 6). To analyze the defect in the liver, liver size, and the angle between the eye and otolith was assessed and compared. No impactful difference was observed in the control group and the other experimental groups. However, the BP-3 group at 4 μg/ml exhibited deference in both the size and angle.

Compared to the size and angle measurements of the control group, the size of the BP-3 group at 4 μg/ml was 65.33 ± 8.93% and the angle, showing a 5° decrease, was 33.5 ± 1.6° (Table S8; Fig. 6G, H, I, M, N). In addition, the EHMC treatment had no significant effect on the angle and, when compared to the control group's liver size, had just a slightly smaller liver size of 84.56± 4.73% (Table S8; Fig. 6D, E, F, M, N). Overall, based on the reports from the other studies, the concentration set up with Daphnia magna, and from the zebrafish experiments, BP-3, combined with EHMC, will eventually affect live organisms. Although EHMC treated groups did not show any phenotypic change, heartbeat counting and the liver size assessments provided the comprehensive net effect can occur when treated with BP-3, simultaneously.

4. Discussion

Contemporary environmental evaluations have mainly analyzed the toxicity of individual substances, but such evaluations fail to

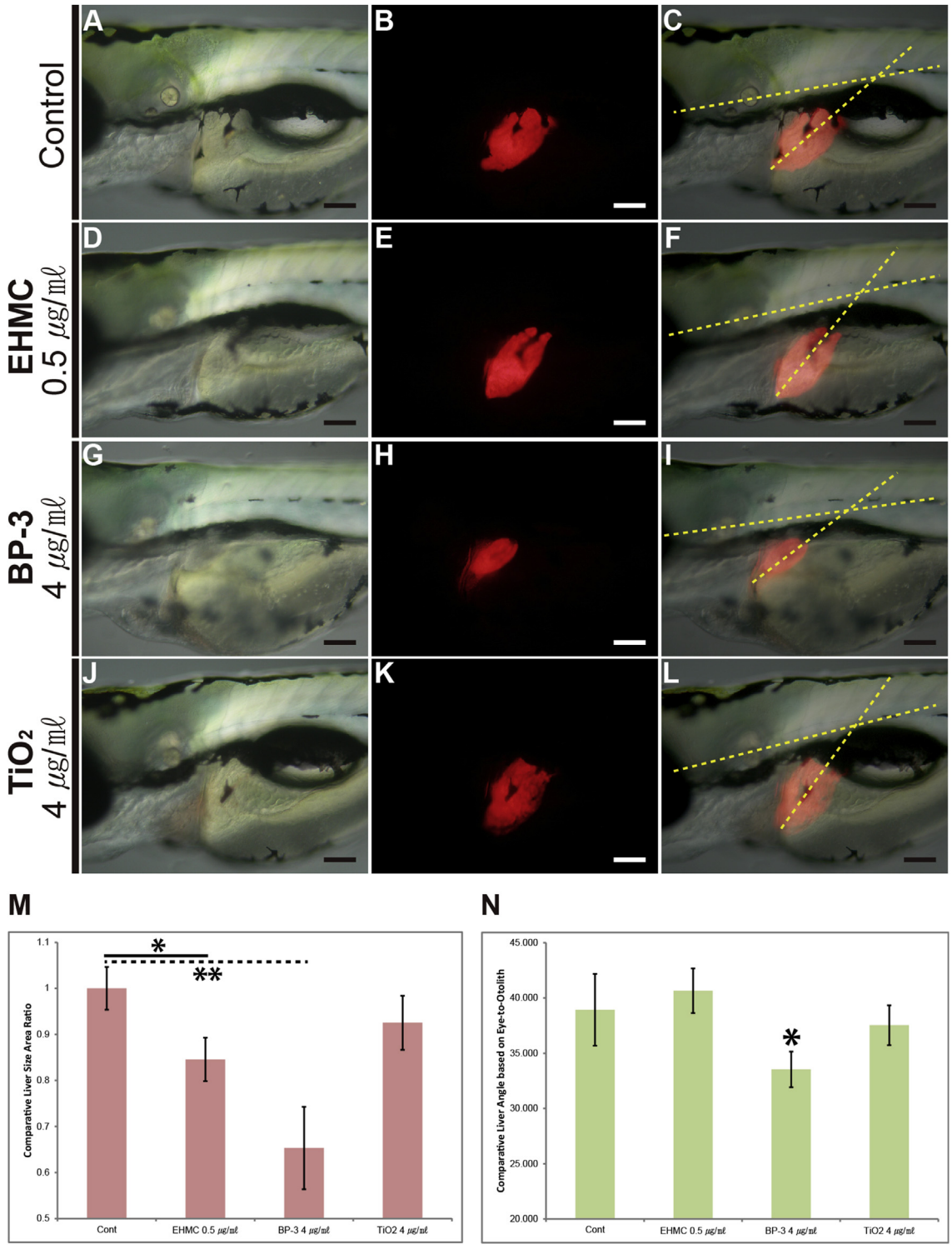

Fig. 6. Observation of liver defect via Tg(lfabp:DsRed) zebrafish upon treatment with each UV-filter substance. To examine potential effects of the UV-filter substances on the development of the zebrafish liver, transgenic zebrafish modified to express DsRed in the liver were treated and observed in 96 hpf (A–L). Minimal differences in the size of the liver are observed between the control group (A–C) and the transgenic zebrafish embryos treated with TiO_2 (J-L, M). On the other hand, those treated with EHMC and BP-3 (D–I) show a smaller liver. BP-3 treated group (G-I, N) exhibited a smaller liver angle compare to that of the control group's (A–C). (M) Treatment with the EHMC and BP-3 chemicals caused smaller liver; in the case of the BP-3 treated group, the larger the drop, the smaller the liver size. (N) Treatment with the BP-3 chemicals showed the smallest comparative liver angle based on eye-to-otolith. Results from the three embryo measurements are compared to that of the control. Scale bar represents 100 μm.

properly understand the harmful effects of the cumulative heterogeneous substances that actually affect the environment. In order to record and evaluate what really goes on when an organism is exposed to a heterogeneous substance, toxicology of substances in mixture forms should be mandatory.

However, current evaluation methods lack a systematic approach to gain a good understanding of the toxicology regarding heterogeneous substances. Since the EU Cosmetic Regulation 1223/

2009 and OECD's regulation have become stricter on animal experiments for environmental evaluation (Henkler et al., 2012; OECD, 2013), new protocols must adhere to the more rigid guidelines of the regulations.

Daphnia magna is a standard animal model that is often used for toxicity evaluations while complying with the strict OECD policy. However, the nearly instantaneous reaction, in terms of death, to the toxic substances, makes this animal model unfit for toxicity evaluation. In this report, we have demonstrated a proof-of-concept with Daphnia magna initially being used to determine the nominal concentrations of the substances of interest, and with zebrafish embryos—which are not considered as animals until 7 days post fertilization—in subsequent detailed analyses for toxicity assessment of the substances.

Almost all Daphnia magna die right after chemical or nanomaterial treatments. Inability to observe toxicity development because of the Daphnia magna's instantaneous death upon treatment with substances has led to search for other aquatic organism which is zebrafish. For this reason, Daphnia magna were only used to determine nominal concentrations at which toxicity starts to show. With the determined nominal concentrations, the three different transgenic zebrafish lines were subsequently used to observe any growth retardation and morphological deformities caused by the mixtures in threshold approach manner. Once zebrafish embryos are treated with the mixtures of EHMC, BP-3, and TiO_2 NP, the first organs that are exposed and affected by the mixture would be of circulatory system, heart and blood vessels, and liver for detoxification foreign materials. Overall, the focus of this study is to propose a new systematic sequential protocol for mixture toxicity evaluation for which was not possible with the conventional protocols.

A more detailed analysis with the transgenic zebrafish embryos was carried out with the prepared mixtures of EHMC, BP-3, and TiO_2 NP. An alternative approach was taken by administering the substances individually to the transgenic model to observe their effect on heart, liver, and blood vessels. The analysis allowed a comprehensive understanding of the combined toxic effects of the mixtures.

The combined toxicity of EHMC and BP-3 UV-filters in D. magna was higher than the estimated EC50 for either EHMC or BP-3 UV-filter alone, suggesting that these results may be due to a synergistic effect of the UV-filters (Fig. 1A, B). Zebrafish embryo also showed an indication of synergistic effect of the mixture 2, 3, and 4, although the result is not as apparent as one that from Daphnia magna. TiO_2 NPs were chosen based on the criteria in which a substance should have negligible toxic effect to avoid an analytical error caused by excessive toxic effects.

The TiO_2 NP used in this study is one of the nanoparticles which have been studied in our previous to investigate nanotoxicity of nanoparticles. TiO_2 NPs and other nanoparticles were tested to compare their nanotoxicities. However, it was still unclear whether each nanoparticle worked alone to cause the nanotoxicity. Moreover, a further question still remains if TiO_2 NP will still have no toxic effect when the nanoparticles are in a mixture with other UV-filter chemicals or nanomaterials. To address these questions, and knowing that TiO_2 NPs are one of the ingredients in cosmetic mixtures, an assay to assess toxicity caused by mixture is needed. The conventional methods evaluate toxicity of substances by independently investigating individual substances. From this standpoint, this study developed a systematic sequential protocol for the toxicity assessment of mixture. A sequel to this study will be toxicology of obscurely hazardous mixtures that might be yet to be uncovered.

The aim of this study was to develop and propose a systematic sequential protocol for substance mixtures which may cause combined toxic effects when they are introduced into ecosystems. Rather than focusing on the characteristics of each substance, this study focused on the ability of the systematic sequential bioassay for assessing the combined toxic effects of the mixtures (Gao et al., 2013; Grabicova et al., 2013; Masoud et al., 2015; Wang et al., 2016). The Daphnia magna, recommended by the OECD, and the zebrafish transgenic models, both abide by the regulations stated by the OECD while providing the means to efficiently carry out an assessment experiment. Although looking at oxidative stress caused by TiO_2 NP, EHMC, and BP-3 in transgenic zebrafish model has been a typical method for toxicity assessment, it is uncertain whether these substances would cause oxidative stress when they are in a mixture. In addition, substantial oxidative stress level deviation depending on each zebrafish embryo and experimental conditions led us to choose the transgenic zebrafish embryos. Thus, instead of looking at oxidative stress, status of the organs, the vasculatures, the heart, and the liver, which are firstly exposed to foreign substance are monitored to demonstrate the combined toxic effects. Based on the results from this study, the three transgenic lines are proven to be used as a new tool for assessment of combined toxicity of mixtures. We believe that the proof-of-concept demonstrated in this study will be employed in toxicology to gain better understanding on substance interactions and combined toxic effect on ecosystem when substances are in a mixture.

5. Conclusions

The purpose of this research was to systematically assess the heterogeneous substances that pervade the ecosystems. Unlike contemporary evaluation protocols that focus mostly on individual substances, we offer a systematic protocol that starts off by utilizing Daphnia magna to find effective toxic concentration of heterogeneous substances, and finishes by utilizing the zebrafish to detect defects caused by the heterogeneous substances. The research goes in-depth by using wild-type zebrafish to identify phenotype defects, and then goes further by using various transgenic zebrafish models to highlight the effects at various organs. The systematic analysis indicates that heterogeneous substance assessments are possible with current technology. In addition, this study suggests the need for toxicology of heterogeneous substances to understand true harmful effects using the in-vivo models in sequence from Daphnia magna to zebrafish embryos for environmental protection.

Competing interests

The authors declare no competing financial interest.

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Author contributions

KHL, GHJ and CBP were involved in the design of the study, participated in the data collection and manuscript preparation. BJK carried out biological based experimentation, statistical analysis, and with revising the manuscript. YJK participated on biological experiments and with revising the manuscript. KHL is PhD supervisor of GHJ and BJK, was involved in the planning and technical aspects of the study, and helped with interpretation of the data and revising the draft manuscript. All authors read and approved the final manuscript. All authors have given approval to the final

version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.06.001.

Abbreviations

hpf	hours post fertilization
BP-3	benzophenone-3 (BP-3)
EHMC	Ethylhexyl methoxycinnamate
TiO_2 NP	Titanium dioxide nanoparticle
GFP	Green Fluorescent Proteins
OECD	Organization for Economic Co-operation and Development
FDA	Food and Drug Administration
EC_{50}	50% effective concentration

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:12 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 8 of 9
Attachments: 72 Sharma DNA Damage cis trans.docx; 73 Ruszklewicz 2017 Neurotoxic Effects Toxicology Reports.pdf; 74 SCCP 1991 complete doc pg 67 EHMC.pdf; 75 Schreurs Toxicol Sci.docx; 76 Boin et al Mutation Research 105()303-308[1982] Genotoxicity of methoxycinnamate.pdf; 77 Shimoi S, Nakamura Y, Tomita I 1988 Effect of UV absorbers on UV-Induced mutagenesis in E. coli Br. Journal of Health Sciences 1-21-24.pdf; 78 European Commission (2000). Reports of the Scientific Committee on Cosmetology. S 28-2-ethylhexyl-4-methoxycinnamate. ISBN 92-828-8951-3. Pp67-72..pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

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Different DNA damage response of cis and trans isomers of commonly used UV filter after the exposure on adult human liver stem cells and human lymphoblastoid cells.

Sharma A1, Bányiová K1, Babica P1, El Yamani N2, Collins AR3, Čupr P4.

Abstract
2-ethylhexyl 4-methoxycinnamate (EHMC), used in many categories of personal care products (PCPs), is one of the most discussed ultraviolet filters because of its endocrine-disrupting effects. EHMC is unstable in sunlight and can be transformed from trans-EHMC to emergent cis-EHMC. Toxicological studies are focusing only on trans-EHMC; thus the toxicological data for cis-EHMC are missing. In this study, the in vitro genotoxic effects of trans- and cis-EHMC on adult human liver stem cells HL1-hT1 and human-derived lymphoblastoid cells TK-6 using a high-throughput comet assay were studied. TK-6 cells treated with cis-EHMC showed a high level of DNA damage when compared to untreated cells in concentrations 1.56 to 25μgmL-1. trans-EHMC showed genotoxicity after exposure to the two highest concentrations 12.5 and 25μgmL-1. The increase in DNA damage on HL1-hT1 cells induced by cis-EHMC and trans-EHMC was detected at the concentration 25μgmL-1. The No observed adverse effect level (NOAEL, mg kg-1bwday-1) was determined using a Quantitative in vitro to in vivo extrapolation (QIVIVE) approach: NOAELtrans-EHMC=3.07, NOAELcis-EHMC=0.30 for TK-6 and NOAELtrans-EHMC=26.46, NOAELcis-EHMC=20.36 for HL1-hT1. The hazard index (HI) was evaluated by comparing the reference dose (RfD, mgkg-1bwday-1) obtained from our experimental data with the chronic daily intake (CDI) of the female population. Using comet assay experimental data with the more sensitive TK-6 cells, HIcis-EHMC was 7 times higher than HItrans-EHMC. In terms of CDI, relative contributions were; dermal exposure route>oral>inhalation. According to our results we recommend the RfDtrans-EHMC=0.20 and RfDcis-EHMC=0.02 for trans-EHMC and cis-EHMC, respectively, to use for human health risk assessment. The significant difference in trans-EHMC and cis-EHMC response points to the need for toxicological reevaluation and application reassessment of both isomers in PCPs.

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Neurotoxic effect of active ingredients in sunscreen products, a contemporary review

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ABSTRACT

Sunscreen application is the main strategy used to prevent the maladies inflicted by ultraviolet (UV) radiation. Despite the continuously increasing frequency of sunscreen use worldwide, the prevalence of certain sun exposure-related pathologies, mainly malignant melanoma, is also on the rise. In the past century, a variety of protective agents against UV exposure have been developed. Physical filters scatter and reflect UV rays and chemical filters absorb those rays. Alongside the evidence for increasing levels of these agents in the environment, which leads to indirect exposure of wildlife and humans, recent studies suggest a toxicological nature for some of these agents. Reviews on the role of these agents in developmental and endocrine impairments (both pathology and related mechanisms) are based on both animal and human studies, yet information regarding the potential neurotoxicity of these agents is scant. In this review, data regarding the neurotoxicity of several organic filters: octyl methoxycinnamate, benzophenone-3 and −4, 4-methylbenzylidene camphor, 3-benzylidene camphor and octocrylene, and two allowed inorganic filters: zinc oxide and titanium dioxide, is presented and discussed. Taken together, this review advocates revisiting the current safety and regulation of specific sunscreens and investing in alternative UV protection technologies.

1. Introduction

Sunscreen application is the main strategy used to prevent the maladies inflicted by the sun since the 1930s. Unfortunately, although global use of sunscreen is continuously on the rise, so is the prevalence of malignant melanoma − a cancer type which is mainly caused by sun exposure [1–4]. There are several types of electromagnetic radiation emitted by the sun. One type − ultraviolet (UV) radiation − is composed of three wavelengths: UVA rays, which range at 320–400 nm and are not absorbed by the ozone layer, UVB rays, which range 290–320 nm and are partially absorbed by the ozone layer, and UVC rays, which are stopped by the ozone layer. The detrimental effects of exposure to UVA and UVB rays, which can cross the epidermis, have been reviewed and it was concluded that such exposure leads to reactive oxygen species (ROS) generation, DNA/protein/lipid damage, activation of various signal transduction pathways, compromised skin defense systems, altered growth, differentiation, senescence and tissue degradation, to name a few [5–7]. Two kinds of UV filters are currently being used in sunscreens for minimization of these adverse effects: organic (chemical) filters, e.g. octyl methoxycinnamate (OMC), benzophenone-3 (BP-3) or octocrylene (Table 1), which absorb light in the UV range, and inorganic (physical) filters, zinc oxide (ZnO) and titanium dioxide (TiO_2), which scatter and reflect UV rays. Sunscreens are usually comprised of more than one of these UV filters: organic, inorganic or a mixture of both types, which gives broad-spectrum of protection. Beyond its debatable efficiency, questions regarding the main ingredients of different sunscreens are being raised in recent years, mainly about the prevalence of these ingredients in the environment and about their potential toxicity.

1.1. Human exposure and detrimental effects

Many factors might influence human exposure to UV filters: geographic location, season, lifestyle, gender or occupation, which means it can be highly individualized. For instance, a study in Australia showed 56% of people apply sunscreens at least 5 days per week, and 27% of

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Table 1
Organic UV filters.

International nomenclature of cosmetic ingredients (INCI)	*United States adopted name (USAN)*	*Other names*
UVB filters		
4-methylbenzylidene camphor*	Enzacamene	
Homosalate	Homosalate	
Isoamyl-*p*-methoxycinnamate	Amiloxate	
Octyl dimethyl PABA	Padimate O	OD-PABA
Octyl methoxycinnamate	Octinoxate	2-ethylhexyl 4-methoxy cinnamate
Octyl salicylate	Octisalate	2-ethylhexyl salicylate
p-aminobenzoic acid	p-aminobenzoic acid	4-aminobenzoic acid, PABA
Triethanolamine	Trolamine salicylate	
UVA filters		
Disodium phenyl dibenzimidazole tetrasulfonate	Bisdisulizole disodium	
Butyl methoxydibenzoylmethane	Avobenzone	
Menthyl anthranilate	Meradimate	
Terephthalylidene dicamphor sulfonic acid	Ecamsule	Mexoryl SX
UVB-UVA filters		
Benzophenone-3	Oxybenzone	2-hydroxy-4-methoxybenzophenone
Benzophenone-4	Sulisobenzone	
Benzophenone-8	Dioxybenzone	
3-Benzylidene camphor[a]		Mexoryl SD
Bis-ethylhexyloxyphenol methoxyphenyl triazine[a]	Bemotrizinol	Tinosorb S
Cinoxate	Cinoxate	
Drometrizole trisiloxane[a]		Mexoryl XL
Methylene bis-benzotriazolyl Tetramethylbutylphenol[a]	Bisoctrizole	Tinosorb M
Octocrylene	Octocrylene	2-ethylhexyl 2-cyano-3,3-diphenylacrylate
Phenylbenzimidazole sulfonic acid	Ensulizole	

[a] Not approved by the Food and Drug Administration, used in other parts of the world.

people use it less frequently − 2 or fewer days per week [8] and a study in Denmark showed 65% of the sunbathers used one or more sunscreens [9].

Dermal exposure is the most relevant entry route of chemicals related to sunscreen use, however considering a common human behavior related to sunscreen application, e.g. eating and drinking with sunscreen applied on hands and lips, gastrointestinal or pulmonary exposure should also be considered [10–12]. The typically recommended mode of application (2 mg/cm^2) [13] implies a single dose of sunscreen product may be as large as 40 g, assuming application on the total body surface (2 m^2 for an average adult male), which for an average adult male weighting 78 kg and a typical concentration of about 10% of active ingredient in a commercial product, means maximum exposure around 50 mg/kg body weight (bw) [14]. Simple calculation suggests that with a maximum skin penetration up to 5% for some organic filters [15], the total amount of compound absorbed from a single application might be up to 200 mg, or 2.56 mg/kg bw, assuming an average bw of 78 kg for adult males. However, with application frequently thinner than recommended, partial body cover and different properties of compounds, these doses are usually much lower. For instance, a study on Australian population showed that the median daily amount of sunscreen applied was 1.5 g/day (range, 0–7.4 g/day) and the median quantity of sunscreen applied was 0.79 mg/cm^2 [8], whereas sunbather in Denmark applied on average 0.5 mg/cm^2 [9], in both cases it was less than half the amount needed to achieve the labeled sun protection factor.

Levels of UV filters found in human samples are usually low. In one epidemiological study, 2517 urine samples from United States (US) general population were analyzed for the presence of benzophenone-3 (BP-3), as part of the 2003–2004 National Health and Nutrition Examination Survey [16]; BP-3 was detected in 97% of the samples, with mean concentration of 22.9 ng/ml and 95th percentile concentration of 1040 ng/ml. In another study, investigating correlation between couples' presence of urinary benzophenone-type UV filters and sex ratio of their offspring, the mean concentrations of these compounds ranged from 0.05 ng/ml to 8.65 ng/ml, with BP-3 as the most predominant among the study population (samples collected between 2005 and 2009 in Michigan and Texas) [17]. Interestingly, about nine times higher than previously reported levels of BP-3 (up to 13000 ng/ml, average around 200 ng/ml) were found in urine samples collected in 2007–2009 from Californian females, which is probably a result of specific demographics [18].

The experimental studies confirm substantial absorption and distribution of organic filters, whereas inorganic filters seem to penetrate the human skin in a minimal degree. When adults applied a sunscreen formulation containing 10% of BP-3, 4-methylbenzylidene camphor (4-MBC) and octyl methoxycinnamate (OMC) on a daily basis (2 mg/cm^2) for a week, the mean urine concentrations for these ingredients were 60, 5, 5 ng/ml for females and 140, 7, 8 ng/ml for males, respectively [19]. At the same time, maximum plasma concentrations for these ingredients, reached 3–4 h after application, were 200, 20, 10 ng/ml for females and 300, 20, 20 ng/ml for males, respectively. Similar findings were reported following a 4-day exposure to these ingredients, which were detectable in the plasma of human males and females merely 2 h following application [20]. More data on human skin penetration and distribution of various UV filters, both organic and inorganic, can be found in recent reviews [21,22,15].

Of importance, some UV filters were also found in human milk samples. In a cohort study between 2004 and 2006, 54 human milk samples were analyzed; UV filters were detectable in 46 samples and levels were positively correlated with the reported usage of UV filter products [23]. Concentrations of ethylhexyl methoxy cinnamate (EHMC), octocrylene (OC), 4-MBC, homosalate (HMS) and BP-3 ranged 2.10–134.95 ng/g lipid, with EHMC and OC being most prevalent (42 and 36 positive samples, respectively) and an average of 7 positive samples for the other three [23]. In other study, levels of BP-3 in maternal urinary samples taken in gestational weeks 6–30 were positively correlated with the overall weight and head circumference of the baby [24]. These reports rise concerns about potential prenatal exposure and developmental toxicity of UV filters.

Besides intentional sunscreen application, additional routes might intensify human contact, namely occupational and environmental exposure. Workplace contact may be a source of substantial exposure to sunscreens, especially inorganic filters − nanoparticles (NPs) of ZnO

and TiO_2, which are frequently manufactured and stored as nanopowder. One study reported the presence of ZnO NPs in the work environment in an industrial scale plant in Japan. Electron microscopy analysis revealed the presence of a large number of submicron and micro-sized aggregated ZnO structures (concentrations not showed) [25]. Occupational exposures to TiO_2 NPs have been reported more frequently and was summarized in a recent review, demonstrating that the respirable TiO_2 concentration in the workers' breathing zone might reach 150 μg/m^3 [26]. Experimental studies suggest that with insufficient protection, inhalation of nanoparticles aerosol might result in pulmonary and systemic alterations. A single 10–30 min inhalation of a high dose (20–42 mg/m^3) of ZnO NPs aerosol increased levels of the inflammatory cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor α (TNF-α) in bronchoalveolar lavage fluid within 3 h after exposure in humans Kuschner et al., 1997. However, chronic, low-concentration exposure is more likely in workplaces, its effects are poorly known. Occupational exposure to sunscreen NPs was discussed more extensively in [12].

Environmental exposure is another way UV filters reach humans. Swimming in or drinking contaminated water might increase the contact, and thus absorption (through dermal and oral route) of these compounds. Recent data reviews indicate that the highest UV filter concentrations were found in rivers, reaching 0.3 mg/l for the benzophenone derivatives (e.g. BP-3), whereas ng to μg/l range were detected in lake and sea water. Moreover, lower levels (few ng/l) of organic UV filters were found in tap and groundwater [27–29,31]. Organic UV filters accumulate in wastewater treatment plants (WWTPs) up to mg/l concentrations, and since conventional WWTPs are not able to remove them, they are consequently released into rivers, lakes and oceans Ramos et al., 2016. Swimming pools are sinks for UV filters and its chlorine byproducts, at the μg/l range, or higher. Analysis by Sharifan et al. suggested that small, urban swimming pools might contain significantly higher, than in natural waters, levels of UV filters: 2.85, 1.9, 1.78 and 0.95 g/l, respectively of EHMC, OC, 4-MBC and BP-3, which question their safety for using them people, especially children [30].

Due to the widespread application of these compounds in many daily-use products and growing awareness of the risk associated with the sun exposure, the market of UV filters increases every year. Thus, increasing usage, persistent input and accumulation in environment is becoming an issue of great concern because of threat to human health, but also to the environment. UV filters were found to be ubiquitous in many aquatic systems and aquatic biota. Occurrence and impact (including toxicity) of UV filters on environment have been reviewed extensively elsewhere [27–29,31]. Aquatic organisms are frequently studied and UV filters were found at ng/g range in many of them, especially in fish and mussels, but also crustacean, mammals and aquatic birds [28]. In a study of the presence of several UV filters in Swiss lakes and rivers (which receive input from waste water treatment plants and recreational activity), and the fish which live inside them, water concentrations of BP-3, 4-MBC, EHMC and OC ranged 2–35 ng/l, while lower limit of detection (LOD) in fish for those compounds was 3–60 ng/g, with concentrations reaching as high as 166 ng/g for 4-MBC [11]. Later study showed even higher levels of BP-3, 4-MBC and EHMC (range 6–68 ng/l) in Swiss river, moreover substantial amount of EHMC was found in fish (up to 337 ng/g) and in cormorants (up to 701 ng/g), suggesting food-chain accumulation [10]. Many ecotoxicological studies addressed the potential damage of sunscreens and their components and *in vitro* experiments suggested that UV filters might be toxic for some aquatic microorganisms. UV filters were detected in nearshore waters around Majorca Island at variable concentrations: 53.6–577.5 ng/l for BP-3, 51.4–113.4 ng/l for 4-MBC, 6.9–37.6 μg/l for Ti, 1.0–3.3 μg/l for Zn, and various popular sunscreen formulations were shown to affect negatively the growth of local phytoplankton *Chaetoceros gracilis*, however at concentrations much higher than those detected in natural waters (EC50: 45–218 mg/l after 72 h treatment) Tovar-Sanchez et al., 2013. This is a typical observation. For instance, EC50 values of selected organic filters (e.g. BP-3, BP-4, EHMC, 4-MBC) in standardized toxicity assays on three aquatic species, *Daphnia magna*, *Raphidocelis subcapitata* and *Vibrio fischeri*, were in the mg/l range for all the species, which suggest minimal risk for these organism in their natural ecosystems [32]. However, like many researchers suggest, toxic effects of chronic, low-dose exposures cannot be ruled out and require further investigations [32]. Moreover, with increased usage and lack of efficient removal, environmental contamination will probably increase in the future. Recent report on coral bleaching showed that environmental contamination with BP-3 already poses a hazard to coral reef. The levels of BP-3 detected in coral reefs in the U.S. Virgin Islands (75–1400 μg/l) and Hawaii (0.8–19.2 μg/l) might lead to death of several local coral species with LC50: 8–340 μg/l and LC20: 0.062–8 μg/l (4 h exposure) [33].

Therefore, while sunscreens have been effective in protecting against a variety of UV-related pathologies, such as sunburns, actinic keratoses, squamous cell carcinomas and melanomas [34], growing popularity and thus, possibility for exposure questions their safety in environment and human health. Available data imply, that sunscreen compounds might block vitamin D synthesis or act as endocrine disruptor and lead to developmental toxicity. The effects of sunscreen on cutaneous synthesis of vitamin D induced by sunlight have been a subject of debate for recent years, however the newest analysis suggests, that normal usage of sunscreen by adults do not decrease cutaneous synthesis of vitamin D [35]. The endocrine disruptive and developmental toxicity of many organic UV filters in experimental models is well established, these filters seem to be associated with altered estrogen, androgen and progesterone activity, reproductive and developmental toxicity and impaired functioning of the thyroid, liver or kidneys, reviewed elsewhere [36,37,1,38,29]. Since many of UV filters were shown to cross the blood-brain barrier (BBB), the risk for neurotoxicity also occurs. In this review, the potential neurotoxicological effects of exposure to sunscreen have been discussed, as literature regarding the neurotoxicity of both organic and inorganic UV filters is presented.

2. Organic filters

Organic or chemical filters are the most popular and widely used in sunscreens and other cosmetic products. Data from 2003 indicate that over 80% sunscreen products contained OMC, 60% contained BP-3, and 20% contained octocrylene (OC) or HMS, whereas inorganic filters were present in around 20% of products [39]. Organic filters can be classified by the type of ultraviolet (UV) radiation they absorb, namely UVB, UVA or UVB-UVA filters (Table 1). As mentioned previously, the main route of human exposure is dermal absorption, however other routes and environmental exposure should be also considered. The last is particularly true for organic filters, which, due to their high lipophilicity could bioaccumulate in aquatic organism and reach humans through the food chain. Thus, they also are emergent as an environmental pollutant [40]. Chemical UV filters are easily absorbed by the skin and reach the systemic circulation, and accumulate in various tissues, as adipose tissue, liver and the brain [41–44]. Their lipophilicity permits them to readily cross the BBB, nonetheless, the effect of organic UV filters in the central nervous system (CNS) has been yet to fully addressed. However, there is a wide range of *in vitro* and *in vivo* studies of the toxic effects of UV filters as endocrine disruptors. And since it is known that other chemicals classified as endocrine disruptors can impair neuronal transmission, synaptic plasticity and produce neurotoxic effects [45], chemical filters might potentially produce similar effect. The documented neurotoxic effects of organic UV filters have been described below and summarized in Table 2.

2.1. Octyl methoxycinnamate

Octyl methoxycinnamate (OMC) is a UVB filter also known as

Table 2
Neurotoxic effects of organic UV filters.

Compound	*Exposure model*	*Experimental design*	*Effect*	*Reference*
Octyl methoxycinnamate	Wistar rats	Oral (gavage) administration during gestation and lactation 500–1000 mg/kg/day	Decreased motor activity in female offspring, increased spatial learning in male offspring.	[46]
	Sprague-Dawley rats, female	Oral (gavage) administration for 5 days 10–1000 mg/kg/day	Non-estrogenic interference within the rodent HPT axis; no changes in pre-proTRH mRNA in mediobasal-hypothalamus.	[47]
	Wistar rats	*In vitro* incubation of hypothalamus isolated from adult rats, 60 min 0.263 μM	Decreased hypothalamic release of GnRH. Increased GABA release and decreased Glu production in males. Decreased Asp and Glu production in females.	[48]
	Wistar rats	*In vitro* incubation of hypothalamus isolated from immature rats, 60 min 0.263 μM	Decreased hypothalamic release of LHRH. Increased GABA release in males, decreased Asp and Glu levels in females.	[49]
	SH-SY5Y neuroblastoma cell line	72 h 10^{-8}–10^{-4} M	Decreased cell viability and increased caspase-3 activity.	[50]
Benzophenone-3	*Danio rerio*	Waterborne 14 days for adult 120 h for embryos 10–600 μg/l	Anti-androgenic activity: decreased expression of *esr1*, *ar* and *cyp19b* expression in the brain of males.	[51]
	Sprague-Dawley rats	Dermal application 30 days 5 mg/kg/day	No changes in behavioral tests (locomotor and motor coordination).	[42]
	Rat primary cortical astrocytes and neurons	1–7 days 1–10 μg/ml	Decreased cell viability of neurons but not of astrocytes.	[42]
	SH-SY5Y neuroblastoma cell line	72 h 10^{-8}–10^{-4} M	Decreased cell viability and increased caspase-3 activity.	[50]
Benzophenone-4	*Danio rerio*	Waterborne 14 days 30–3000 μg/l	Upregulated estrogenic-related genes: *vtg1*, *vtg3*, *cyp19b* in the brain of males.	[52]
4-methylbenzyli-dine camphor	Long Evans rats	Oral (in diet) administration during mating, pregnancy, lactation, until adulthood of offspring 7, 24, 47 mg/kg/day	Impaired female proceptive and receptive sexual behavior. Altered expression of oestrogen- related gens in a sex- and region −dependent manner.	[53–55]
	Wistar rats	Subcutaneous administration during pregnancy 20–500 mg/kg/day	Altered hypothalamic release of Glu and Asp in male offspring. Inhibited testicular axis in male offspring during the pre-pubertal stage and stimulated during peri-pubertal stage.	[56]
	Danio rerio	Embryos exposed in medium 68 h 1–50 μM	Inhibited AChE activity, impaired early muscular and neuronal development.	[57]
	Neuro-2a mouse neuroblastoma cell line	45 min 0.1–100 μM	Inhibited AChE activity.	[57]
	SH-SY5Y neuroblastoma cell line	72 h 10^{-8}–10^{-4} M	Decreased cell viability and increased caspase-3 activity.	[50]
3-benzylidene camphor	Long Evans rats	Oral (in food) administration during mating, pregnancy, lactation, until adulthood of offspring 0.24–7 mg/kg/day	Impaired proceptive and receptive sexual behavior and disturbed estrous cycles of female offspring. Altered expression of oestrogen- related gens in a sex- and region-dependent manner.	[55]
Octocrylene	*Danio rerio*	Waterborne 14 days 22–383 μg/l	Impaired expression of genes related with development and metabolism in the brain.	[58]

Abbreviations: AChE: acetylcholine esterase; ar: androgen receptor; Asp: aspartate; cyp19b: cytochrome P450 aromatase b; esr1: estrogen receptor; GABA: gamma amino butyric acid; Glu: glutamate; GnRH: gonadotrophin-releasing hormone; HPT: hypothalamo-pituitary-thyroid; pre-proTRH: pre-pro-thyrotrophin-releasing hormone; vtg1, vitellogin 1; vtg3: vitellogin 3.

octinoxate and 2-ethylhexyl 4-methoxy cinnamate. This compound is approved as a cosmetic ingredient in US and in European Union (EU) in concentrations of 7.5–10% [1].

Dermal penetration of OMC has been measured *in vitro*, with values ranged from 0.2% to 4.5% of the applied dose, depending on the experimental conditions, however systemic absorption seems to be much lower. In humans, when a cream containing 10% OMC was applied to the entire body (40 g), OMC was absorbed through the skin and is detectable in blood (maximum concentrations 10 ng/ml in females and 20 ng/ml in males) and in urine (5 ng/ml in females and 8 ng/ml in males). Taking the highest detectable concentration (20 ng/ml) and assuming 4.7 l of blood, the systemic absorption represents only 0.002% of the applied dose [19].

Several studies indicated that OMC acts as an endocrine disruptor due to the ability to interfere with endocrine system at different levels [47,59,60]. *In vitro* and *in vivo* studies in rodents have shown that OMC have estrogen activity [61,62]. In humans OMC exposure has minor, but statistically significant effects on the levels of testosterone and estradiol [19]. Moreover, some studies suggested that OMC can interact with the hypothalamo-pituitary-thyroid (HPT) axis [63]. Ovariectomized rats exposed to 57.5 mg/20 g body weight of OMC applied via food presented a decrease in thyroxine (T_4) levels without changes in triiodothyronine (T_3) or thyroid-stimulating hormone (TSH) levels [60].

OMC has also a non-estrogenic endocrine disrupting activity in the HPT axis absent altering the expression of pre-pro-thyrotrophin-releasing hormone (pre-proTRH) in the mediobasal hypothalamus, but affecting the axis in other points, when was administrated orally (10–1000 mg/kg/day) for 5 consecutive days [47]. Experiments with rats showed that OMC (0.263 μM) decreases the hypothalamic release of gonadotrophin-releasing hormone (GnRH) [48] and luteinizing hormone-releasing hormone (LHRH) [49] *in vitro*. Furthermore, *in vitro* experiments in hypothalamic cells from male and female adult rats showed that the same dose of OMC inhibited the release of

neurotransmitters aspartate (Asp) and glutamate (Glu), but not gamma-aminobutyric acid (GABA) in females, whereas in males decreased Glu and increased GABA release [48]. Similar results were found in hypothalamus isolated from immature rats (pre-pubertal and peri-pubertal males and females) [49]. These results indicate that OMC disrupts the normal neuroendocrine mechanism in a sex-dependent manner. Moreover, a study of offspring of dams treated with OMC (500–1000 mg/kg/day) showed sex-dependent behavioral changes, namely decreased motor activity in females, but not in males, and improved spatial learning in males, suggesting that OMC can affect neuronal development, however the doses used in these experiments were extremely high, not relevant to possible human exposure [46]. Corroborating these observations, recent studies in neuroblastoma cell line (SH-SY5Y) demonstrated that exposure to high concentrations of OMC (0.01–100 μM) decreased cell viability and increased apoptosis, however effective concentrations were not observed *in vivo* [50].

2.2. Benzophenone-3

Benzophenone-3 (BP-3, oxybenzone) is a common organic filter used in sunscreens and other personal care products (nail polish, lotions, lipsticks) in a maximum allowed concentration of 6% in US. It is used as broad-spectrum UV filter due to absorption of both UVB and short UVA rays [1].

BP-3 applied topically in human can cross the skin by direct penetration through the intercellular laminae of the stratum corneum (SC) or by passive diffusion by high-concentration gradient and then reach the blood [64]. When 25 volunteers applied a commercially available sunscreen containing 4% BP-3 for 5 days, their urine samples showed that approximately 4% of BP-3 is absorbed into the system [65]. BP-3 was detected in more than 80% of urine samples of healthy Danish children and adolescents (median concentration 0.92 ng/ml) [66]. Repeated (4 days) topical applications (2 mg/cm^2 of sunscreen formulation) of BP-3 resulted in urine levels up to 81 ng/ml and plasma levels up to 238 ng/ml [20]. Moreover, another concern relates to the fact that the simultaneous application of some insect repellents components such as *N, N*-diethyl-*m*-toulamide (DEET) and BP-3 can enhance skin penetration of each other when jointly applied [42]. Once BP-3 is in the systemic circulation, it is transported to different organs. BP-3 is a highly lipophilic, and in rats it has been detected in liver [41,42,44,43] and in brain (15.5–34.1 ng/g) [42]. High concentrations of BP-3 were also detected in adipose tissue after topical administration [41].

High, not environmentally relevant concentration of BP-3 (up to 1000 μg/l) were shown to disrupt the neuro-endocrine system in fish [67,68]. BP-3 (waterbone exposure 10–600 μg/l, where the lowest concentration represents the worst-case, environmentally relevant concentration) impaired the sexual behavior of *Danio rerio* zebrafish adult males by decreasing the expression of androgenic genes: estrogen receptor 1 (*esr1*), androgen receptor (*ar*) and cytochrome P450 aromatase B (*cyp19b*) in the brain at concentration 84 μg/l [51]. Whereas topical administration of BP-3, at dosage which mimics possible human exposure (5 mg/kg/day for 30 days) in male and female Sprague-Dawley rats did not affect locomotor activity and behavioral test, nor did it produce neurological deficits [42]. Moreover, no effect on rat primary cortical astrocyte cultures were detected when cells were incubated with low, physiological concentrations (0.1–10 μg/ml) of BP-3 for up to 7 days [42]. However, studies in rat primary cortical neuronal cultures [42] and SH-SY5Y neuroblastoma cell line [50] showed decreased cell viability after BP-3 treatment at moderate concentrations (e.g. 1–10 μg/ml).

2.3. Benzophenone-4

Benzophenone-4 (BP-4, sulisobenzone) is frequently used as UV absorber at concentration up to 10% [1].

BP-4 was found in human placenta (0.25–5.41 ng/g), suggesting efficient skin penetration and accumulation, which may lead to exposure of human embryos and fetuses [69]. BP-4, like BP-3 is a benzophenone derivative, yet its potency as an estrogenic disruptor has been not well defined.

In zebrafish, adult males exposed to high concentrations (3000 μg/l) of BP-4 for 14 days displayed estrogenic activity by up-regulation of estrogenic-related genes: vitellogin 1 (*vtg1*), vitellogin 3 (*vtg3*) and the *cyp19b* in the brain, however lower dosages did not induce changes. In contrast, in the liver, some of these genes (*vtg1*, *vtg3*) were down-regulated [52]. No other effects in the nervous system were reported.

2.4. 4-methylbenzylidene camphor

4-methylbenzylidene camphor (4-MBC) or enzacamene is an organic camphor derivative used as a UVB filter in sunscreen and other cosmetic products. Although the compound is not approved by the Food and Drug Administration (FDA), other countries allow it usage at maximum concentration of 4% [1].

4-MBC is a high lipophilic component which can be absorbed through the skin and was found in human tissues, including placenta [70]. Repeated (4 days) topical applications (2 mg/cm^2 of sunscreen formulation) of 4-MBC resulted in urine levels up to 4 ng/ml and plasma levels up to 18 ng/ml [20]. When orally administrated to rats, 4-MBC reaches the liver where is metabolized to 3-(4-carboxybenzylidene) camphor and 3-(4-carboxybenzylidene) hydroxycamphor [71]. 4-MBC exhibits a toxic activity as estrogenic endocrine disruptor [62,59,68]. Moreover, *in vivo* studies suggest that 4-MBC affected the thyroid axis [63].

Several studies described the effects of 4-MBC on developing neuroendocrine system. Rats exposed to 4-MBC (7–47 mg/kg) in diet before mating, during pregnancy and lactation, and in the offspring until adulthood, showed a region- and sex-dependent alteration in the oestrogenic genes in the brain [55,54,53]. For instance, the expression of progesterone receptor (PR) was decreased in the ventromedial hypothalamic area of 4-MBC-treated females, but not in males [55]. In addition, females showed impaired proceptive and a non-receptive sexual behavior after 4-MBC exposure [55]. Female sexual behavior was significantly impaired at the lowest doses studied 7 mg/kg/day, which resulted in rat milk concentration of 208.6 ng/g lipid, which is over 10 times higher than value (19 ng/g lipid) found in human milk [55]. Subcutaneous administration of high dosages (up to 500 mg/kg/day) during pregnancy and lactation altered the hypothalamic secretion of excitatory amino acids Glu and Asp in male offspring. These neurotransmitters play a role as stimulators of gonadal axis, thus the observed changes are consistent with alterations in sexual development of male offspring, affecting pre-pubertal stage, but stimulating the peri-pubertal stage [56]. In addition, 4-MBC has been reported to have an acetylcholinesterase (AChE) inhibitory activity. Zebrafish embryos exposed to 15 μM 4-MBC for 3 days showed abnormal axial curvature and exhibited impaired motility. 4-MBC also impaired muscle development and axon pathfinding [57]; however, the dose used in the study was significantly higher than those detected in environmental aquatic media. Inhibition of AChE activity was also observed in mammalian Neuro-2a cells exposed to 10 and 100 μM for 45 min, indicating a possible mechanism for the 4-MBC-induced muscular and neuronal defects [57]. 4-MBC (up to 100 μM) has been shown recently to decrease cell viability and induce apoptosis in neuroblastoma cell line (SH-SY5Y), suggesting possible neurotoxic effects, however again, effective concentrations were not observed *in vivo* [50].

2.5. 3-benzylidene camphor

3-benzylidene camphor (3-BC) is a lipophilic compound closely related to 4-MBC. It is used in sunscreen products in EU, at a maximal concentration of 2% [1].

After topical application to rats for 65 days (60–540 mg/kg/day) 3-

BC was detected in all analyzed tissues, including the brain (concentration 0.13–1.2 μg/g), suggesting that similar disposition and distribution may occur in humans [72]. Though not detectable in urine of Danish children [66], the compound was found in human placenta [70].

Analogous to 4-MBC, 3-BC has also been described as an estrogenic disruptor [59,73]. Moreover, it has been reported that 3-BC can affect the CNS. Rats pre- and postnatally treated with 3-BC (0.24–7 mg/kg/day) showed region- and sex-specific response in expression of genes involved in sexual behavior: PR, estrogen receptors (ERa, ERb), and steroid receptor coactivator-1 (SRC-1) in the brain [55].

2.6. Octocrylene

Octocrylene (OC) is an ester belonging to the cinnamates family and is present in sunscreen and daily care cosmetic products at a maximal concentration of 10%. It can absorb UVB and high energy components of UVA radiation [74]. To date there are few studies on its accumulation and toxicity, especially in aquatic organism [75–77].

Zebrafish embryos and adult male exposed to environmentally relevant concentrations of OC in water (22–925 μg/l) absorbed and accumulated this compound. Moreover, the microarray analysis from adult zebrafish male exposed to OC (383 μg/l) showed major impairment in the expression of 628 genes in the brain regulating mainly developmental processes and 136 genes in the liver, responsible mainly for metabolism [58].

3. Inorganic filters

Inorganic (physical) ingredients used in modern sunscreens include metal oxide particles, typically titanium dioxide (TiO_2) and zinc oxide (ZnO), which occurs typically at 5–10% concentration (maximum allowed is 25%). While chemical filters still dominate in sunscreen products, the usage of physical compounds is constantly growing. One of the reasons is that they have a higher spectrum of protection − TiO_2 is very effective in absorbing UVB, while ZnO absorbs mainly the UVA range, and the combination of both particles provides a broad UV protection. Other advantages of physical filters are lack of skin sensitization and limited skin penetration [78]. However, these mineral filters, when in normal pigment size range (200–400 nm for ZnO, 150–300 nm for TiO_2) have poor particle dispersion, which makes them difficult to apply; they also reflect and scatter light, which result in undesirable visible white film on the skin. With nanotechnology, these materials can be reduced to nanoparticles (NPs) (< 100 nm) which are easier to apply and are transparent on the skin [12]. Nevertheless, with micronization some properties are changed − they may be more bioreactive and easier penetrate the skin and other tissues, leading to concerns about their safety use. Moreover, part of the absorbed UV radiation can generate free radicals on the surface of metal oxides in the presence of water and this photocatalytic activity increases with decreasing NPs size. NP-induced cyto- and genotoxicity has been associated with increased photocatalytic activity, leading to increased production of free radicals [79]. Despite increased awareness of nanomaterials toxicity, the nanoneurotoxicity is a relatively new field with numerous data gaps awaiting improvements. One of the main reasons for this is the lack of reliable methods for NPs detection and quantification. Only estimates and predictions about NPs concentration in natural environments are available, and they suggest that TiO_2 might be present in the range 0.7–24.5 μg/l, whereas ZnO might reach higher levels, up to 76 μg/l [80]. Analogously, NPs accumulation and physiological concentrations are difficult to assess; thus, most studies report changes in Zn and Ti ion levels only. This also raises questions regarding the relevance of predominantly high-dose exposures used in toxicological studies. To date, most studies attesting to neurotoxic effect of NPs have been carried out in acutely high concentration exposure scenarios, and their relevance to “real-life” exposure scenarios needs to be further assessed.

3.1. Zinc oxide

Zinc oxide nanoparticles (ZnO NPs) are used not only in sunscreens, but also in pigments (UV-absorbers, paintings) and electronic equipment (thin film transistors, semi-conductors, liquid crystal displays, light-emitting diodes) due to their exceptional optoelectronic, piezoelectric, ferromagnetic and optical properties. Moreover, their antiseptic activity makes them potentially useful in treatment of bacteria-related infections or diseases [81]. As their commercial utilization has increased, wider application raises the potential risk of human exposure [82].

3.1.1. ZnO NPs absorption and transport across the BBB

Several *in vitro* and *in vivo* studies evaluated the fate and toxicity of ZnO NPs from different exposures: dermal, gastrointestinal or pulmonary. Dermal absorption is a major route of ZnO NPs exposure from sunscreen application. Most studies demonstrated that ZnO NPs did not penetrate into deeper layers of the skin (SC) [83,84,22,85,86]. However, some data indicated that ZnO NPs penetrated the skin to a limited extent. A small increase of zinc ions (Zn^{2+}) in the blood and urine was observed in humans exposed to ZnO NPs-containing sunscreen products for five constitutive days *via* healthy skin [87]. Human skin *in vitro* was shown to absorb 0.34% of ZnO NP after 72 h [88]. In general, penetration ability of NPs increases when the skin barrier is damaged, pursuant to sunburn, skin disease or physical damage. ZnO NPs were found to better penetrate tape-stripped, lesioned or wounded, rather than healthy human skin [85,89]. Moderate skin sunburn increased the penetration of ZnO NPs in pigs, however transdermal absorption was not detected [90]. *In vitro* studies reported similar findings, only a limited number of ZnO NPs were found on the outer surface of the SC, and no particles were observed in the deeper SC layers [83,84]. Generally, the risk of ZnO NPs exposure from dermal absorption is rather low, however, considering a common human behavior related to sunscreen application, e.g. eating and drinking with sunscreen applied on hands and lips, gastrointestinal or pulmonary exposure should also be considered, moreover, as mentioned previously, the occupational exposure might be of high concern for some people [12].

Inhalation might be specifically associated with increased brain exposure, since the olfactory nerves can directly transport particles into the brain. In fact, Kao et al. observed the translocation of ZnO NPs into the brain following nasal administration (6 h airborne exposure) in a Sprague Dawley rats [91]. In healthy human adults inhaling 500 $\mu g/m^3$ of ZnO NPs for 2 h, the results were below the threshold for acute systemic effects on the respiratory, hematologic, and cardiovascular endpoints [92]. Other studies have shown that various NPs can enter the brain across the BBB [81,93]; however, a limited number of studies address this issue for ZnO NPs. The BBB was found to be intact in rats after repeated oral administrations of ZnO NPs for 28 days (500 mg/kg) [94], however the presence of ZnO NPs in the rat brain was observed after oral administration for 21 days (500 mg/kg) [95]. Moreover, Yeh et al. (2012) showed increased ^{65}Zn accumulation in the mouse brain up to 10 days after single-dose (120 g) intravenous injection of small (10 nm) ^{65}ZnO NPs [96]. In adult mice, neuronal NPs localization was observed for several days after single oral (gavage) administration of 3 mg of fluorescent ZnO NP. Decreased fluorescent signal over time is consistent with biodegradation or elimination of NPs from the brain [97]. Additional studies are needed to investigate the brain penetration capacity of ZnO NPs.

Other reviews discuss absorption, distribution, metabolism and excretion of ZnO NPs in humans and experimental models more extensively [12,79,82]. To date, data available indicate that ZnO NPs can be absorbed *via* different routes and distributed to a range of organs, including the brain and placenta. Distribution depends on the size of ZnO NPs, the dose, time and route of exposure. The fate of ZnO NPs

Table 3
Neurotoxic effects of ZnO NPs.

Compound	*Exposure model*	*Experimental design*	*Effect*	*Reference*
ZnO NPs	Wistar rats	Intraperitoneal injection biweekly, 8 weeks 4 mg/kg	Attenuated spatial cognition capability, enhanced long-term potentiation.	[99]
	Wistar rats	Intravenous injection single dose 25 mg/kg	Increased brain Zn concentrations; no changes in neurotransmitter levels, locomotor activity, exploratory behavior or spatial working memory.	[100]
	Wistar rats, male	Intraperitoneal injection, 10 days 25 mg/kg/day	Decreased iron and calcium, but not Zn, sodium and potassium levels in rat brain homogenates; unchanged emotional behavior.	[101]
	Wistar rats, male	Oral (gavage) 7 days 600 mg/kg	Elevated TNF-α, IL-1β, IL-6, CRP, MDA, decreased GSH and SOD levels, CAT, and GPx activity.	[102]
	Sprague-Dawley rats	Oral 13 weeks 134.2, 268.4, 536.8 mg/kg/day	Increased Zn levels in the brain of male rats.	[103]
	C57BL/6J mice, male	Intraperitoneal injection 3 times per week, 4 weeks 5.6 mg/kg	Impaired learning and memory abilities, suppression of cAMP/CREB signaling pathway.	[104]
	Swiss albino mice, male	Intraperitoneal injection every other day, 8 times 5.6 mg/kg	Improved behavioral and cognitive impairment in mice with depressive-like behaviors.	[105]
	Swiss albino mice, male	Oral 21 days 500 mg/kg	Elevated ROS levels, altered antioxidant system, increased DA and NE levels, presence of ZnO NPs in neurons.	[95]
	ICR mice, pregnant female	Subcutaneous at GD 5, 8, 11, 14, 17 100 μg/day	Changed DA, 5-HT and their metabolites levels in a 6-week old offspring.	[106]
	Cyprinus carpio	Waterborne 1–14 days 0.5, 5, 50 mg/l	Changed CAT, SOD, GPx activity, GSH levels and lipid peroxidation.	[107]
	Prochilodus lineatus	Waterborne 5, 30 days 7, 70, 700 μg/l	Increased protein oxidative damage, decreased AChE activity.	[108]
	Apis mellifera carnica	Oral (food) 10 days 0.8 mg Zn/ml	Decreased brain weight and increased brain AChE and GST activity.	[109]
	Isolated rat neurons	1 mg/ml	Increased the opening number of sodium channels, delayed rectifier potassium channels, enhanced excitability of neurons.	[110]
	Rat primary neurons	24 h 1–100 μg/ml	Concntration-dependent cytotoxicity, disrupted cell membranes, DNA damage.	[111]
	Mouse neural stem cells	24 h 3–24 ppm	Concentration-dependent decrease in cell viability; apoptosis, necrosis, release of zinc ions.	[112]
	RCS96 rat Schwann cells	6–48 h 4–400 μg/ml	Concentration- and time-dependent decrease in cell viability; apoptosis and necrosis, G2/M phase cell cycle arrest, release of Zn ions.	[113]
	Human olfactory neurosphere-derived cells	2–24 h 10–80 μg/ml	Decreased cell viability, activation of numerous pathways associated with stress, inflammation and apoptosis.	[114]
	RCG-5 rat retinal ganglion cells	4–72 h 2.5–10 μg/ml	Concentration- and time-dependent decrease in cell proliferation; cell cycle arrest, ROS generation, increased caspase-12, decreased bcl-2 and caspase-9.	[115]
	RCG-5 rat retinal ganglion cells	6–72 h 2.5–10 μg/ml	Decreased mitochondrial membrane potential, increased ROS production, increased caspase-12.	[116]
	RCG-5 rat retinal ganglion cells	4–72 h 2.5–10 μg/ml	Decreased expression and activity of the plasma membrane calcium ATPase, disrupted intracellular calcium homeostasis, increased ROS production.	[117]
	PC12 rat pheochromocytoma and SH-SY5Y human neuroblastoma	24 h 10–10000 μM	Decreased cell viability, mitochondrial impairment, internalization of ZnO NPs in membrane-bound vesicles.	[91]
	SH-SY5Y human neuroblastoma	6, 12, 24 h 5–30 mg/ml	Concentration- and time-dependent decrease in cell viability; apoptosis via the PI3 K/Akt/caspase-3/7 pathway and necrosis by LOX-mediated ROS production.	[118]
	SH-SY5Y human neuroblastoma	3–48 h 10–80 μg/ml	Concentration- and time-dependent decrease of cell viability, apoptosis and cell cycle alterations, genotoxicity: micronuclei, H2AX phosphorylation, DNA damage.	[119]
	U87 human brain tumor	24 h 1–200 μg/ml	Concentration-dependent cytotoxicity e.g. increased formation of micronuclei.	[120]
	Rat primary astrocytes	6, 12, 24 h 4, 8, 12 μg/ml	Reduced cell viability, increased LDH release, stimulated ROS generation, caspase-3 activation, decreased MMP, phosphorylated JNK, ERK, p38 MAPK.	[121]
	C6 glia cells	3, 6, 24 h 5–80 μg/ml	Time- and concentration-dependent cytotoxicity, apoptosis and increased ROS production.	[122]
	A172, U87, LNZ308, LN18, LN229 glioma cell lines and normal human astrocytes	24 h 1, 5, 10 mmol/l	Cytotoxicity and ROS generation in glioma lines, but not in normal human astrocytes.	[123]
	N9 mouse microglial cell line	5–60 min; 1–24 h 1–100 μg/ml	Increased intracellular calcium and ROS levels, decreased intracellular ATP level, upregulated apoptosis markers.	[124]

(continued on next page)

Table 3 *(continued)*

Compound	*Exposure model*	*Experimental design*	*Effect*	*Reference*
	BV-2 mice microglia cell line	2–24 h 10 μg/ml	Increased cytotoxicity; activated PINK1/parkin-mediated mitophagy.	[125]

Abbreviations: 5-HT: 5-hydroxytriptamine; Akt: protein kinase B; cAMP: cyclic adenosine monophosphate; CAT: catalase; CREB: cAMP response element binding protein; CRP: c-reactive protein; DA: dopamine; ERK: extracellular signal-related kinase; GSH: glutathione; GPx: glutathione peroxidase; GST: glutathione-S-transferase; H2AX: H2A histone family member X; IL-1β: interleukin-1β; IL-6: interleukin-6; JNK: c-Jun N-terminal kinase; LDH: lactate dehydrogenase; LOX: lipoxygenase; MDA: malondialdehyde; MMP: mitochondrial membrane potential; NE: norepinephrine; NPs: nanoparticles; p38 MAPK: p38 mitogen-activated protein kinase; PINK1: PTEN-induced putative kinase 1; PI3 K: phosphoinositide 3-kinase; ROS: reactive oxygen species; SOD: superoxide dismutase; TNF-α: tumor necrosis factor α; Zn: zinc; ZnO: zinc oxide.

remains unclear; most data suggest that ZnO NPs decompose in medium or in cells and release Zn^{2+} which are responsible for toxic effects. However, this issue, together with the risk of long-term exposure and absorption *via* healthy *vs*. damaged skin remain to be established.

3.1.2. Neurotoxic effects in vivo

Although increasing number of studies aimed to investigate the potential toxicity of ZnO NPs in different cell types and animal systems [98,82,12], little is known about their neurotoxic effects (Table 3), especially *in vivo*. ZnO NPs exposure was shown to induce neurobehavioral changes in experimental animals. Impaired learning and memory abilities, and hippocampal pathological changes were demonstrated in old (18 months) mice following ZnO NPs exposure (intraperitoneally, i.p., 5.6 mg/kg, three times per week for four weeks) [104]. The spatial learning and memory ability was attenuated in ZnO NPs-treated (i.p. 4 mg/kg, biweekly for 8 weeks) Wistar rats. The exposed animals exhibited prolonged escape latency in the Morris water maze (MWM), and enhanced long-term potentiation (LTP), but not sufficient depotentiation in the dentate gyrus (DG) region of the hippocampus [99]. ZnO NPs administered i.p. for several days ameliorated the behavioral and cognitive impairment in young Swiss male mice with depressive-like behaviors, suggesting that they may affect neuronal synaptic plasticity [105]. Subcutaneous administration of ZnO NPs in pregnant ICR mice at gestation day (GD) 5, 8, 11, 14 and 17 (100 μg/day) affected dopamine (DA), 5-hydroxytriptamine (5-HT) and their metabolites' levels in a 6-week old offspring [106]. This observation questions the safety of ZnO NPs exposure during pregnancy, potential transfer through placenta and the effect on developing brain. In contrast, single intravenous injection of ZnO NPs (25 mg/kg) did not affect locomotor activity, exploratory behavior, spatial working memory or neurotransmitter: norepinephrine (NE), epinephrine (EPI), DA, and 5-HT levels in adult male Wistar rats 14 days after injection, despite the plasma and brain Zn^{2+} levels increased in treated group [100]. Sub-acute ZnO NPs treatment (25 mg/kg, 10 days) resulted in minimal effect on emotional behavior (e.g. unaffected anxious index), but showed alteration in trace elements homeostasis in rat brain homogenates: decreased levels of iron (Fe^{2+}) and calcium (Ca^{2+}), while Zn^{2+}, sodium (Na^{+}) and potassium (K^{+}) concentrations remained unchanged [101].

Disrupted ion homeostasis is an important pathomechanisms of neurotoxicity, and ZnO NPs might affect it. Long-term (13 weeks) oral ZnO NPs administration (134.2, 268.4, 536.8 mg/kg/day), resulted in detection of slightly, but significantly higher Zn^{2+} levels in the brain of male rats (but not in female) [103]. In isolated rat hippocampal CA3 pyramidal neurons the ZnO NPs solution (1 mg/ml) was shown to enhance the current amplitudes of I_{Na} and I_K by increasing the opening number of sodium channels, delaying rectifier potassium channels, and enhancing the excitability of neurons, leading to intracellular Na^{+} accumulation and K^{+} efflux. These might disturb the ionic homeostasis and the physiological functions of neurons [110].

Oxidative stress and disrupted antioxidant system is another effect observed in brains of ZnO NPs-treated animals. Oral ZnO NPs (500 mg/kg) administration for 21 consecutive days resulted in elevated ROS levels and altered antioxidants: glutathione (GSH) levels, superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase activity (GST), in both the brain and liver of male Swiss albino mice [95]. Combined with increased DA and NE levels in the cerebral cortex, these results suggest a neurotoxic potential for ZnO NPs [95]. Changes in CAT, SOD, GPx activity, GSH levels and lipid peroxidation was also observed in the brain and other organs of juvenile carp (*Cyprinus carpio*) exposed to waterborne ZnO NPs (0.5, 5, 50 mg/l) for 1, 3, 7, 10 and 14 days [107]. Exposure to environmentally relevant concentrations of ZnO NPs (7, 70, 700 μg/l) for 5 and 30 days led to increased protein oxidative damage in the brain and gills, but not in the liver, and decreased AChE activity in the brain and muscle of *Prochilodus lineatus* juvenile fish [108]. Honey bees (*Apis mellifera carnica*) exposed to ZnO NPs (0.8 mg Zn/ml) in food for 10 days showed decreased brain weight and increased brain AChE and GST activity [109]. Week-long oral administration of ZnO NPs (600 mg/kg) to male Wistar rats resulted in decreased brain CAT, GPx, and GR activities, decreased GSH and SOD levels, but elevated malondialdehyde (MDA) level and inflammatory markers: TNF-α, IL-1β, IL-6, C-reactive protein (CRP). The neurotoxic effects were partially reversed by the antioxidant and anti-inflammatory compound, hesperidin [102]. The pro-oxidant and pro-inflammatory effect of ZnO NPs was also observed in the serum and the brain of mice injected with ZnO NPs (i.p., 5.6 mg/kg) three times per week for four weeks [104]. In this study the suppression of cAMP/CREB signaling pathway was also identified: the contents of hippocampal cyclic adenosine monophosphate (cAMP), cAMP response element binding protein (CREB), phosphorylated CREB and synapsin I, were decreased in ZnO NPs-treated mice in an age-dependent manner [104].

3.1.3. Neurotoxic effects in vitro

The neurotoxic effect of ZnO NPs *in vitro* has been also evaluated, demonstrating oxidative stress- and apoptosis-related cytotoxicity. Deng et al. [112] have demonstrated that ZnO NPs impaired viability of neural stem cells (NSCs) in a concentration-, but not size-dependent manner. Twenty-four-hour exposure of concentrations higher than 12 ppm induced apoptosis and necrosis in the NSCs. Authors suggested that observed changes might result from the Zn^{2+} dissolved in solution or intracellularly, rather than from NPs, since ZnO NPs were not detectable in apoptotic cells, and similar cytotoxicity was observed after treatment with $ZnCl_2$ [112]. In primary rat astrocytes ZnO NPs exposure (4, 8, 12 μg/ml for 6–24 h) was found to reduce cell viability, increase lactate dehydrogenase (LDH) release, stimulate ROS generation, and elicit caspase-3 activation in a concentration- and time-dependent manner [121]. Apoptosis was shown by nuclear condensation and poly(ADP-ribose) polymerase-1 (PARP) cleavage. ZnO NPs stimulated the phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), and p38 mitogen-activated protein kinase (p38 MAPK). A decrease in mitochondrial membrane potential (MMP) and increase in the expression of Bax/Bcl-2 ratio was also observed, suggesting mitochondria-mediated apoptosis [121]. ZnO NPs (1–100 μg/ml, 24 h) induced concentration-dependent cytotoxicity, disrupted cell membranes and DNA damage in rat primary neuronal cells, human fibroblasts and A549 cells, but not in HepG2 cells and human skin keratinocytes [111]. A time- and concentration-dependent cytotoxicity characterized by apoptosis and increased ROS production

was also observed in ZnO NPs-treated (5–80 μg/ml) C6 glial cells [122]. ZnO NPs exposure (1–100 μg/ml) resulted in increased intracellular Ca^{2+} and ROS levels, decreased intracellular ATP level and upregulated apoptosis markers in mouse microglial cell line [124]. ZnO NPs (1, 5, 10 mmol/l, 24 h) evoked cytotoxicity in the human glioma cell lines (A172, U87, LNZ308, LN18, LN229), but not in normal human astrocytes. Cytotoxicity observed in the glioma cells was related to increased ROS generation, and *N*-acetyl-L-cysteine (NAC) treatment decreased the cytotoxic effect of the ZnO NPs in these cells [123]. ZnO NP-induced cytotoxicity was also observed in microglia (BV-2 cells) exposed to 10 μg/ml ZnO NPs for 2–24 h [125]. ZnO NPs induced parkin protein translocation from the cytoplasm to the mitochondria, implying the involvement of mitophagy in ZnO NPs-induced toxicity [125].

The neurotoxicity of ZnO with four different hierarchical architecture: monodispersed spherical NPs (35 nm), hollow ZnO microspheres (2.7 mm), and larger, prism- and flower-like structures, was evaluated in RSC96 rat Schwann cells [113]. Cells were treated with ZnO at doses 4, 8, 40, 80, 400 μg/ml for 6, 12, 24, and 48 h. ZnO NPs and microspheres displayed significant cytotoxic effects on Schwann cells in concentration- and time-dependent manners, whereas no or low cytotoxic effect was observed when the cells were treated with the prism-like and flower-like ZnO. Cell apoptosis and G2/M cell cycle arrest were observed when RSC96 Schwann cells were exposed to ZnO nanoparticles and microspheres at a dose of 80 μg/ml for 12 h. The time-dependent increase of Zn^{2+} concentration in the culture media suggests that the cytotoxic effects were associated with the decomposition of ZnO hierarchical architecture and the subsequent release of Zn^{2+}, and not exclusively to the nanoparticulated fraction [113].

Neurotoxicity of ZnO NPs was examined in rat pheochromocytoma PC12 and human neuroblastoma SH-SY5Y cells, showing significant cell loss after 24 h treatment at concentration of 0.1 mM in PC12 cells and 1 mM in SH-SY5Y cells [91]. Moreover, when the PC12 cells were treated with 1 mM (81.4 μg/ml) ZnO NPs for 10 min, the endocytosis of ZnO NPs was observed and increased cellular Zn^{2+} levels indicated that ZnO NPs may be converted to Zn^{2+} in endosomes, and then be mobilized into the cytoplasm, leading to Zn^{2+} dyshomeostasis [91]. The cytotoxic and genotoxic effects of ZnO NPs in SHSY5Y cells under different exposure conditions were also investigated by Valdiglesias et al. [119]. Despite the results showed that ZnO NPs (10–80 μg/ml) do not enter the neuronal cells, their presence in the medium induced decrease in cell viability, apoptosis, cell cycle alterations, and genotoxicity, including micronuclei production, H2AX (H2A histone family, member X) phosphorylation and DNA damage (primary and oxidative) in a concentration- and time-dependent manner. Unlike in previously described studies, free Zn^{2+} released from the ZnO NPs was not responsible for the viability decrease [119]. Exposure of SH-SY5Y cells to ZnO NPs (10, 15, 20, 25, 30 mg/ml) resulted in neurotoxicity, as confirmed by LDH activity assay, mitochondria toxicity test (MTT) and Muse™ cell viability assay. Allopurinol, NAC and α-tocopherol protected from ZnO NP-induced cytotoxicity. Electron microscopy revealed typical necrotic characteristics, such as swelling or loss of cell organelles and rupture of the cytosolic or nuclear membrane at 12 h and 24 h after ZnO NPs exposure. Apoptotic changes (annexin V and caspase-3/7 activities) were evident at 12 h and 24 h, but not 6 h after exposure to 15 mg/ml ZnO NPs. PI3 kinase (PI3 K) and p-Akt/Akt (protein kinase B) activities induced by ZnO NPs were significantly decreased by esculetin (antioxidant) or LY294002 (PI3 K inhibitor). Esculetin reduced the production of ROS and the depletion of antioxidant enzymes induced by ZnO NPs. ZnO NPs induced apoptosis via the PI3 K/Akt/caspase-3/7 pathway and necrosis by lipoxygenase (LOX)-mediated ROS production [118]. Treatment with ZnO NPs (1–200 μg/ml) induced cytotoxicity (e.g. increased formation of micronuclei) in the human brain tumor U87 cells in a concentration-dependent manner, but did not affect normal human HEK cells [120]. Different types of ZnO NPs (coated < 200 nm and uncoated < 30 nm) (10–80 μg/ml, incubated for 2, 6 or 24 h) induced cytotoxicity in human olfactory neurosphere-derived (hONS) cells *via* mechanisms associated with cell stress, inflammation and apoptosis [114]. Changes in cytokines IL-6 and IL-8 secretion, increase in caspase-3/7 activity, and phosphorylation of key proteins involved in signaling pathways: MAPK/ERK (pMEK, pERK, pJNK, p-cJUN, p-p38), Akt (pAkt, pBAD) and NF-κB (pNF-κB, pI-κB) has been demonstrated. Microarray RNA analysis revealed that short-term (2 h) exposure to ZnO NPs activated pathways involved in cellular stress responses (e.g. upregulation of Nrf2-mediated oxidative stress response pathway), whereas longer (6 h) exposure affected pathways more related to cell injury and repair. Of note, the cellular response was dependent on NPs surface coatings [114]. ZnO NPs (2.5–10 μg/ml) induced cytotoxicity in rat retinal ganglion cells (RGC-5) and inhibited cell proliferation in a time- and concentration-dependent manner. Moreover, ZnO NPs treatment led to cell cycle arrest of S and G2/M phases, ROS production and increased level of caspase-12 and decreased levels of bcl-2 and caspase-9 [115]. Further, the same group showed that ZnO NPs decreased the MMP in RGC-5 cells [116], ZnO NPs (2.5–10 μg/ml) have been shown to decrease the expression and activity of the plasma membrane calcium ATPase, increase intracellular Ca^{2+} level and disrupt the intracellular calcium homeostasis which might trigger mitochondrial dysfunction, ROS production and cell death [117].

3.2. Titanium dioxide

Titanium dioxide (TiO_2) is widely used as a white pigment in paint, ink, plastic, and paper and as food additive, while the nanosized TiO_2 is also used for its photocatalytic activity in self-cleaning materials and for its UV absorption capacity in sunscreen. Moreover, TiO_2 is included in the list of inactive ingredients by the FDA, considering it safe to be used in dental paste, oral capsules, suspensions, tablets, dermal preparations and non-parenteral medicines. TiO_2 particles are believed to possess low toxicity and thus are widely used in biomedical applications for their excellent biocompatibility. The range of light that is scattered as well as other properties of TiO_2 depend on the particle size. It naturally exists in three crystal structures: anatase (tetragonal), rutile (tetragonal), and brookite (orthorhombic). Anatase and rutile TiO_2 both have a tetragonal structure, while the TiO_6 octahedron of anatase TiO_2 is distorted to be larger than that of the rutile phase [126]. When the size of TiO_2 is diminished to nanoscale (diameter < 100 nm), the bioactivity and physiochemical properties of nano-sized TiO_2 are significantly different from the properties of their bulk analogue [127,128]. Nanoparticles of TiO_2 (TiO_2 NPs) are allowed as sunscreen additives in concentrations of up to 25% [1]. The increased use of nanosized materials has led to an increased burden of TiO_2 NPs in aquatic environments. It is, however, unclear how high levels might occur in environment and if they are harmful to organisms [129]. Analogous to ZnO NPs, the increased demand for products containing TiO_2 is met by increased occupational exposure. Apart from the NIOSH 2011 current intelligence bulletin, to date, no occupational or environmental exposure limits for TiO_2 NPs have been set by any other regulatory agency. The number of workers currently exposed to TiO_2 dust is not available.

Often when a product is so attractive to industry, the understanding about its risk assessments is insufficient and lags behind their rapid advancement and widespread applications [130]. In the case of TiO_2 NPs, it is not yet clear how they are transported into or out of the brain, how they accumulate or what kind of behavioral or cognitive dysfunction they may cause, however the evidence summarized in this (Table 4) and other review articles [130,128,79,149–154] may indicate that their toxic potential remains to be fully elucidated

3.2.1. TiO_2 absorption and transport across the BBB

Dermal absorption is the most relevant entry route of chemicals related to sunscreen use. Several studies have analyzed TiO_2 penetrance into intact or damaged skin using different models. On the whole,

Table 4
Neurotoxic effects of TiO_2 NPs.

Compound	*Exposure model*	*Experimental design*	*Effect*	*Reference*
TiO_2 NPs	Mice	Intratracheal instillations once per week for 4 weeks 13.2 mg/kg	Inflammatory cell aggregation and neuron necrosis. Ti level in the brain 3 days after a single instillation was upregulated by 100%.	[131]
	Wistar rats, male	Intratracheal 0.1, 1.0, 10.0 mg/kg	Ti accumulation in the brain and dose-dependent injury. TiO_2 NPs with diameter of 200 nm did not cause significant alterations in the brain.	[132]
	BBB model based on rat primary endothelial cells (BECs) and astrocytes	Acute exposure: 24 h, 0–500 μg/ml Chronic exposure: 5 days, 0–100 μg/ml	Reduced expression of P-gp, claudin 5, caveolin-1, and caveolin-2 associated with BBB integrity.	[133]
	Fisher F344 rats, male	Intravenous single dose 1 mg/kg	Upregulation of tight junction proteins, modulation of P-gp mRNA expression and persistent brain inflammation markers: IL-1β, IP-10, GFAP and CXCL1. No Ti accumulation in the brain after 24	[134]
	Mice	Intranasal 90 days 2.5, 5.0, 10 mg/kg	Ti accumulation in the brain. Oxidative stress, high levels of lipid, protein, and DNA peroxidation, overproliferation of glial cells, tissue necrosis, hippocampal cell apoptosis. Microarray showed significant alterations of 249 genes expression.	[135]
	Mice, female	Intranasal instillation every other day for 2, 10, 20, 30 days 500 μg	Ti accumulation in hippocampus after 30 days of rutile exposure. Irregular arrangement and loss of neurons, morphological changes and oxidative damage in hippocampus. Increased TNF-α and IL-1β levels.	[136]
	Mice, female	Intranasal instillation every other day for 2, 10, 20, 30 days 500 μg	Imbalance of monoaminergic neurotransmitters, increased NE and 5-HT, while levels of DA, DOPAC, HVA and 5-HIAA were decreased.	[137]
	Wistar rats, male	Intragastrical 60 days 50, 100, 200 mg/kg	Downregulated AChE activity. Increased plasmatic and brain IL-6. Increased GFAP expression.	[138]
	Zebrafish embryos	96 hpf 0.1, 1, 10 μg/ml	Hatching time was decreased, with increase in malformation rate. Accumulation in brain with ROS and cell death in hypothalamus. Alterations in behavior and PD-related genes.	[139]
	Caenorhabditis elegans	24 h 7.7, 38.5 μg/ml	GC–MS-based metabolomics perturbations mainly occurred in TCA cycle, glyoxalate, tricarboxylate, inositol phosphate, Gly, Ser, Thr, Gln, and Glu metabolism.	[140]
	Caenorhabditis elegans	96 h under dark or light conditions 1–100 mg/l	Light exposure induced the production of ROS and increased toxicity from a median effect concentration of more than 100 mg/l to 53 mg/l.	[141]
	D384 glial cell line and SH-SY5Y human neuroblastoma	24 h 15, 31 μg/ml,	Concentration- and time-dependent alterations of mitochondrial function, cell membrane damage, inhibition of cell proliferation. Effects dependent on TiO_2 size. Neuronal cells were more sensitive than glial cells.	[142]
	U373 human glial cells and C6 rat glial cells	24–96 h 2.5–40 μg/cm^2	DNA fragmentation assessed in U373 cells, but not in C6 cells. Morphological changes associated with depolymerization of F-actin, apoptotic cell death.	[143]
	U373 human glial cells and C6 rat glial cells	2–24 h 20 μg/cm^2	Increased expression of antioxidant enzymes: GPx, CAT, SOD2, lipid peroxidation and mitochondrial depolarization.	[144]
	PC12 rat pheochromocytoma	6–48 h 1–100 μg/ml for	Apoptosis prevented by a ROS scavenger, N-MPG.	[145]
	Co-culture of PC12 cells with primary rat microglia	24–48 h 0.25–0.5 mg/ml	Supernatant from TiO_2 NPs treated microglia caused significant cytotoxicity in PC12 cells.	[146]
	PC12 cell line	24 h 1–125 μg/ml	Decreased cell viability, mitochondrial impairment and decreased DA levels.	[139]
	BV2 microgial cells	6, 18 h 2.5–120 ppm	Release of ROS, mitochondrial hyperpolarization	[147]
	BV2 microgial cells, N27 neurons, primary cultures of rat striatum	2, 6, 24, 48 h 2.5–120 ppm	Microglia generated ROS damages neurons in complex primary cultures. No cytotoxicity in isolated N27 neurons	[148]

Abbreviations: 5-HIAA: 5-hydroxyindole; 5-HT: 5-hydroxytriptamine; AchE: acetylcholine estarese; BBB: blood-brain barrier; CAT: catalase; CXCL1: chemokine C-X-C motif ligand 1; DA: dopamine; DOPAC: 3,4-dihydrophenylacetic acid; GC–MS: gas chromatography mass spectrometry; GFAP: glial fibrillary acidic protein; Gly: glycine; Gln: glutamine: Glu: glutamate; GPx: glutathione peroxidase; HVA: homovanillic acid; hpf: hours post fertilization; IL-1β: interleukin-1β; IL-6: interleukin-6; IP-10: interferon gamma-induced protein 10; NE: norepinephrine; N-MPG: *N*-(2-mercaptopropionyl)glycine; NPs: nanoparticles; PD: Parkinson's disease; P-gp: P-glycoprotein; ROS: reactive oxygen species; Ser: serine; SOD2: superoxide dismutase 2; TCA: tricarboxylic acid cycle; TNF-α: tumor necrosis factor α; Thr: threonine; Ti: titanium; TiO_2: titanium dioxide.

studies demonstrated that TiO_2 NPs cannot permeate intact and damaged skin and can be found only in the stratum corneum and epidermis, without reaching the brain or peripheral organs [155–158]. Furthermore, low cytotoxicity observed in human HaCaT keratinocytes, suggests a low toxic potential of these nano-compounds at the skin level. These results can be explained by the great stability and low ionizing capacity of these particles and are in accordance with several studies in the literature [159–161]. However, studies simulating real-world scenarios on sunburned skin, with UV exposure in long-term chronic exposure conditions need to be conducted to assure the safety of TiO_2 in sunscreen.

Long-term intake of TiO_2 NPs at low doses was assayed in rats. Animals received 1 or 2 mg/kg TiO_2 suspension per day for 5 consecutive days. On the sixth day their gut tissue was analyzed for TiO_2 content and possible adverse effects. A sex-specific effect on villus cells proliferation was observed in male rats, indicating a potential role for the endocrine system in this process. Oxidative stress in intestinal cells was transient and decreased after 24 h [162].

NPs have the ability to cross the BBB. While this may be desirable for drug-delivery systems [163], it can also pose a risk of unwanted accumulation of potentially harmful chemicals in the brain. In an *in vivo* study by Li et al. (2010), mice were chronically exposed to TiO_2 NPs of

3 nm diameter (4 mg/kg) *via* intratracheal instillations. After 4 weeks, inflammatory cell aggregation and neuron necrosis were present. The amount of Ti in the brain was measured by inductively coupled plasma mass spectrometry (ICP-MS) 3 days after a single instillation of 4 mg/kg TiO_2 and found to be upregulated by 100% (120 ng/g Ti in controls, compared to 240 ng/g in treated animals) [131].

3.1.2. TiO_2 neurotoxic effects in vivo

The diameter of TiO_2 NPs seems to be important for its carriage. Rats were treated with TiO_2 NPs (0.1, 1, 10 mg/kg) suspension of different diameters (10, 20, and 200 nm) through intratracheal treatment. Seventy-two hours later, TiO_2 NPs with diameters of 10 and 20 nm were both transported into the brain, inducing dose-dependent alteration in pro-inflammatory markers (TNF-α, IL-1β and IL-10). However, TiO_2 NPs with diameter of 200 nm did not cause significant alterations in the brain [132]. In a BBB model based on rat primary endothelial cells (BECs) and astrocytes, TiO_2 NPs (acute exposure for 24 h with 0–500 μg/ml or chronic exposure for 5 days with 0–100 μg/ml) could not only pass through the BBB but also disrupt its integrity by reducing the expression of P-glycoprotein (P-gp), claudin 5, caveolin-1, and caveolin-2, which are associated with the BBB integrity [133].

The effects of TiO_2 NPs on the brain may not occur by a direct interaction between the chemical and the BBB. [134] described the *in vivo* uptake and clearance of TiO_2 NPs by BECs and demonstrated a Ti burden in the liver, spleen and lungs up to a year after intravenous (i.v.) administration of TiO_2 NPs (1 mg/kg) to rats, with a very low clearance rate. At this dose, the authors did not observe Ti accumulation in the brain, however upregulation of tight junction proteins, modulation of P-gp mRNA expression and persistent brain inflammation markers such as IL-1β, IP-10 (interferon gamma-induced protein 10), GFAP (glial fibrillary acidic protein) and CXCL1 (chemokine C-X-C motif ligand 1) were observed. The authors suggested that TiO_2 NPs can exert an indirect effect on the CNS that seems dependent on circulating biomarkers potentially released by organs accumulating Ti [134].

Brain levels of 0.05–0.15 μg/ml were detected after intranasal administration of 2.5–10 mg/kg TiO_2 NPs for 90 consecutive days in association with oxidative stress, high levels of lipid, protein, and DNA peroxidation, overproliferation of glial cells, tissue necrosis, hippocampal cell apoptosis in mice. Microarray showed significant alterations of 249 genes expression involved in oxidative stress, apoptosis, memory and learning, brain development, lipid metabolism, DNA repair, signal transduction, immune response and response to stimulus in the brain-injured mice. Some of these genes may be potential biomarkers of brain toxicity caused by TiO_2 NPs exposure [135].

Female mice were intranasally instilled with 500 μg of two types of well-characterized TiO_2 NPs (i.e. 80 nm, rutile or 155 nm, anatase) every other day for 2, 10, 20 or 30 days. High Ti accumulation (ranging from 0.13 to 0.3 μg/ml) was more pronounced in hippocampus after 30 days of rutile exposure, compared to other brain regions (cerebellum, olfactory bulb or cortex). Histological analysis revealed irregular arrangement and loss of neurons, morphological changes and oxidative damage in the hippocampus. Increased TNF-α and IL-1β levels were also observed [136]. Translocated TiO_2 NPs (500 μg) caused imbalance of monoaminergic neurotransmitters, with significantly increased NE and 5-HT levels, while levels of DA, 3,4-dihydrophenylacetic acid (DOPAC), homovanillic (HVA), and 5-hydroxyindole acetic acid (5-HIAA) were decreased [137].

Acetylcholinesterase activity was evaluated in plasma and brain of rats after 60 days intragastric treatment with anatase TiO_2 NPs (50, 100, 200 mg/kg). Plasmatic AChE activity was decreased with the increasing TiO_2 NPs doses. The higher doses of TiO_2 NPS caused a significant decrease in the AChE activity in the brain. These effects were accompanied by IL-6 increase in the brain and plasma and increased levels of GFAP in cerebral cortex, suggesting neuroinflammation [138]. Cognitive function may have also been compromised in this model, but behavioral experiments are lacking. Studies that describe the specific proteins that carry TiO_2 to and/or from the brain are lacking.

In zebrafish larvae exposed to environmentally relevant concentrations (1–10 μg/ml) of TiO_2 NPs induced Parkinson's disease (PD)-like symptoms, with locomotor alteration, reduced DA, Lewy bodies formation and alterations in mRNA levels of *pink1, parkin* and *α-syn,* that were significantly increased in a dose-dependent manner. The authors observed TiO_2 accumulation in brain and oxidative stress, with cell death in hypothalamus. To further investigate TiO_2 effects on DAergic cells, the authors exposed PC12 cells to 1–125 μg/ ml TiO_2 NPs for 24 h. Cell viability was decreased at the higher dose and similarly to zebrafish, DA levels were decreased. This study suggests a role for TiO_2 exposure in the development of PD [139].

Caenorhabditis elegans (*C. elegans*) is an excellent biological model organism for environmental risk assessment. Gas chromatography mass spectrometry (GC–MS)-based metabolomics approach was used to understand the toxicity of sub-lethal concentrations (7.7 and 38.5 μg/ml) of TiO_2 NPs (< 25 nm). Most of the significant perturbations occurred in organic acids (citric, lactic, fumaric, succinic and malic acids) and amino acids. Differential marker metabolites identified from the metabolomic analysis suggested that the disturbances, mainly occurred in metabolism of: glyoxalate, inositol phosphate, tricarboxylate, glycine (Gly), serine (Ser) threonine (Thr) glutamine (Gln) and Glu [140]. Toxicity of bulk-scale (~160 nm) and nanoscale (21 nm) TiO_2 was tested under dark and light conditions. Light exposure induced the production of ROS by nanoscale TiO_2 and increased toxicity of the nanomaterial from a median effect concentration of more than 100 mg/l to 53 mg/l. The observation that light increased the toxicity of the highly photoactive nanoscale TiO_2 suggests that ROS play a role in the photoactivated toxicity of the nanomaterial. No evidence of intracellular oxidative stress was found. Because TiO_2 accumulated in worm intestines, as observed by microscopy, the authors suggested that ROS were formed extracellularly in the apical surface of the worms' intestinal cells [141].

3.2.3. TiO_2 neurotoxic effects in vitro

In vitro human cell models may represent a valid instrument to investigate TiO_2 NPs effects on CNS and to determine their underlying mechanistic processes, providing information about doses of exposure. [142] demonstrated concentration- and time-dependent alterations of the mitochondrial function on D384 (glial cell line) and SH-SY5Y (neuronal cell line) cells starting at the dose of 31 and 15 μg/ml TiO_2 (15–69 nm in diameter, anatase isoform), respectively, after 24 h exposure. Neuronal cells were more sensitive than glial cells. These effects were more pronounced in cells exposed to NPs compared to TiO_2 bulk, where with the latter effects appeared only at the highest doses (125 and 250 μg/ml) after 24 and 48 h, similarly in both cerebral cell lines. Cell membrane damage was present in both cell lines starting at 125 μg/ml after 24 h exposure and also dependent on TiO_2 size. TiO_2 NPs were potent inhibitors of cell proliferation in human CNS cells after prolonged exposure (up to 10 days) at doses ranging from 0.1 to 1.5 μg/ml [142].

TiO_2 NPs induced apoptosis in both human (U373) and rat (C6) glial cells at 96 h of treatment, evidenced by active caspase-3 starting at 5 $\mu g/cm^2$. At this concentration, DNA fragmentation assessed with the TUNEL assay was observed in U373 cells, but not in C6 cells. Morphological changes associated with depolymerization of F-actin were found, accompanied by apoptotic cell death [143]. In a similar protocol of exposure, TiO_2 NPs induced oxidative stress in U373 and C6 glial cells, mediating changes in the cellular redox state, which was correlated with increase in antioxidant enzyme expression (GPx, catalase and SOD2) and lipoperoxidation. Mitochondrial depolarization was also observed. These effects occurred within 24 h exposure to 20 $\mu g/cm^2$ TiO_2 NPs [144]. Oxidative stress was also present in rat PC12 cells exposed to TiO_2 NPs 50 μg/ml for 24 h (P25 type, 21 nm in average size) and *N*-(2-mercaptopropionyl)-glycine (N-MPG), a kind of ROS scavenger, prevented apoptosis in this model [145], indicating that

oxidative stress is an important factor in TiO_2 NPs-induced neurotoxicity.

P25 (an uncoated photo-active, largely anatase form of nanosize TiO_2, not used in sunscreen) stimulates ROS in BV2 microglia at 2.5–120 ppm, starting at 5 min exposure [147] and was later found to be nontoxic to isolated N27 neurons. However, P25 rapidly damages neurons at low concentrations (5 ppm, 6 h) in complex brain cultures of striatal cells, suggesting that microglial generated ROS damages neurons [148]. Ability of activated microglia to induce death of target cells was studied by Xue et al. (2012) in co-culture with PC12 cells. Supernatant from TiO_2 NPs-treated (0.25–0.5 mg/ml) microglia caused significant cytotoxicity in PC12 cells. The authors suggested that TiO_2 NPs stimulated microglia produced inflammatory factors, which caused PC12 cells cytotoxicity [146].

Recent studies report endoplasmic reticulum stress (ER stress) as a common response to NPs related toxicity. The ER stress also known as unfolded protein response (UPR) refers to an important cellular self-protection mechanism, which can be activated to counteract the cell situation of stress (overloading proteins or direct ER damage). ER stress was observed in human epidermal keratinocytes (HEKn) and human umbilical vein endothelial cells (HUVEC) exposed to up to 20 $\mu g/cm^2$ for 16–24 h [164], [165]. Analogous to neural cells, oxidative stress was also observed in different cell types, demonstrating that TiO_2 can affect a wide range of tissues. For example, the lung is a primary target of NPs exposure, especially in occupational settings. In the case of TiO_2 inhalation, nanoscale particles may deposit in the lung interstitium and cause inflammation [166]. Several excellent reports are available on TiO_2 effects on peripheral tissues, such as skin [167], kidney [168], liver [169], lung [170] and vascular endothelial cells [171].

4. Conclusions and future perspectives

Although some UV-related pathologies could be prevented by applying sunscreen, the efficiency and safety of these products is questionable. As the use of sunscreen is continuously increasing worldwide, so do the levels of environmental accumulation and human, and wildlife exposure. Whether concentration resulted from daily use and/or environmental contact possesses a realistic hazard to humans and other organisms is still unknown. Numerous studies raised concerns about the association between exposure to substances commonly found in sunscreens and endocrine and developmental impairments. In this review, the potential neurotoxicity of such substances is presented and the question of cost-benefit is raised regarding large scale use of sunscreen in its current composition. Although most studies reviewed in this paper reported adverse neurotoxic effects of UV filters at concentrations substantially higher that those observed in environment and human tissues, these studies should not be disregarded, as they afford potential pathomechanisms which might occur in other conditions or sensitive populations. It is noteworthy, that gene x environment interactions *vis-à-vis* toxicity of sunscreen components has yet to be studied. Unfortunately, the effects of repeated, long-term and low-dose exposures to single compounds and mixtures of various UV filters is also poorly studied. More studies are needed to evaluate the realistic hazard of contemporary sunscreens. Furthermore, it is also timely and meritorious to advance studies on alternative, safer and more efficient UV filters.

Conflicts of interest

The authors declare no conflict of interest.

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Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays.

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Abstract
Two important ingredients of personal care products, namely polycyclic musk fragrances and UV filters, can be found in the environment and in humans. In previous studies, several compounds of both classes have been tested for their interaction with the estrogen receptor. Two polycyclic musk fragrances, namely AHTN and HHCB, turned out to be anti-estrogenic both in vitro and in vivo in a transgenic zebrafish assay. Several UV filters have been shown to exert estrogenic effects in vitro and in some in vivo studies. Here, we assessed the interaction of five polycyclic musk compounds and seven UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone (PR) receptor, using sensitive and specific reporter gene cell lines. Four polycyclic musks (AHTN, HHCB, AETT, and AHMI) were found to be antagonists toward the ERbeta, AR and PR. The UV filters that showed estrogenic effects (benzophenone-3, Bp-3; 3-benzylidene camphor, 3-BC; homosalate, HMS; and 4-methylbenzylidene camphor, 4-MBC) were found to be antagonists toward the AR and PR. The ERalpha agonistic UV filter octyl-dimethyl-p-aminobenzoic acid (OD-PABA) did not show activity toward the AR and PR. Octyl methoxy cinnamate (OMC) showed weak ER alpha agonism, but potent PR antagonism. Butyl methoxydibenzoylmethane (B-MDM) only showed weak ERalpha agonism and weak AR antagonism. Most effects were observed at relatively high concentrations (above 1 muM); however, the anti-progestagenic effects of the polycyclic musks AHMI and AHTN were detected at concentrations as low as 0.01 muM. The activity of anti-progestagenic xenobiotics at low concentrations indicates the need to undertake more research to find out about the potential endocrine disrupting effects of these compounds in vivo.

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UV-absorbing and other sun-protecting substances: genotoxicity of 2-ethylhexyl *P*-methoxycinnamate

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Summary

The mutagenicity of 2-ethylhexyl *p*-methoxycinnamate was demonstrated when 25 sunscreen ingredients were tested in the Salmonella/microsome assay. This substance also increased the frequency of sex-linked recessive lethals in *Drosophila melanogaster*. A trace contaminant may be implicated because many samples were obtained from several sources and the results were batch-related.

Sunscreen agents have been extensively tested for acute toxicity to humans, and yet there is little information on their chronic effects. We have recently undertaken a study of some UV-absorbing chemicals and other ingredients of sunscreen formulations for evidence of mutagenic activity in the Salmonella/microsome assay. The reason for this was not to challenge the basic assumptions behind the use of sunscreens. There can be little doubt that most sunscreens, if used sensibly, will reduce the risk of sunlight-induced skin cancer. Instead, we sought to determine whether any of these substances, themselves, had mutagenic or carcinogenic activity. While all UV-absorbing chemicals may protect users from most of the harmful effects of UV light, not all such chemicals may be entirely safe.

Salmonella mutagenesis assays, using the standard plate incorporation test, were conducted according to established methods (Ames et al., 1975), with the standard bacterial strains TA100, TA98, TA1535, TA1537 and TA1538. Chemicals were prepared freshly in the solvent dimethyl sulphoxide (DMSO) and a minimum of 2 plates per dose point was used. Both toxicity and true genotypic reversion of mutant

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colonies were carefully monitored. All chemicals were tested in the presence and absence of S9 mix, prepared from the livers of Aroclor 1254-induced male (150 – 200 g) Sprague – Dawley rats.

Table 1 lists the names of substances screened for Salmonella mutagenicity. All, with the exception of 2-ethylhexyl *p*-methoxycinnamate (2-EHMC), gave negative results. The mutagenicity results obtained with this substance are shown in Table 2. It can be seen that samples 67/79, 74/79 and 53/81 were essentially negative, whereas the remaining 7 were consistently positive on repeated testing. Although the mutagenicity of this substance was previously reported (Lane Brown et al., 1980a,c), the failure to reproduce this result in another sample (Baker et al., 1980) seemed to implicate an impurity. Further samples were then tested to investigate whether

TABLE 1

SUBSTANCES SCREENED FOR SALMONELLA MUTAGENICITY

Substance	Source
2-Ethylhexyl *p*-methoxycinnamate	Various[a]
2-Hydroxy-4-methoxybenzophenone	Merck
Homomenthyl salicylate	Rockes
p-Aminobenzoic acid	Prosana Labs
2-Ethylhexyl ester of 4-dimethylamin benzoic acid	Bronson and Jacobs
Ethyl dihydroxypropyl *p*-aminobenzoic acid	Bronson and Jacobs
Octyl-*N,N*-dimethyl *p*-aminobenzoic acid	M.M. Lane-Brown
p-Chloro-*m*-xylenol	M.M. Lane-Brown
Trichlorcarbanilide	C.H. Gallagher
3,4′,5-Tribromosalicylanilide	R.T. Vanderbilt
2,2′,4,4′-Tetrahydroxybenzophenone	B.A.S.F.
4-*tert*-Butyl-4′-methoxy-dibenzoylmethane	Givaudan
3-(4′-Methylbenzylidene)-2-oxo-bornylidene	Merck
2-Phenyl-benzimidazole 5-sulphuric acid	Merck
5-(3,3-Dimethyl-2-norbornylidene)-3-penten-2-one	Dragoco
Diethanolamine salt of *p*-methoxycinnamic acid	Givaudan
Ethyl 2-cyano-3,3-diphenylacrylate	B.A.S.F.
2-Hydroxy-4-methoxybenzophenone-5-sulphuric acid	B.A.S.F.
2,2′-Dihydroxy-4,4′-dimethoxybenzophenone-5-sodium sulphonate	B.A.S.F.
2,4-Dihydroxybenzophenone	B.A.S.F.
2-Ethylhexyl-2-cyano-3,3-diphenylacrylate	B.A.S.F.
Dihydroxy acetone	Merck/B.D.H.
Dibenzaldehydamine	Merck/B.D.H.
3,4-Dimethoxyphenylglyoxilic acid	Merck/B.D.H.
2-Ethylhexyl-4′-phenylbenzophenone, 2-carboxylate	Merck/B.D.H.

[a] See Table 2.

TABLE 2

MUTAGENICITY OF 2-ETHYLHEXYL *p*-METHOXYCINNAMATE IN *Salmonella typhimurium* STRAIN TA1538 IN THE ABSENCE OF S9 MIX

Laboratory chemical number	Date of assay	Volume of chemical/plate				Solvent[a] control
		100 μl	32 μl	10 μl	3.2 μl	
67/79	30.7.79	n.d.[b]	n.d.	27,24	25,28	32,24
	11.4.80	n.d.	n.d.	19,22	15,17	18,22
72/79	6.8.79	44,39[c]	27,29	22,19	16,17	14,15
	6.5.81	62	48,57	25,27	15,19	10,10
74/79	7.8.79	21,25	19,20	17,18	19,20	18,25
	6.5.81	12,17	15,17	15,15	11,11	10,10
	15.6.81	4,12	9,14	13	8,12	5,8,13
	7.7.81	4,8	12,13	6,6	7,8	6,6,6
14/80	29.4.80	34,38	33,37	21,24	19,21	13,15,17
	6.5.81	27,28	37,37	20,32	17,22	10,10
	15.6.81	18,20	16,21	14,18	11,14	5,8,13
	7.7.81	26,27	22,23	15,22	7,5	6,6,6
21/80	7.4.81	16,18	6,9	8,10	8,10	8,9
	6.5.81	23,24	20,33	10,26	13,22	10,10
	15.6.81	22,24	17,22	8,17	8,9	5,8,13
	7.7.81	13,27	13,21	17,18	5,10	6,6,6
3/81	1.4.81	69,78	59,72	34,32	23,30	8,8
	15.6.81	52,54	44,52	32,34	15,19	5,8,13
	7.7.81	75,93	61,63	31,42	15,19	6,6,6
48/81	6.5.81	102,106	109,114	63,66	42,50	10,10
	15.6.81	98,101	100,103	63,76	33,43	5,8,13
	7.7.81	107,132	81,102	82,83	42,42	6,6,6
49/81	6.5.81	61,80	75,76	41,43	15,26	10,10
	15.6.81	108,112	65,79	48,59	27,28	5,8,13
	7.7.81	101,123	65,75	56,59	26,34	6,6,6
52/81	15.6.81	113,122	107,113	73,84	44,41	5,8,13
	7.7.81	120,137	102,108	61,68	33,35	6,6,6
53/81	15.6.81	14,18	13,14	11,16	7,9	5,8,13
	7.7.81	12,13	8,14	7,12	7,10	6,6,6

[a] 4-Nitroquinoline-1-oxide (0.1 μg/plate) was used to test for the revertability of TA1538, and consistently induced a 3-fold increase of revertant colonies, compared to solvent controls.

[b] n.d., not determined.

[c] Underlined results are those showing at least a doubling of background control-plate colony numbers.

Manufacturers: Haarman and Reimer, 67/79, 52/81; Givaudan, 74/79, 14/80, 21/80, 3/81, 48/81, 53/81.

Secondary sources (manufacturer unknown): Greiter A.G., 72/79.

Containers: Metal covered glass, metal lid, 21/80; Brown glass, metal lid, 67/79, 74/79; Brown glass, bakelite lid, 14/80, 49/81, 52/81, 53/81; Clear glass, bakelite lid, 3/81; All-plastic, 72/79, 48/81.

correlations existed between mutagenicity and individual batches, storage conditions and containers. Several features of this investigation are worth noting. All positive samples were detected with the same Salmonella strain, TA1538, and in the absence of S9 mix. The finding of mutagenic activity in both glass and plastic containers dispels the possibility that the material in which samples were stored may have been responsible, but clearly implicates an unidentified impurity.

Preliminary studies of one sample (49/81) of 2-EHMC in Chinese hamster ovary (CHO) cell cultures showed that the substance had a marked inhibitory effect on cell division without being overtly toxic at concentrations in excess of 0.01 M. However, in a single experiment, at a concentration of 0.18 M and under modified conditions of prolonged incubation in the presence of 10 μM BrdU (48 h), 2-EHMC gave 28.4 $\pm$ 11.0 sister-chromatid exchanges (SCE)/metaphase compared to control values of 14.3 $\pm$ 3.1 SCE/metaphase. S9 mix was not required. Differentiation of sister chromatids was obtained by staining the slides according to the fluorescence plus Giemsa technique (Perry and Wolff, 1974).

TABLE 3

INDUCTION OF SEX-LINKED RECESSIVE LETHALS IN *Drosophila melanogaster* BY 2-ETHYLHEXYL *p*-METHOXYCINNAMATE

Sample	Stage	Sex-linked recessive lethals[a] Brood			Mutation frequency (%)	Probability[b]
		A	B	Total		
72/79	Adult[c]	1/63	1/63	2/126	1.6	0.0752
	Adult[c]	2/114	9/248	11/362	3	0.0001
49/81	Larval[d]	10/636	5/886	15/1522	0.99	0.0002
Negative control	Adult	0/280	1/351	1/631	0.16	
Positive control (EMS)[e]	Adult	60/180		60/180	33	<0.0001
Negative control	Larval	0/800	1/833	1/1633	0.06	

[a] Mutants were scored by mating 20 treated 4–7-day-old Newcastle strain males to *Basc* females, 3 females to each male, with a successive brood from fresh virgin females after a 72-h interval. All cultures were maintained at 25°C and suspected lethals were checked in the F_3 generation. No mutant clusters were detected.

[b] Probability calculated by 1-tail Fisher Exact test.

[c] Adult males were fed on 2.5×10^{-2} M 2-EHMC in 10% DMSO, 10% sucrose for 48 h.

[d] Third instar male larvae were fed on 5×10^{-3} 2-EHMC in 10% DMSO, 1% sucrose for 48 h prior to pupation.

[e] Positive control adults were fed 2.5×10^{-2} M ethyl methanesulphonate (EMS).

2 of the samples which were mutagenic in Salmonella were tested for their ability to induce sex-linked recessive lethals in *Drosophila melanogaster* and the results are shown in Table 3. Sample 72/79 was tested in adult flies (Abrahamson and Lewis, 1971) but a more sensitive larval assay (Clark, 1979; Angus et al., 1981) was employed to test sample 49/81. Both samples of 2-EHMC were found to be mutagenic for Drosophila.

If structure – activity relationships can be used as a guide in evaluating a compound's potential carcinogenic and mutagenic hazard, it is of interest to note that, (a) 2-ethylhexyl acrylate, a chemical analogue, has recently been reported to induce a low incidence of skin tumours in mice (T. Tyler, personal communication); (b) di-(2-ethylhexyl) phthalate and di-2-ethylhexyl-adipate, 2 other chemically related substances, have been reported to be mutagenic and the former also caused significant growth inhibition in human diploid cell cultures (Fishbein, 1979).

The findings reported here present a matter for concern, particularly since 2-EHMC is commonly used at concentrations of 5 – 10% w/v in sunscreen formulations and under conditions of moderate exposure to human populations. All involved with the substance should be made aware of the preliminary mutagenesis data, and of the possible implications if mouse skin tests (currently being conducted) should reveal the samples to be carcinogenic or cocarcinogenic.

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E. coli B/r 株における紫外線吸収剤の UV 誘発突然変異に対する影響

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Effect of UV Absorbers on UV-Induced Mutagenesis in *E. coli* B/r

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Effects of cosmetic UV absorbers on UV-induced mutagenesis in *E. coli* B/r strains were studied. Among 8 substances tested, cinoxate (2-ethoxyethyl *p*-methoxycinnamate) enhanced UV (254 nm)-induced mutation at non-lethal toxic doses. This adverse effect was also observed when the mid and near-UV (295 nm—400 nm) irradiated cells of WP 2 were used.

This effect, however, was not observed in WP 2 s *uvrA*, a DNA excision repair deficient strain of *E. coli* B/r. It is thus assumed that cinoxate inhibited DNA excision repair system and consequently increased UV-induced mutation.

Keywords——cosmetic UV absorbers; UV-induced mutation; DNA excision repair; cinoxate

緒 言

紫外線は我々に多大な恩恵を与える一方で我々の皮膚に日焼けなどの炎症を起こすなど，危害をももたらす．強い太陽光線に皮膚が長時間繰り返し暴露されると，皮膚の早期老化が起こったり皮膚癌が発生したりする.[1] 以前から細胞に紫外線を照射すると DNA 上に cyclobutane pyrimidine dimers や pyrimidine pyrimidone(6-4) photoproducts などの核酸塩基の損傷が生じ，それが原因となって細胞の致死や突然変異がひき起こされ，更には皮膚癌に至ると言われている.[2,3]

紫外線のこのような有害な作用に対し，それを防止する目的で紫外線遮断剤 (sunscreens：サンスクリーン) が用いられる．サンスクリーンには光のエネルギーを吸収する紫外線吸収剤 (ultraviolet absorbers) と光を散乱させることにより紫外線の害を少なくする紫外線散乱剤 (ultraviolet scattering agents) とがある．我々の皮膚にはメラニン，ウロカニン酸などが存在し，紫外線吸収の役目を担っている．香粧品中のいわゆる合成紫外線吸収剤として利用されているものにはその化学構造から，ベンゾフェノン系，安息香酸系，サリチル酸系，ケイ皮酸系などに分類される化合物がある.[4-6]

これら紫外線吸収剤のほとんどは，それ自体変異原性を示さず,[7,8] 太陽光による皮膚癌発生を阻止する効果は大きいと思われるが，今回，著者らは *E. coli* B/r 株を用いて紫外線照射後に突然変異が固定化される過程への吸収剤の影響を検討し，若干の知見を得たので報告する．

実 験 方 法

1. 試薬及び培地 紫外線吸収剤——化合物名，構造式及び製造元を Table I に示す．これらはすべてジメチルスルホキシド (DMSO) に溶解して用いた．

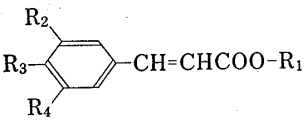
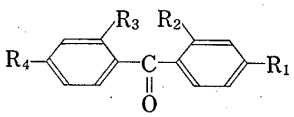
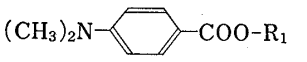
NB 培地——Difco nutrient broth 8 g, NaCl 5 g を蒸留水 1 l に溶解しオートクレーブで滅菌した．

SEM 寒天培地——$(NH_4)_2SO_4$ 1 g, KH_2PO_4 10 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, trisodium citrate$\cdot 2H_2O$ 0.5 g を蒸留水に溶解後，KOH で pH 7.0 に調整して全量を 400 ml とし，glucose 4 g, Difco nutrient broth 0.16g, Difco agar 15 g に蒸留水 600 ml を適量に分けて加えてそれぞれオートクレーブで滅菌した．その後，それらを混合して 1 l としプレートに 30 ml ずつ分注した．

Soft agar——NaCl 0.6 g, Difco agar 0.7 g を蒸留水 100 ml に加え，オートクレーブで滅菌した．

PB (リン酸緩衝液)——1/15 M KH_2PO_4 200 ml と 1/15 M Na_2HPO_4 800 ml を混和し pH 7.4 に調整した後，

TABLE I. UV Absorbers Tested for Their Effects on UV-Induced Mutagenicity

Compounds	Structural formula	Source
1. 2-Ethoxyethyl-*p*-methoxycinnamate		Givaudan
2. Methyl-2,5-diisopropylcinnamate		Haarmann & Reimer, Japan
3. 2-Ethylhexyl-*p*-methoxycinnamate		Givaudan
4. 2-Hydroxy-4-methoxybenzophenone		Kimura Sangyo
5. 2,2′-Dihydroxy-4,4′-dimethoxybenzophenone		〃
6. Mixture of No. 5 and other benzophenone		〃
7. Amyl-*p*-*N*,*N*-dimethylaminobenzoate		Ina Trading Co.
8. 2-Ethylhexyl-*p*-*N*,*N*-dimethylaminobenzoate		〃

1. $R_1=CH_2CH_2OCH_2CH_3$, $R_2=H$, $R_3=CH_3O$, $R_4=H$ 2. $R_1=CH_3$, $R_2, R_4=CH(CH_3)CH_3$, $R_3=H$
3. $R_1=CH_2CH(C_2H_5)CH_2CH_2CH_2CH_3$, $R_2, R_4=H$, $R_3=CH_3O$ 4. $R_1, R_2=H$, $R_3=OH$, $R_4=CH_3O$
5. $R_1, R_4=CH_3O$, $R_2, R_3=OH$ 7. $R_1=C_5H_{11}$ 8. $R_1=CH_2CH(C_2H_5)CH_2CH_2CH_2CH_3$.

オートクレーブで滅菌した.

2. 使用菌株 *E. coli* B/r WP 2 *trpE* 65[9] 及びその DNA 除去修復欠損株である WP 2 s *uvrA* 155 *trpE* 65[10] を用いた.

3. 紫外線照射条件 遠紫外線 (254 nm) 源には殺菌用ランプ 15 W, 4 W (東芝) を, 中, 近紫外線 (295—400 nm) 源には健康線用蛍光ランプ：sunlamp (FL20S. E, 東芝) を用いた (295 nm 以下の紫外線を除くために Falcon のペトリ皿のカバーを照射時に使用). そのエネルギー強度は各々 1.36, 0.101, 0.4 J/m²/s で, UV ラジオメーター UVR-254, UVR-365 (東京光学機械) により測定した.

4. 抗変異原性試験 NB 培地 (各試験管 5 ml ずつ) で14時間培養した対数後期の菌 20 ml を, 遠心分離 (5000 rpm, 10分) により 2 回洗浄して 5 ml の PB に再浮遊させた ($1-2\times10^9$ cells/ml). この菌浮遊液 5 ml を内径 9 cm のペトリ皿に入れ, 15秒間 5 秒おきに攪拌しながら照射 (中, 近紫外線の場合は45分) し, PB により復帰変異体数計測のため 10^{-1} に, 生存菌数計測のため 10^{-6} に希釈した. 試験管に PB 0.5 ml, 試料 50 μl (コントロールには DMSO を使用), 紫外線を照射した菌の各希釈液 0.2 ml, ソフトアガー 2 ml を入れてよく混合し, SEM 寒天培地に注ぎ固化させた. 37℃ で 2 日間培養した後, Trp^+ の復帰変異体数及び生存菌数を計数した. また, 試料の致死毒性, 変異原性を調べるため未照射の菌についても全く同様に行った. なお, 液体保持 (liquid holding : LH)[11] を行った場合は, 各試験管に PB 0.5 ml, 試料 50 μl, 及び紫外線照射菌液 0.2 ml を入れ37℃のインキュベーター中に置き, 30分, 60分, 90分後にそれぞれソフトアガーを加えた.

それぞれの結果はすべて 2 回の実験の平均値で示した.

TABLE II. Effects of UV Absorbers on Survival and Mutation Induction in *E. coli* B/r WP2

Compounds	Trp^+ revertants/plate	Viable cells/plate	Survival (%)
Control (DMSO)	6	415	100
1.[a]	5	405	97.6
2.	6	409	98.6
3.	7	414	99.8
4.	5	381	91.8
5.	8	424	102
6.[b]	5	391	94.2
7.	4	389	93.7
8.	3	373	89.9

Dose of UV absorbers : 1 (*a*) 2, *b*) 0.25) mg/plate.

実験結果及び考察

Table III に 8 種の紫外線吸収剤の UV (紫外線) 誘発突然変異への影響を一括して示した. 今回用いた各紫外線吸収剤の濃度ではほとんど致死毒性を示さず, また, それ自体に変異原性も認められなかった (Table II). これより高い濃度では結晶が析出し実験が不可能であった. Cinoxate (2-ethoxyethyl *p*-methoxycinnamate) に突然変異増強作用が認められた. 2 mg/plate において誘発突然変異頻度が 176 revertants/10^7 cells から 336 revertants/10^7 cells となり約 2 倍に上昇した. このような効果は DNA 除去修復欠損株である WP 2s *uvrA* においては認められなかった. したがって, この突然変異増強作用は DNA 除去修復機構——細胞分裂が始まる前に速やかにしかも正確に DNA 上の損傷を切り出して修復する[12,13]——に cinoxate が作用した結果によると推定される. そこで液体保持 (LH) を行って,

TABLE III. Effects of UV Absorbers on UV (254 nm)-Induced Mutagenesis in *E. coli* B/r WP2 and WP2*suvrA*

Compounds	Dose (mg/plate)	Trp+ revertants/plate	Viable cells/plate	Trp+ revertants/10^7 cells
Control (DMSO)		374 (560)	212 (144)	176 (389)
1.	0.25	427	219	195
	0.5	385 (569)	191 (153)	202 (372)
	1.0	548 (558)	186 (133)	295 (420)
	2.0	545 (581)	162 (140)	336 (415)
2.	0.5	370	181	204
	1.0	370	183	202
3.	0.5	414	224	185
	1.0	380	224	170
4.	0.5	364	192	190
	1.0	349	182	192
5.	0.5	356	186	191
	1.0	343	188	182
6.	0.1	341	192	178
	0.25	267	168	159
7.	0.5	395 (455)	202 (118)	196 (386)
	1.0	476 (471)	197 (114)	242 (413)
8.	0.5	380 (526)	209 (154)	182 (342)
	1.0	448 (540)	220 (148)	204 (365)

( ): results from WP2*suvrA*, UV dose: 20.4 J/m^2.

cinoxate の突然変異誘発への影響を調べてみた．液体保持とは非栄養培地中に一定時間 DNA 損傷を受けた菌を保持することで，この間に DNA 除去修復が進行すると言われている.[11] Fig. 1 に示したように，UV 照射した菌を非栄養培地である PB 中に保持するとコントロール (DMSO) の場合，revertant 数は速やかに減少し30分後には 1/5 になった．しかし，cinoxate を添加した場合，revertant 数は濃度に依存して減少が見られなかった．この結果から cinoxate が DNA 除去修復を阻害することが更に支持された．

Cinoxate はケイ皮酸系の化合物である．以前に我々はケイ皮酸メチルエステル誘導体である methyl sinapate (methyl-3,5-dimethoxy 4-hydroxycinnamate) が，UV，4NQO (4-nitro-quinoline 1-oxide) 誘発突然変異を特異的に増強し，その作用が，*uvr*$^-$ 株では見られないこと，*uvr*$^+$ 株の UV 致死性を高めること，LHR (liquid holding recovery) を阻害すること，更に thymine dimers の除去率を有意に減少させることなどの理由から DNA 除去修復を阻害して UV 誘発突然変異を増強することを報告した.[14] Cinoxate は methyl sinapate と類似した化学構造を持っており，同じような作用で DNA 除去修復機構に働いているものと思われる．

以上は 254 nm の遠紫外線に対する結果であるが，実際の太陽光の紫外線の波長域は 290—400 nm であり突然変異や発癌に寄与しているのは 320 nm 前後の紫外線であると言われている.[1,15] そこでサンランプを用いて中，近紫外線による誘発突然変異への影響も調べ，その結果を Table IV に示した．この表から明らかなとおり cinoxate には 254 nm による時と同様の効果が認めら

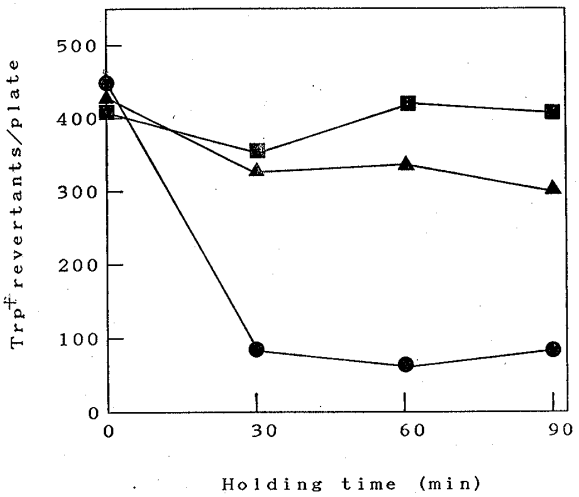


Fig. 1. Inhibitory Effect of Cinoxate on UV-Induced Mutation Reduction during Liquid Holding

After UV-irradiation (20.4 J/m^2), the cells were kept in phosphate buffer in the presence or absence of cinoxate (—●—: DMSO, —▲—: 25 μg/tube, —■—: 40 μg/tube) for indicated time and then poured onto the SEM plates with soft agar.

TABLE IV. Effects of UV Absorbers on Mid and Near UV (295—400 nm)-Induced Mutagenesis in *E. coli* B/r WP2

Compounds	Dose (mg/plate)	Trp^+ revertants/plate	Viable cells/plate	Trp^+ revertants/10^7 cells
Control (DMSO)		160	270	59
1.	0.25	163	286	57
	0.5	198	277	71
	1.0	255	250	102
	2.0	219	235	93
7.	0.5	170	269	63
	1.0	204	254	80
8.	0.5	170	259	66
	1.0	170	262	65

UV dose : 1080 J/m^2.

れた.

8種の紫外線吸収剤のうち *E. coli* B/r 株において，UV 誘発突然変異増強作用が認められた cinoxate は現在，香粧品に5％以下の配合濃度で使用されており，表示指定成分である.[16] この増強作用はそれほど強いものではないが，cinoxate が UV と直接反応して変異原物質を生成するのではなく，UV によって損傷した DNA が修復される過程で作用するものであること，ヒトの常染色体劣性遺伝病で，太陽光により皮膚癌を多発する色素性乾皮症 (Xeroderma pigmentosum : XP) の患者由来の細胞が DNA 除去修復を欠損していること,[2,3] また，最近，テニス，ゴルフなどの戸外でのスポーツを行う人が増加しており，したがって，紫外線吸収剤を含有した香粧品を使用する機会も増加していると思われることなどを考えると，今後，動物細胞あるいは動物個体における影響について検討がなされるべきであろう．そして，その結果によってはこの種の紫外線吸収剤の添加された香粧品は使用に際して注意が必要ではないかと思われる.

謝辞 紫外線吸収剤の入手について御便宜をおはかりくださいました東京薬科大学の永山富雄先生に深く感謝致します.

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FOREWORD

The Scientific Committee on Cosmetology was set up by Commission Decision 78/45/EEC of 19 December 1977 (OJ L13 of 17 January 1978, p. 24) in order to provide the Commission with informed opinions on scientific and technical matters related to cosmetic products, and in particular to the substances used in the preparation of cosmetic products and to their composition and conditions of use of these products.

The members of the Committee are independent scientists highly qualified in the fields of medicine, toxicology, biology, chemistry or other similar disciplines. The chairman is elected by its members and the secretariat is provided by the Commission.

The SCC expresses its opinions on answers to specific questions from the Commission, and these are published. The opinions expressed reflect the present state of knowledge concerning aspects of cosmetic products and other related aspects in regards to Community legislation, and in particular :

Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (OJ L262, 27.9.76, p.126), as amended for the last time by Council Directive 93/35/EEC of 14 June 1993 (OJ L151, 23.6.93, p. 32).

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OPINIONS ADOPTED DURING THE 46TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 19 February 1991

A 19: *2,7-DIHYDROXYNAPHTHALENE*

1. General

1.1 Primary name

2,7-dihydroxynaphthalene

1.2 Chemical names

2,7-dihydroxynaphthalene
2,7-naphthalenediol

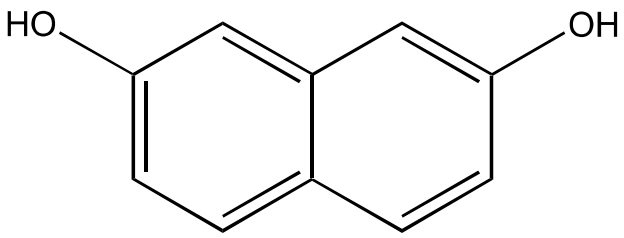
1.3 Trade names and abbreviations

Ro 575

1.4 CAS no.

582-17-2

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_8O_2$
Mol weight: 160.2

2. Function and uses

Oxidative hair dye; max. use: 1 %; 0.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: mice CD1, oral: 720 (655-792) mg/kg b.w.
rat, oral: >5000 mg/kg b.w.
(1 % of 2,7-dihydroxynaphthalene containing formulation)

3.2 Acute dermal toxicity

0.93 % of 2,7- dihydroxynaphthalene equivalents was absorbed through the skin of rats over a period of 24 hours after 30 minutes of dermal application to intact, clipped skin of male and female rats with a formulation containing ^{14}C-2,7-dihydroxynaphthalene (21.76 mg).

3.7 Subchronic oral toxicity

2,7-dihydroxynaphthalene was administered daily by oral gavage, over a period of 12 weeks to 15 male and 15 female Wistar rats (Mu Ra Han 67 SPF) for each group, at dose levels of 0-20-60-180 (5.5 weeks)/360 (6.5 weeks) mg/kg b.w./day (10 ml/kg in aqueous suspension). The highest test dose produced a weight increase of liver, spleen and kidney, liver's pigmentation, increase hematopoiesis in the spleen, and hyaline deposition in the kidney. The other doses (20 and 60 mg/kg/day) did not show clinical, biochemical and pathological-anatomical signs of a systemic cumulative toxicity. The dose of 60 mg/kg/day represents the dose with the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied (500 µl in gauze patches) as a 10 % (w/v) solution in 2 % carboxymethylcellulose (pH=8-10) for four hours on the clipped skin of rabbits resulted mild irritating. The compound applied twice daily for 5 days, as 10 % (w/v) aqueous solution, to the same of the back skin area of male hairless mice resulted not irritating. The compound containing in a formulation (1 %) resulted not irritating to rabbit's skin when applied under occlusion for 4 hours. The compound containing in a formulation (1 %) resulted not irritating to the mouse' skin when applied daily (30 minutes for application) for 5 days.

4.2 Irritation (mucous membranes)

The compound applied as 5 % (w/v) water solution (100 µl) on rabbit's eyes resulted not irritating for the cornea and iris in all animals. The conjunctiva 2 hours after instillation showed mild or severe redness in all animals, with mild oedema (1 rabbit) and exudation (2 rabbits), that disappears 72 hours after treatment. The compound containing formulation (1 %) resulted slightly irritating for the rabbit's eyes.

5. Sensitization

It was induced in guinea pigs by intradermal injection of 5 % (w/v) test compound in propylene glycol, Freund's complete adjuvant (FCA) and 1:1 (v/v) mixture of the above solution on day 0, and 7 days later by dermal application of 5 % (w/w) test compound in vaseline, under occlusion, for 48 hours. 14 days later the guinea pigs were challenged by a dermal application, under occlusion at a new skin site, of the 10 % (w/v) test compound in propylene glycol. The compound resulted non-sensitizer in guinea pigs.

The compound containing formulation (1 %) resulted non-sensitizer in guinea pigs after two different challenge exposures (open epicutaneous at day 21, and dermal administration at day 28).

6. Teratogenicity

2,7-dihydroxynaphthalene administered daily by oral gavage to groups of 30 pregnant CD-Sprague Dawley rats from day 5 to 15 of gestation at doses of 0-20-60-360 mg/kg showed in the highest test dose slight retardation of the average body weights during the treatment. No other differences have been observed for other teratogenicity and embryotoxicity parameters. The dose of 60 mg/kg resulted the dose with the NOAEL.

Embryotoxicity: The compound tested in the Hen Egg Test resulted moderately toxic: LD_{50}: 5.1 mg/egg (1 day) and 2.05 mg/egg (5 days). The compound did not show evidence of a teratogenic potential in this system.

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C-2,7-dihydroxynaphthalene applied subcutaneously (20 mg in distilled water) or oral (60 mg in distilled water) to male and female Wistar rats (SPF-TNO) showed that the radioactivity was excreted within 24 hours: in the urine (partly as glucuronide or sulphate) and feces after subcutaneous treatment (more than 95 %) and in the urine after oral administration. In the expired air no radioactivity has been found after subcutaneous test. In the subcutaneous test no parent compound was revealed in the urine. In the oral treatment the test substance was completely absorbed by the intestine.

Dermal absorption: 0.93 % of 2,7-dihydroxynaphthalene equivalents was absorbed through the skin of rats over a period of 24 hours after 30 min of dermal application to intact, clipped skin of male and female rats with a formulation containing ^{14}C-2,7-dihydroxynaphthalene (21.76 mg).

8. Mutagenicity

Mutagenicity and genotoxicity studies have shown that 2,7-dihydroxynaphthalene does not induce: (1) gene mutations on five strains of *Salmonella typhimurium* in the absence and in the presence of Phenobarbital or Aroclor induced rat liver enzymes; (2) micronuclea in CD-1 mice (bone marrow cells) treated by oral gavage (2 equal doses separated by an interval of 24 hours) with total doses of 0-60-300-600 mg/kg b.w.

11. Conclusions

The SCC requires a cytogenetic and a mouse lymphoma gene mutation *in vitro* study with full specifications of the compound tested and the nature and quantity of impurities eventually present, including mono, di, and trioxide naphthalene.

Classification: B

A 22: *P - METHYLAMINOPHENOL*

1. General

1.1 Primary name

p-methylaminophenol

1.2 Chemical names

p-methylaminophenol
1-hydroxy-4-methylamino-benzene
Phenol, p-(methylamino)-benzene
N-methyl-p-aminophenol
4-(methylamino)-phenol
N-(methyl-4-aminophenol)
p-hydroxy-N-methylaniline
N-methyl-p-hydroxyaniline
N-methyl-4-hydroxyaniline
4-hydroxy-N-methylaniline

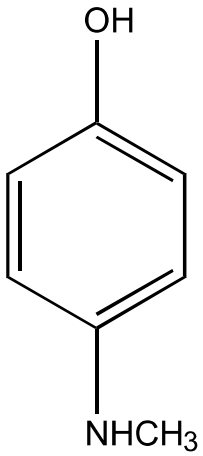
1.3 Trade names and abbreviations

IFG 62/78

1.4 CAS no.

150-75-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123; 134 (as sulphate $^1/_2$ H_2O)

1.7 Purity, composition and substance codes

The compound is usually used as sulphate.

2. Function and uses

Oxidative hair dye; max. use 3 %, 1.5 % with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: male mice, oral: 380 mg/kg (320-440 mg/kg).

3.4 Repeated dose oral toxicity

The compound was administered daily (7 days/week) for 30 days (males) and 31 days (females) by gastric intubation to 10 male and 10 female Sprague-Dawley OFA rats per group at doses of 0, 10, 30, 90 mg/kg b.w. (as sulphate) in 10 ml sterile water/kg b.w. The macroscopical histopathological analysis showed discoloration of spleen in 9 females (90 mg/kg) and acute tubular necrosis (30 and 90 mg/kg). Pigments and cells in the urines (30 and 90 mg/kg) have been observed at the urinary analysis. The hematology examination revealed signs of anaemia in females (90 mg/kg). No adverse effects have been revealed at the doses of 10 mg/kg/day. It is concluded that the dose of 10 mg/kg represents the NOAEL for p-methylaminophenol after oral treatment of rats.

3.8 Subchronic dermal toxicity

N-methyl-p-aminophenol sulphate in two formulations (0.05 % and 0.1 % in water) were tested on shaven intact or abraded skin of New Zealand rabbits by topical applications: no toxic effects at 3, 7 and 13 weeks were observed after treatment by means of histopathological analyses.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied, under occlusion, to intact (left flank) and abraded (right flank) skin of 3 male and 3 female albino Bouscat rabbits, as 2 % sulphate in 0.5 ml aqueous solution for 24 hours, resulted slightly irritating after a reading at 24 or 72 hours: primary cutaneous irritation index = 0.74/8.

4.2 Irritation (mucous membranes)

The compound instilled into the conjunctival sac of one eye, without rinsing, of 6 male albino rabbits, as 2 % sulphate salt in aqueous solution (0.1 ml/animal) resulted practically not irritating after a reading at 1 day, 2, 3, 4 and 7 days after treatment.

5. Sensitization

It was tested in 10 male and 10 female Albino Hartley Guinea pigs treated with 0.5 g of the pure compound by topical occlusive applications behind the right shoulder blade, 3 times/week, with a 2-day interval for 3 weeks (treatments of 48 h) and once at the start of the 4th week. The animals received also an intradermal injection of 50 % saline Freund's complete adjuvant on days 1 and 10 of induction phase. At challenge phase, 12 days after induction, the untreated left flank received 0.5 g of test compound for 48 hours under occlusion. The compound showed no reaction after macroscopical and histological examinations at 1 hour, 6, 24 and 48 hours after the removal of the patch.

6. Teratogenicity

The compound (as sulphate) administered orally to pregnant rats on days 6-15 of gestation at the doses of 0, 10, 30, 70 and 150 mg/kg/day (0.5 ml/kg b.w. in sterile water) did not show embryotoxic or teratogenic activity at doses up to 70 mg/kg/day; the dose of 150 mg/kg/day produced adverse clinical signs and mortality in the dams.

No teratogenicity effects were observed on rats dermally treated with formulations containing the compound (0.05 % and 0.1 % in water) as sulphate.

A multigeneration reproduction study on rats with a formulation containing the compound (1.0 % in water) has produced negative results.

7. Toxicokinetics (incl. Percutaneous Absorption)

In Vitro **absorption:** It has been studied on abdominal human epidermis plus finely cut human hair (10 mg) with a commercial hair dye formulation (1.5 g N-methyl-p-aminophenol: 1.34 g Resorcinol), containing the test compound (0.2475 mg), mixed 1:1 with hydrogen peroxide. After application of 33 mg of test solution on 1.65 cm^2 and rinsing off after 30 min, any amount of the test compound was revealed by HPLC in the resulting chamber (4 ml NaCl 0.9 %, detection limit = 20 x 10-^{9} g/ml) during 4 h and 30 min observation period, thus indicating a value of absorption of less than 0.05 mg/cm^2.

8. Mutagenicity

The compound was tested for gene mutations and found negative in the *Salmonella* (spot and plate tests), in the yeast *S. pombe* P1 (forward mutation assay) and in *Drosophila melanogaster* (sex-linked recessive lethals test SLRL). The compound has been also evaluated for the induction of chromosome aberrations *in vitro* on CHO cells with negative results. In the micronucleus test performed by i.p. injections on mice (2 doses separated by an interval of 24 hours, 10 ml/kg) at doses of 2x 50 -75 -100 mg/kg b.w. negative results have been obtained.

9. Carcinogenicity

A long term study was carried out with two formulations containing the test compound (0.05 and 1.0 % in water, as sulphate) by dermal topical applications on mice once a week for 21 or 23 months (0.5 ml per application): no biologically significant differences were observed between treated and control groups.

Other studies, performed on rats treated dermally, by topical applications (0.2 ml, increases by 0.1 ml to 0.5 ml, 2 times/week per 2 years) from the time of weaning to the weaning of their young, with two formulations containing 0.05 % or 0.1 % of test compound as sulphate, have produced negative results.

11. Conclusions

The SCC requires an *in vitro* mouse lymphoma gene mutation study and a dermal absorption study on rats. Data on contamination of this compound are also required (with nitrosamine?).

Classification: B

A 54: *4 - ETHOXY-M-PHENYLENEDIAMINE*

1. General

1.1 Primary name

4-ethoxy-m-phenylenediamine

1.2 Chemical names

1-ethoxy-2,4-diamino-benzene
1,3-bezenediamine-4-ethoxy
m-phenylenediamine-4-ethoxy
4-ethoxy-m-phenylenediamine
Diamino-phenetol

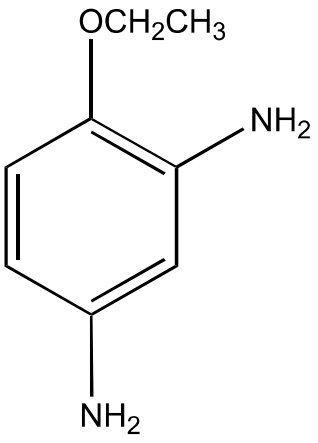
1.3 Trade names and abbreviations

Rodol Cox
C.I. Oxidation Base 14

1.4 CAS no.

5862-77-1
C.I.: 76055

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}N_2O$
Mol weight: 250

2. Function and uses

Oxidative hair dye; max. use 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rats, oral 2540 (3090-2090) mg/kg

3.4 Repeated dose oral toxicity

The compound as sulphate was administered daily for 28 days by oral gavage at doses of 0, 25, 100, 400 and 1200 mg/kg b.w./day to 5 male and 5 female CD rats per group. All rats treated at highest test dose died or were killed during the first 4 days of treatment. Haematological examination revealed reductions in the red cell characteristics of blood sample at doses of 400 (male and female) and 100 mg/kg (female). In the 400 mg/kg group increases in blood urea nitrogen, alkaline phosphatase and glutamate-oxaloacetate transaminase were revealed. A darkened appearance of thyroids, spleens and kidneys were observed in rats treated with 400 mg/kg/day. The dose of 25 mg/kg represents the dose with the NOAEL.

The compound as sulphate was administered daily to CD rats by oral gavage at doses of 0, 30, 100 and 300 mg/kg/day for 13 weeks. The macroscopic examination revealed a dose related discoloration of the internal organs (thyroids, liver and spleen). Ophthalmic examination showed a slight increase in the incidence of keratitis at the 300 mg/kg/day group after 4 weeks, no more evident after 8 and 12 weeks. A broadly dose related reduction in values of haemoglobin, erythrocytes count and haematocrit were revealed in all treated animals and at all examinations. The mean cell volume was increased in the highest test dose and in the females treated with 30 mg/kg/day. The reticulocyte count was increased at the highest test dose after 12 weeks. The microscopic examination showed at all doses a yellow/brown pigment in different tissues (adrenal, duodenum, kidney, liver, lung, spleen and thyroid) in a dose related manner. The highest test dose showed this pigment in cervical and mesenteric lymph nodes. The presence of the pigment was associated with a dose related hypertrophy of the thyroid at the two highest test doses.

The pigment noted in treated rats was lipofuscin-like, in the tissues analyzed the pigment was positive for haemosiderin and in kidney it was also positive for haemoglobin. This study has not permitted to establish a “non-toxic dose level” for the test compound in rats.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 10 % water solution, applied on intact and abraded rabbit’s skin (0.5 ml) under occlusion for 24 hours, resulted mildly irritating after reading at 0, 24 and 72 hours.

4.2 Irritation (mucous membranes)

The compound as 10 % water solution, instilled into one eye of each of sex Albino rabbits at doses of 1 ml without rinsing off, resulted not irritating after 1, 2, 3, 4, 7, 14 and 21 days of treatment.

5. Sensitization

It was induced in 10 guinea pigs by three pairs simultaneously intradermal injections of Freund's complete adjuvant (FCA, 1:1 in water), 0.1 % water solution of test compound and a 1:1 mixture of the above solution in shoulder area. One week later 0.4 ml of a 50 % suspension of test substance in Paraffin perliquidum was topically applied in a filter paper, under occlusion, on the same area for 48 hours. 14 days later the guinea pigs were challenged by a single topical application of 0.1 ml of a 10 % water solution of test substance, under occlusion, on the left flank for 48 hours. The results evaluated after 24, 48 and 72 hours showed no reaction in 8 out of 9 animals. It has been reported that one animal died from lung infection.

A 0.5 % water solution of test substance applied by patch test on 22 human volunteers for 3 weeks has given a positive response in only 1 subject during the treatment and the challenge application.

6. Teratogenicity

Groups of 25, 28 and 25 inseminated NMRI mice were treated on day 5 to 7, day 8 to 10 or day 11 to 14 of pregnancy subcutaneously at a volume of 0.1 ml/30 g/mouse with a formulation containing test compound (1.35 g 2,5-diaminophenetolsulphate, 0.1 g Sodium sulphite, 4.0 ml Ammonia 23 % and 10.0 ml Isopropanole in 100 ml of water: 45 mg/kg). The 25 control mice received subcutaneously the same volume of distilled water on day 4 to 15 of pregnancy. The average number of resorption sites (1.9 vs. 1.4) and the number of foetuses with malformations (external: 1.9 % vs. 0 %, skeletal: 2.1 % vs. 0 % and visceral: 4.3 % vs. 1.5 %) were increased on day 8 to 10 of pregnancy. No differences were observed between other treated groups and control. It is concluded that the compound at the dose of 45 mg/kg shows teratogenic effects on mice from 8 to 10 days of pregnancy.

Metabolites: The blood plasma samples showing high 'metabolite' peaks from rats treated for 13 weeks (see subacute oral toxicity studies) were pooled and analyzed by HPLC and Mass Spectrometry for identify the metabolite of 4-ethoxy-m-phenylenediamine. The results showed that this metabolite is an N-acetyl derivative. Probably this metabolite would also be conjugated as a glucuronide or aryl sulphate at the remaining free amino group.

8. Mutagenicity

The compound as sulphate (2 studies) and as hydrochloride (1 study) has been tested and found positive for gene mutation *in vitro* on *Salmonella typhimurium*. The compound was unable to induce micronuclea *in vivo* on CD-1 mice treated by oral gavage (2 equal doses separated by an interval of 24 hours) at total doses of 0-50-100-200 mg/kg b.w. However, an increase in the normochromatic to polychromatic erythrocyte ratio (x2.48, 1.96 vs. 0.79) has been observed at the highest tested dose indicating a bone marrow depression.

11. Conclusions

In view of the concerns in a number of areas, namely systemic toxicity, mutagenic potential, teratogenicity, the SCC believes that this compound should not be used in cosmetic.

Classification: D

A 74: *1-HYDROXY-3-METHYL-4-AMINOBENZENE*

1. General

1.1 Primary name

1-hydroxy-3-methyl-4-aminobenzene

1.2 Chemical names

1-hydroxy-3-methyl-4-aminobenzene
4-amino-3-methyl-phenol
4-amino-m-cresol
2-methyl-4-hydroxy-aniline
2-amino-5-hydroxy-toluene
6-amino-3-hydroxy-toluene

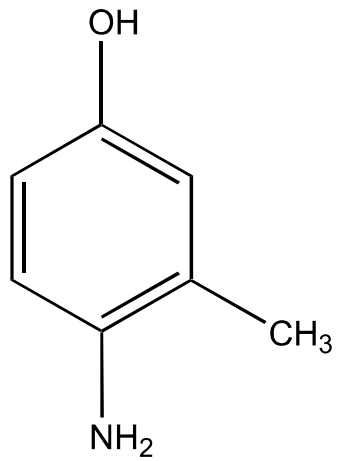
1.3 Trade names and abbreviations

Oxyrot

1.4 CAS no.

2835-99-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123

1.7 Purity, composition and substance codes

It exists as free base, hydrochloride and hemisulfate.

2. Function and uses

Oxidative hair dye; max. use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: female rats, oral (stomach intubation) 1010 mg/kg
male rats, oral (stomach intubation) 870 mg/kg
female mice, oral (stomach intubation) 908 mg/kg

3.7 Subchronic oral toxicity

Oxyrot (1-hydroxy-3-methyl-4-aminobenzol-sulfat) administered daily by stomach intubation, over a period of 13 weeks to Wistar rats at dose levels of 15, 60 and 120 mg/kg b.w. to 20 males and 20 females per dose, showed no specific finding at 15 mg/kg b.w., dark discoloured urines in both sexes (8 to 13 wks.) at 60 and 120 mg/kg b.w.; increase in spleen weights (males and females) and in creatine values (females) at 120 mg/kg b.w. after 13 weeks. The dose of 60 mg/kg represents the NOAEL. For evaluating the recovery 5 males and 5 females were similarly treated only at 120 mg/kg b.w. and examined during 4 following weeks without treatment: no other clinical signs, no difference between control and treated group in spleen weights and in creatine-values at the end of observation period.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 3% aqueous suspension applied on clipped skin (3x4cm, without washing) of guinea pigs once a day on 5 consecutive days resulted 5 hours after each treatment not irritanting (no erythemas or oedemas).

4.2 Irritation (mucous membranes)

The compound as 1.5 % (0.1ml) in 50 % ethylene glycol, instilled into one eye (without washing) of guinea pigs showed after 24 and 48 h (examinations with 0.1 % fluoroscein sodium solution) no pathological lesions on conjunctiva, iris, cornea and the found of the eyes.

5. Sensitization

3-methyl-4-aminophenol-hemisulfat (Oxyrot) showed no delayed contact hypersensitivity in a maximization-test after repeated intradermal injection (1st and 2nd injection in craniodorsal area: 3 % in water, 0.05 ml; 3rd injection, 48 h after the first two injections: 3 %, 0.05 ml in Freund's Adjuvant complete (FCA diluted in oleum arachidis 1:1) and closed dermal topical applications (3 % in 0.5 ml white Vaseline, 6-8 h after the first two injections) on the clipped shoulder area to guinea pigs (pretreatment with 10 % sodium lauryl sulfate). Challenge

reaction by closed patch test on day 14 after the last exposure with 1 %, 2 % and 3 % (0.05 ml in FCA diluted in oleum arachidis 1:1). Reading at 24 and 48 hours.

6. Teratogenicity

1-hydroxy-3-methyl-4-amino-benzene sulphate administered orally by stomach intubation to groups of 24 pregnant BOR:WISW-SPF rats from day 5 to 15 of gestation at doses of 10, 40 and 80 mg/kg b.w. in deionized water (1 ml/100 g b.w.) did not show any signs of maternal toxicity or adverse effects to the fetal development after autopsy of dams on day 20 of gestation. NOEL = 80 mg/kg b.w.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: [^{14}C]-4-amino-3-methyl phenol hemisulfate (radiochemical purity 96%) applied in DMSO (150 mg/ml, 0.1 ml per animal for 0.5 h) and as ingredient of hair dye products (134.4 mg, 1 g mixture/animal for 24 h) on dorso-lumbar region of PVG rats (15 mg/animal, 1.667 mg/cm^2, 200 µCi) showed after 72 h that 0.42 % (0.25% urine, 0.02% faeces and 0.15% expired air) of the applied dose in hair dye product and 7.47 % (6.54% urine, 0.42% faeces, 0.38% cages washing and 0.13% expired air) of the solutions in DMSO were excreted and that 87.77 % in hair product and 89.24 % in DMSO solution were recovered from dressing, washing and application sites. No significant radioactivity levels were found in tissues.

8. Mutagenicity

The compound has been tested and found negative: (1) for gene mutation *in vitro* on *Salmonella typhimurium* performed with and without hydrogen peroxide; (2) for chromosome aberrations *in vivo* by the micronucleus test on CD1 (up to 2x500 mg/kg/day oral gavage) and NMRI (doses up to 1000 mg/kg b.w. in DMSO by stomach intubation) mice; (3) and for sister chromatid exchanges *in vivo* in male chinese hamsters (tested as hemisulphate, doses up to 400 mg/kg i.p. and up to 2000 mg/kg oral).

11. Conclusions

In the absence of the carcinogenicity data and due to the structural similarity to known mutagens, the SCC requires the submission of data from *in vitro* cytogenetic lymphocytes and gene mutation on mouse lymphoma studies.

Classification: B

A 75: *2-AMINO-5-METHYLPHENOL*

1. General

1.1 Primary name

2-amino-5-methylphenol

1.2 Chemical names

1-hydroxy-2-amino-5-methyl-benzene
2-hydroxy-4-methyl-aniline
4-amino-3-hydroxy-toluol
6-amino-m-cresol
2-amino-5-methylphenol

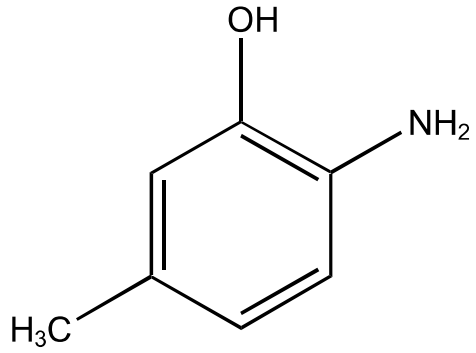
1.3 Trade names and abbreviations

Oxygelb

1.4 CAS no.

2835-98-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123

1.7 Purity, composition and substance codes

It exists as free base and hemisulfate.

2. Function and uses

Oxidative hair dye; max. use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	female rats, oral	1225 mg/kg
	male rats, oral	1375 mg/kg
	female CF 1 mice, oral	1225 mg/kg
	male CF 1 mice, oral	1020 mg/kg
	female CBL mice, oral	750 mg/kg

3.7 Subchronic oral toxicity

The compound (98 % purity) as 10 % suspension in 5 % gum Arabic was administered orally by stomach intubation for 90 days to 10 male and 10 female albino rats at dose of 800 mg/kg/day b.w. reduced at 500 mg/kg/day after 5 weeks (5 ml/kg). 2 rats died during the treatment. Tyrosine crystal were revealed in urine, and liver, kidney and spleen weights were reduced. Increased in Bilirubin and iron concentrations in males, reduction in T4 with no histopathological change in thyroids. The NOAEL <500 mg/kg.

Oxygelb as 0.5% in carboxymethylcellulose administered orally by stomach tube at doses of 0, 50, 250 and 500 mg/kg/day to 15 male and 15 female rats per dose (1 ml/100 g b.w.) for 4 weeks showed these results: 250 mg/kg: slightly increased activity for 10 min. post treatment during 3rd and 4th week; increased urine excretion (yellow-orange discoloured); significative alterations of hematology and clinical chemistry values (reduction in erythrocytes and hemoglobin in males and females and iron in females; increase in reticulocytes and hematocrit in males and females); increase in liver, kidney and spleen weights. 500 mg/kg: moderate reduced activity during the 1st treatment week and later moderated increased activity for 10 min. post treatment; significant increase in water consumption; increased urine excretion (yellow-orange discoloured); significant alterations of hematology and clinical chemistry values (reduction in erythrocytes, hemoglobin, hematocrit and iron in males and females; increase in reticulocytes in males and females and MCV and Prothrombin time in females; significant increase in liver, kidney and spleen weights; dark discoloured spleens at autopsy. No significant histopathological alterations were observed at all doses. The oral dose of 50 mg/kg/day x 28 days represents the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 1 % aqueous solution (thickened with methyl cellulose) was applied on abraded skin area (3x4 cm, washed out after 20 min.) of albino guinea pigs 3 times daily on two consecutive days. A negligible erythema on the first day, not recognizable (only skin area stained) on the second day, was observed; no edemas and crusts were revealed, during further observation.

4.2 Irritation (mucous membranes)

The compound as 1 % aqueous solution instilled into one eye (0.1 ml) of 10 female Pirbright white guinea pigs, resulted not irritating after 24 hours observation period (eye reactions evaluated at 0.5, 1, 2, 3, 4, 6, 7 and 24 hours).

5. Sensitization

Sensitization was tested in 15 female Pirbright white Guinea pigs treated with 3 % in aqueous test suspension of test compound applied epicutaneously without occlusion on abraded flanks, once a day on 5 days/week for 3 wks, using the method of Magnusson and Kligman. The compound did not show any erythemas or edema 24, 48 and 72 hours after challenge reaction.

6. Teratogenicity

1-hydroxy-2-amino-5-methylbenzene administered oral by gastric intubation to 23-26 pregnant Sprague-Dawley rats from day 8 to 15 of gestation at doses of 5, 50 and 200 mg/kg b.w./day in distilled water (10 ml/kg b.w.) not showed embryotoxicity and no sign for embryolethality or teratogenicity. NOAEL > 200 mg/kg b.w.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: [^{14}C]-2-amino-5-methylphenol hemisulfate (radiochemical purity 96 %) in DMSO (150 mg/ml, 0.1ml animal for 0.5 h) and as ingredient of hair dye products (133.14 mg, 1 g mixture animal for 24 h) applied on dorso lumbar region of PVG rats under occlusion (15 mg animal, 1.667 mg/cm^2, 190 Ci) showed after 72 h that 0.58 % (0.41 % urine, 0.09 % faeces, 0.15 % expired air and 0.02 % cage washing) of the applied dose as the hair dye product and 14.25 % (121.83 urine, 0.82 % faeces and 0.60 % cages washing) of the solutions in DMSO were excreted and that 82.78 % as the hair dye product and 74.48 % in DMSO solution were recovered from dressing, washing and application sites. No significant radioactivity levels were found in tissues.

Human-skin absorption: 1-hydroxy-2-amino-5-methylbenzene (mean = 54.1 mg, i.e. 0.06 %) containing in hair dye product was epicutaneously applied (mean = 90.02 g) on five healthy female volunteers by a professional hairdresser for 29-31 min. and blood samples were taken at 0, 10, 20, 30, 45 and 60 min and 2, 3, and 24 h. after applications. The results showed within the range of the sensitivity of method (10 ng/ml, HPLC technique and fluorescence photometer) neither the hair dye nor a possible metabolites was detected in the serum; therefore the volunteers (64.70 kg mean b.w.), presuming a whole body distribution and absorption of at least 0.647 mg (on the bases of method sensitivity) per volunteers, absorbed nothing or less than 1.198 % of the applied dose of the test compound.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have demonstrated that 1-hydroxy-2-amino-5-methylbenzene does induce gene mutations *in vitro* in *Salmonella* (+S9mix ± H_2O_2; -S9mix - H_2O_2). The compound (tested as hemisulfate in *in vitro* test) have been found negative for: (1) gene mutations on mouse lymphoma L5178Y (Na^+/K^+ ATPase and HPRT loci, fluctuation test)

in vitro; (2) chromosome aberrations *in vitro* on human peripheral lymphocytes and (3) *in vivo* by micronucleus test (up to 2x750 mg/kg oral; increase in the frequency of micronuclei when compared with negative control (up to 0.6% mean per 2000 cells vs. 0.2 %), neither significant nor dose-related) on bone marrow cells of CD-1 mice: this study was however inadequate, because the positive control (Cyclophosphamide 100 mg/kg) was not able to induce a significant increase in percentage of micronuclea in this *in vivo* test (mean per 2000 cells: 0.6 % vs. 0.2 % negative control); several genetic damage *in vitro* on *S. cerevisiae* D7 (mitotic crossing over, mutation, gene conversion or aneuploidy) and sister chromatid exchange *in vivo* on mouse (up to 600 mg/kg oral).

11. Conclusions

Since several studies have shown that this compound has produced positive results in *in vitro* mutagenicity studies, the SCC requires a study for the *in vivo* induction of UDS.

Classification: B

A 79: *1,3-BIS-(2,4-DIAMINOPHENOXY)-PROPANE*

1. General

1.1 Primary name

1,3-bis-(2,4-diaminophenoxy)-propane

1.2 Chemical names

1,3-bis-(2,4-diaminophenoxy)-propane
4,4'-1,3-propanediylbis(oxy)-bis-2,4-benzeneamine

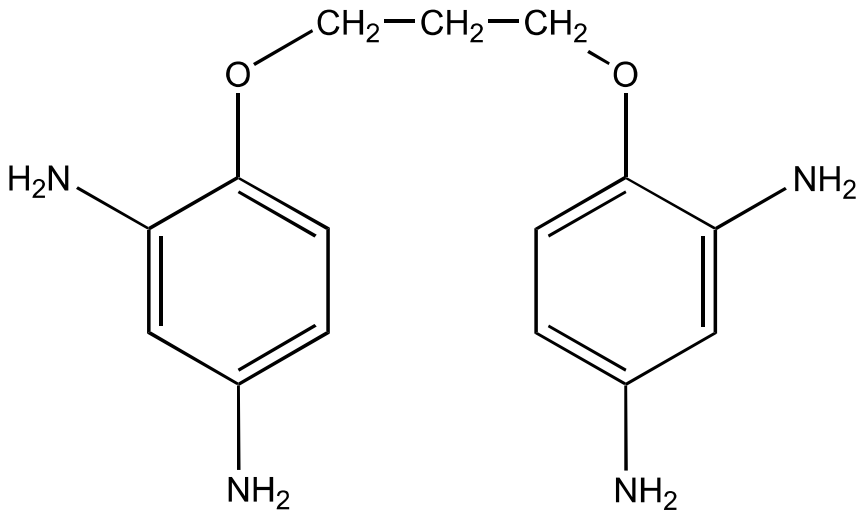
1.3 Trade names and abbreviations

Ro 463

1.4 CAS no.

74918-21-1

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{20}N_4O_2$
Mol weight: 288.3

2. Function and uses

Oxidative hair dye; max. use: 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} rat, oral: 3570 (3170-4002) mg/kg;

rat, oral: >5000 mg/kg (2)
(2 % test compound containing formulation)

3.7 Subchronic oral toxicity

The compound was administered daily by oral gavage, over a period of 13 weeks, to male and female Wistar rats (Mu Ra Han 67 SPF) at doses of 0-5-10-15 mg/kg b.w. in aqueous suspension (10 ml/kg). These results were obtained: 5 mg/kg d.: the thyroid glands of all rats were free of pigments; 10 mg/kg d.: slight pigmentation of the thyroid glands (in a few females) and pigmented macrophages in the small intestine (in a few females and males); 15 mg/kg d.: reddish discolouration of the thyroid gland at macroscopical level, a pigmentation of the thyroidal epithelia and pigment depositions in the small intestine (all rats). The dose of 5 mg/kg day represents the dose with the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied (500 ml in gauze patches) as a 10 % (w/v in water) solution (pH=8-10) resulted non irritant.

The compound applied twice daily for 5 days, as 10 % (w/v) aqueous solution (10 ml), to the same skin area of male hairless mice resulted not irritating.

The containing formulation (2 %) resulted non-irritating to rabbit's skin.

The compound containing formulation (2 %) resulted not irritating to the mouse' skin when applied daily (30 min for application) for 5 days.

4.2 Irritation (mucous membranes)

The compound applied as 5 % (w/v) water solution on rabbit's eyes showed no irritation of the cornea and iris, and mild to severe redness of the conjunctiva in 3 animals (2 and 6 hours) that disappears 24 hours after instillation.

The compound containing formulation (2 %) resulted slightly irritating for the rabbit's eyes.

5. Sensitization

It was induced in guinea pigs by intradermal injection of 5 % (w/v) test compound in aqueous solution, Freund's complete Adjuvant (FCA) and 1:1 (v/v) mixture of the above solution on day 0, and 7 days later by dermal application of 5 % (w/w) test compound in vaseline, under

occlusion, for 48 hours. Challenge exposures were carried out at day 21 (closed patch, 24 hours) and at day 28 (open dermal) at a new skin site. The compound resulted non-sensitizer in guinea pigs.

The compound containing formulation (2 %) resulted non-sensitizer in guinea pigs.

6. Teratogenicity

Embryotoxicity: The compound administered daily by oral gavage to groups of 41-43 pregnant Wistar TNO rats from day 6 to 19 of gestation at the dose of 0-100 mg/kg b.w. (10 ml/kg in water) showed 4/352 (treatment) vs. 0/300 (control) foetuses with visible malformations at analysis of the dams on day 20 of gestation. The other fetal and maternal parameters did not reveal an embryotoxic or maternal toxic effect.

The compound was administered daily by oral gavage to groups of 20 pregnant Sprague-Dawley CD rats from day 6 to 15 of pregnancy at the doses of 0-20-60-180 mg/kg b.w. (10 ml/kg in distilled water). The results showed a slight increase in the number and type of foetal variation in all test groups not treatment related. The other maternal and foetal parameters did not show indications of maternal toxicity, embryotoxic or teratogenic effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C-1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride applied subcutaneously (10 mg/kg b.w.) to 4 male Wistar rats (SPF-TNO) showed more than 88 % of the radioactivity was found in feces (65 %) and urine 24 hours after treatment. The radioactivity in expired air, in the carcass, liver and kidney was very low over an observation period of 144 hours.

^{14}C-1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride administered at oral doses of 10-100-1000 mg/kg b.w. showed an excretion range of 57-79 % in the feces and 23-34 % in the urine over an observation period of 120 hours.

These studies demonstrated that the compound was eliminated with the bile.

^{14}C-1,3-bis-(2,4-diaminophenoxy)-tetrahydrochloride was applied intraperitoneally to male and female Wistar rats at a single dose of 20 mg/kg b.w. and the organ distribution was evaluated by whole body autoradiography at 0.5-2-6-24-96 hours after treatment. The results showed that the compound was principally excreted by the gastrointestinal tract and a minor amount by the kidney. The decrease of radioactivity in the liver was faster than in the kidney. The compound was still revealed in the spleen, thymus, kidney and in the Hardarian gland 96 hours after treatment.

Dermal absorption: 0.63 % of the test compound equivalents were absorbed through the skin of rats over a period of 72 hours after dermal application to intact, clipped skin of male and female rats with a hair dye basic cream containing 0.23 % of ^{14}C-1,3-bis-(2,4-diaminophenoxy)-propanetetrahydrochloride (17.25 mg), without a developer. The radioactivity was revealed both in the urine and in the feces.

A maximum of 0.079 % of test compound equivalents was absorbed through the skin of rats over a period of 72 hours after 30 min of dermal application to intact, clipped skin of male and

female rats with oxidative formulation containing 0.23 % ^{14}C-1,3-bis-(2,4-diaminophenoxy)-propanetetrahydrochloride (34.5 mg). The radioactivity was revealed principally in the feces.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have shown that 1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride induces gene mutation *in vitro* on *Salmonella typhimurium* in the presence of metabolic activation.

Other studies have shown that the compound did not produce: gene mutation *in vitro* on CHO-K1 and V79 hamster cells line (HPRT: 6-TG resistance), *in vivo*/*vitro* by the urinary assay (Salmonella-rat: 100 mg/kg b.w. on the clipped dorsal skin, 24-hours urine sample), and *in vivo* by SLRL test on *D. melanogaster* and spot test in mice with oral doses up to 125 mg/kg b.w.; chromosome aberrations by micronucleus test on mice at oral doses of 100-2500-5000 mg/kg b.w. (in two 2 equal doses separated by an interval of 24 hours); genotoxicity *in vitro* by the mitotic gene conversion on the yeast *S. cerevisiae* and UDS on rat hepatocytes and, *in vivo*/*vitro* by urinary assay (*S. cerevisiae* D4-rats: mitotic gene conversion) with oral doses up to 250 mg/kg.

11. Conclusions

In the absence of carcinogenicity data, the SCC requires an *in vitro* cytogenetic study and an *in vivo* UDS study.

Classification: B

A 80: *OXYTOL B*

1. General

1.1 Primary name

Oxytol B

1.2 Chemical names

1-ß-hydroxyethyl-2,5-diaminobenzene
1,4-diamino-2-ß-hydroxyethyl-benzene
2,5-diamino-phenylethylalcohol

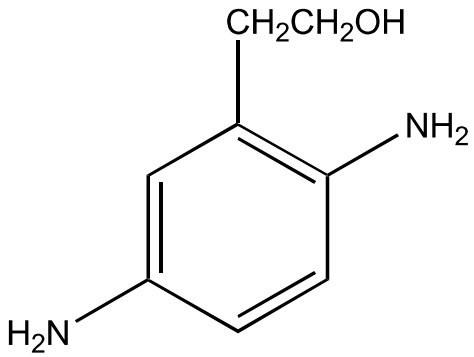
1.3 Trade names and abbreviations

Oxytol B

1.4 CAS no.

93841-25-9

1.5 Structural formula



(sulphate)

1.6 Empirical formula

Emp. formula: $C_8H_{12}N_2O$
Mol weight: 152

1.7 Purity, composition and substance codes

It exists as free base, as dihydrochloride and sulphate. It is used as a sulphate.

2. Function and uses

Oxidative hair dye; max. use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	male and female rats, oral	150 mg/kg
	female CD1 mice, oral	90 mg/kg

3.7 Subchronic oral toxicity

The compound, as sulphate, administered orally to groups of 10 male and 10 female Sprague Dawley rats for 90 days at dose levels of 0, 5, 25, 40 and 40 (recovery) mg/kg/day (10 ml/kg in water) showed a NOAEL at 25 mg/kg b.w. Orange-coloured urine from 11th to 13th weeks dose-related, weight deviations and macroscopic changes of the organs, and increasing of the mean GOT and GTP values after 13 weeks at the highest test dose were observed.

The compound, as hydrochloride, administered daily by stomach tube to 12 male and 12 female SPW Wistar rats for 12 weeks at dose level of 25 mg/kg b.w. showed to all examens (food and water consumption; hematological, clinico-chemical changes and ophthalmoscopical changes; urine; macroscopical finding; and complementary examination of the organs of 5 males and 5 females) no difference between treated and control group (5 ml/kg b.w. water). The dose of 25 mg/kg/b.w. represents the NOAEL (90-day oral study on rats).

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as dihydrochloride (3 % in aqueous solution) applied daily for 5 days to the clipped skin area (3x4 cm), without washing off, of 15 female Pirbright White guinea pigs resulted not irritating (skin reactions evaluated daily 5 h post treatment).

4.2 Irritation (mucous membranes)

The compound as dihydrochloride instilled (1.5 % in water, 0.1ml) into the conjunctival sac of one eye (without washing) of 5 female Pirbright guinea pigs resulted not irritating after 24 hours (examinations with 0.1 % fluoroscein sodium solution) observation period (eye reactions evaluated at 0.5, 1, 2, 3, 4, 5, 6, 7, and 24 hours).

5. Sensitization

Sensitization was tested in male and female Pirbright guinea pigs treated with 3 % intradermal injections and closed dermal topical application (including Freund's complete adjuvant FCA) of the test compound on the clipped shoulder area. Challenge reaction by closed patch test on day 14 after the last exposure with 1 %, 2 % and 3 % in distilled water. The compound showed no skin reactions (reading at 24 and 48 hours).

6. Teratogenicity

1-(ß-hydroxyethyl)-2,5-diaminobenzene-sulphate administered daily by gastric intubation to 25 mated female Sprague-Dawley rats from day 6 to 15 of gestation at oral doses of 10 mg/kg/day (10 ml/kg in distilled water) did not show embryotoxicity and teratogenicity on day 20 of gestation.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human-skin absorption: 1-(ß-hydroxyethyl)-2,5-diaminobenzene (mean = 1855.20 mg, i.e. 2.4 %) contained in a hair dye product was epicutaneously applied (mean = 77.3 g) to five healthy female volunteers by a professional hairdresser for 24-32 min. and blood samples were taken from 4 volunteers at 0, 10, 20, 30, 45 and 60 min and 2, 3, and 24 h. after application. The results showed that within the sensitivity range of the method (25 ng/ml, HPLC technique and fluorescence photometer) neither the hair dye nor a possible metabolite was detected in the serum; therefore the volunteers (64.18 kg mean b.w.) – presuming a whole body distribution and absorption of at least 1.604 mg (on the bases of method sensitivity) per volunteer – absorbed nothing or less than 0.086 % of the applied dose of the test compound.

8. Mutagenicity

The compound tested as sulphate has been found negative for: (1) gene mutation *in vitro* on *Salmonella* (tested only in the presence of metabolic activation) and in mouse lymphoma 6-TG^R fluctuation assay; (2) chromosome aberrations *in vitro* on CHO cells and *in vivo* by micronucleus test on mice (up to 200 mg/kg oral); (3) and sister chromatid exchange *in vivo* in the bone marrow cells of rats (up to 80 mg/kg i.p. and p.o. or 5x128 mg/kg epicutaneous).

11. Conclusions

The SCC requires an adequate study for the induction of gene mutations in Salmonella assay.

Classification: B

A 81: *1-(-HYDROXYETHYL-2,4-DIAMINOBENZENE*

1. General

1.1 Primary name

1-ß-hydroxyethyl-2,4-diaminobenzene

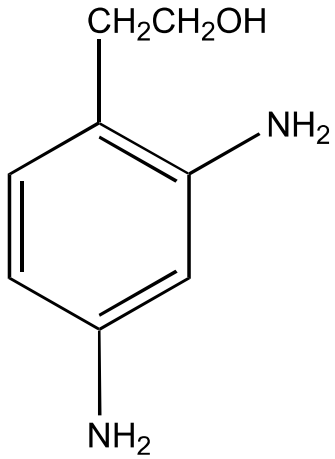
1.2 Chemical names

1-ß-hydroxyethyl-2,4-diaminobenzene
3-amino-4-ß-hydroxyethyl-aniline
2,4-diamino-phenylethylalcohol

1.3 Trade names and abbreviations

Oxyblau

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}N_2O$
Mol weight: 152

1.7 Purity, composition and substance codes

It exists as free base (unstable), as sulphate and dihydrochloride.

2. Function and uses

Oxidative hair dye; max. use 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}	Female Wistar rats, oral	1150 mg/kg
	Female CF1 mice, oral	1450 mg/kg
	Female CBL mice, oral	1125 mg/kg

3.7 Subchronic oral toxicity

The compound, as sulphate, administered orally by stomach intubation to groups of 25 male and 25 female SPF Wistar (TNO/W) rats for 90 days at dose levels of 0, 20, 100, 400 and 400 (10 males and 10 females, reversibility effects) mg/kg/day (10 ml/100 g b.w. in water) showed a ***NOAEL* < 20mg/kg b.w. (1st trial).** Due high mortality (400 and 200 mg/kg) reversibility examinations could not be made. The compound resulted toxic in several organs (thyroids, livers, kidney and spleens) on morphological level; it induces dosedependent changes at lymphatic nodes, hearths, gonads and uteri, histomorphological finding correlated with hematological, clinico-chemical values (most dose-related) and organ weights, and adverse effects on the erythrogenic system. At 20 mg/kg a slight thyroids discolorations and organ weights increase (liver and kidney in the females only) with no histomorphological or functional organs alterations.

The compound, as sulphate, administered orally by stomac intubation to groups of 20 male and 20 female Wistar-TNO/W rats for 90 days at dose levels of 0, 5 mg/kg/day (10 ml/kg in water) showed no specific finding for clinical sign, body weights food comsumption, hematology, clinical chemistry and urinalysis and organ weights (2nd trial). Slightly significantly ($P< 0.05$) increased in liver weights in the males not test compound related, because in the previous trial at 20 mg/kg no difference were observed. ***NOAEL = 5 mg/kg b.w.***

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as dihydrochloride (3 % in aqueous solution) applied daily for 5 days to the clipped skin area (3 x 4 cm), without washing off, of 15 female Pirbright White guinea pigs resulted not irritating (skin reactions evaluated daily 5 h. post treatment).

4.2 Irritation (mucous membranes)

The compound as sulphate instilled (1 % in water, 0.1 ml) into the conjunctival sac of one eye (whitout washing) of 10 female Pirbright guinea pigs resulted practically not irritating after 24 hours (examinations with 0.1 % fluoroscein sodium solution) observation period (eye reactions evaluated at 0.5, 1, 2, 3, 4, 5, 6 and 7 hours).

5. Sensitization

The compound, as sulphate (Oxyblau), showed no delayed contact hypersensitivity after repeated intradermal injection (1st and 2nd injection in craniodorsal area: 3 % in aqua, 0.05 ml;

3a injection, 48 h after the first two injections: 3 %**,** 0.05 ml in Freund's Adjuvant complete (FCA diluted in oleum arachidis 1:1) and closed dermal topical applications (3 % in 0.5 ml white vaseline, 6-8 h after the first two injections) on the clipped shoulder area to guinea pigs (pretreatment with 10 % sodium lauryl sulfate). Challenge reaction by closed patch test on day 14 after the last exposure with 1 %, 2 % and 3 % (0.05 ml in FCA diluted in oleum arachidis 1:1). Reading at 24 and 48 hours.

6. Teratogenicity

1-(ß-Hydroxyethyl)-2,4-diaminobenzene-sulphate administered daily by gastric intubation to 25 mated female Sprague-Dawley rats from day 6 to 15 of gestation at oral doses of 10 mg/kg/day (10 ml/kg in distilled water) did not show embryotoxicity or teratogenicity on day 20 of gestation. ***NOAEL= 10 mg/kg b.w.***

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption. 1-(2-hydroxyethyl)-2,4-diamino-(U^{14}C-benzene) (^{14}C-Oxyblue, radio-chemical purity 96 %) in DMSO (10 % w/v solution, specific activity 1.603 µCi/mg) and as ingredient of hair dye formulation (119.9 mg, specific activity 2.543 µCi/mg, 1:1 with H_2O_2), applied on shaven back skin of male and female Long-Evan rats (15 mg per animal, 9 cm^2) for 30 min (hair dye formulation) or 24 h (136 µl DMSO solution) showed after 72 h, that 0.2 % (male, urine), 0.4 % (female, urine), 0.1 % (male, faeces) and 0.2 % (female, faeces) of the applied dose as the hair dye product and 17.79 % (male, urine), 17.12 % (female, urine), 5.86 % (male, faeces) and 6.30 % (female, faeces) of DMSO solution were excreted. After washing and dressed were recovered 90.30 % (male) and 92.48 % (female) of the applied dose as hair dye formulation and 57.27 % (male) and 52.83 % (female) of the solution in DMSO. In the site of application were revealed 12.8 % (male) and 13.85 % (female) as DMSO solution and 2.17 % (male) and 2.89 % (female) as hair dye product of the applied dose. The results showed that more than ***20 % as DMSO*** solution and less than ***1.2%*** as hair dye formulation of the applied dose of ^{14}C-Oxyblue was absorbed.

Human-skin absorption. 1-(ß-hydroxyethyl)-2,4-diaminobenzene (mean = 873.31 mg, i.e. 1.150 %) contained in hair dye product was epicutaneously applied (mean = 75.94 g) on five healthy female volunteers by professional hairdresser for 27-48 min. and blood samples were taken at 0, 10, 20, 30, 45 and 60 min and 2, 3 and 24 h. after applications. The results showed within the range of the sensitivity of method (50 ng/ml, HPLC technique and fluorescence photometer) neither the hair dye nor a possible six chemical modification was detected in the serum; therefore the volunteers (64.48 kg mean b.w.), presuming a whole body distribution and absorption of at least 3.2 mg (on the bases of method sensitivity) per volunteers, absorbed nothing or less than 0.366 % of the applied dose of test compound.

8. Mutagenicity

The compound tested as dihydrochloride (1.35 % solution + 200 µl 25 % NH3 + 2 ml isopropanol warmed in 10 ml distilled water) was able to induce gene mutation *in vitro* on TA1537, TA1538 and TA98 strains of *Salmonella typhimurium* in the absence and in the presence of ral liver metabolic activation with a clear dose-related effect (the increase of the

no. of revertants over the control was up to: 5 (2702 µg/p, TA1537 -S9mix); 10. 1 (4053 µg/p., TA1538 -S9mix); 47.5 (4053 µg/p., TA98 -S9mix); 50.5 (2702 µg/p., toxic, TA1537 +S9mix); 114.7 (1720 µg/p., TA1538 +S9mix); and 116.7 (1351 µg/p., TA98 +S9mix). LEDs dose (Lowest effective dose): 13.5 µg/p. (TA1538, +S9mix); 27 µg/p. (TA98, +S9mix); 1351 µg/pl. (TA1537, +S9mix; TA 98, -S9mix); 2702 µg/pl. (TA1537, TA1538, -S9mix).

The compound tested *as sulphate* (code BW 16 01) has been found negative for: (1) chromosome aberrations *in vivo* by micronucleus test on mice (up to 2 x 4000 mg/kg b.w., oral gavage); (2) sister chromatid exchange *in vitro* on CHO-K1-BH4 (±S9mix) cells of chinese hamster; and (3) sister chromatid exchange *in vivo* on rats bone marrow cells (up to 600 mg/kg b.w.).

11. Conclusions

In view of the concerns in the areas of systemic toxicity and mutagenicity the SCC believes that this compound should not be used in cosmetics.

Classification: D

Revision: October 30, 1990

A 84: *1-METHOXY-2-AMINO-4-ß-HYDROXYETHYL-AMINO-BENZENE*

1. General

1.1 Primary name

1-methoxy-2-amino-4-ß-hydroxyethyl-amino-benzene

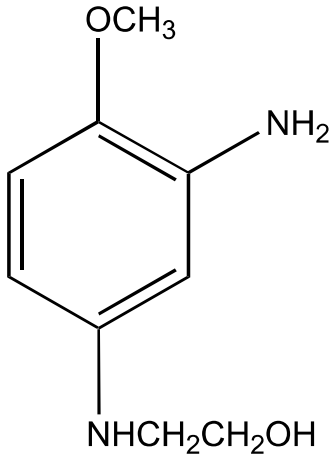
1.2 Chemical names

1-methoxy-2-amino-4-ß-hydroxyethyl-amino-benzene
2-amino-4-ß-hydroxyethyl-amino-anisole

1.4 CAS no.

83763-47-7

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{14}N_2O_2$
Mol weight: 182

1.7 Purity, composition and substance codes

The compound exists as free base (oxidizing), as hydrochloride, as dihydrochloride, and as sulphate.

2. Function and uses

Oxidative hair dye; maximum use 3 % (included as salt); 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	female CF 1 mice, oral	538 mg/kg
	female Wistar rats, oral	588 mg/kg
	male Wistar rats, oral	475 mg/kg

3.4 Repeated dose oral toxicity

The compound as dihydrochloride (0.5 % in distilled water), was administered orally by stomach intubation, 5 days a week, for 3-4 weeks, to groups of 9 male and 9 female SPF Wistar (TNO/W.74) rats at dose levels of 0, 10, 25 mg/kg b.w. day (5 ml/kg). A slight and not clear activation of the thyroid epithelium was observed in rats treated with 10 mg/kg, but not in rats treated with 25 mg/kg. In females treated with 10 mg/kg, a slight reduction in food consumption during the first week and an increase of total number of leucocytes were observed. In rats treated with 25 mg/kg a slight lymphocytosis was revealed. In one rat treated with the highest dose discoloration of the thyroid was observed, without neither pigment sedimentation nor thyroid epithelium sedimentation.

The dose lower than 10 mg/kg represents the NOAEL.

3.5 Repeated dose dermal toxicity

The compound was dermally applied to a clipped area on the back (3x4 cm) of Pirbright white guinea pigs (5 male and 5 female/group), 7 days a week, for 4 weeks at doses of 50, 150, 300 mg/kg b.w. (5, 15, 30 % in water). The treated skin did not show any sign of irritation. No adverse effects were revealed up to a dose of 300 mg/kg b.w.

3.7 Subchronic oral toxicity

The compound, as sulphate, was administered daily by stomach tube to 25 male and 25 female SPW Wistar rats for 13 weeks at dose levels of 0, 2, 50, 100 mg/kg b.w. in distilled water (1 ml/100 g b.w.). The dose of 100 mg/kg was increased until 1380 mg/kg b.w. The reversibility effects were evaluated after 4 weeks without treatment, in 40 additional rats both from the control group (10 males and 10 females) and the highest test group (10 males and 10 females). The dose of 50 mg/kg showed rough pelages, pigmentation of the thyroid gland and in the duodenum. At the end of treatment in males treated with 50 mg/kg dark discoloured urine and increased liver weights were observed. The dose of 100 mg/kg showed rough pelages, pale grey skin and mucosae, dark urine, reduction in activity and body weight (only in males). The weight of thyroid glands, livers, kidneys, spleens and suprarenal bodies (of males) was reduced at the highest test dose. The 100-1380 mg/kg dose showed pigmentation in thyroid glands, intestinal tracts, epididymides, livers and kidneys. The highest dose reduced erythrocytes, haemoglobin and heamatocrit values, and increased reticulocytes, MCV (mean corpuscolar volume of erythrocytes), MCH (mean corpuscolar haemoglobin), ß-globulin and bilirubine. The 2 mg/kg b.w. dose represents the NOAEL.

3.8 Subchronic dermal toxicity

A hair dye formulation ("Koleston 2000"), containing 3 dose levels of test compound as sulphate (1.2 %, 1.8 % and 2.4 %), mixed 1:1 with hydrogen peroxide, was dermally applied (0.05 ml) to the back of mice (75 males and 75 females for each group), 3 times a week, for 12 months. Negative control received 0.05 ml of deionized water in the same way. In all treated animals alopecia and epithelial lesions of treated skin area were found. In females treated with the highest test dose the body weight gains were reduced. No morphological changes in thyroids were observed. The formulation contained other dyes too.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as sulphate (1 % suspended in 10 % Arabic gum) was applied, both to the clipped right (5 animals) and left flank (5 animals) of 10 female albino guinea pigs, 3 times for 2 consecutive days. Treated areas (3x4 cm) were washed off after 20 min. The skin reactions were evaluated during, and three days after treatment. 2 of 10 animals showed a very slight erythema of the clipped scarified skin. All animals were free from symptoms on the last day of the study. The compound resulted not irritant for the skin of guinea pigs.

Human skin irritation: A hair dye formulation ("Koleston 2000", shade blue-black), containing the compound (2.25 %), mixed 1:1 with 9 % H_2O_2 and water, was applied topically to the skin of 40 persons by patch-test, under occlusive condition, for 24 hours. No irritation on the treated skin was found 24, 48 and 72 hours after application.

4.2 Irritation (mucous membranes)

The compound as sulphate, was instilled (1 % aqueous solution, 0.1 ml) into the conjunctival sac of one eye of 10 female Pirbright guinea pigs. The compound was not washed off. The eye of all animals was washed with 1 % fluoroscein sodium solution 24 hours after instillation. The eye reactions were evaluated at 0.5, 1, 2, 3, 4, 5, 6, 7 and 24 hours during treatment. The compound resulted "practically not irritating" in guinea pigs.

5. Sensitization

The compound as dihydrochloride (1 % aqueous solution) was intracutaneously injected to 15 female guinea pigs for induction phase (0.1 ml), 3 times a day for 5 days. 4 weeks later the challenge reaction was performed with different dilutions (1:10, 1:100, 1:500 and 1:1000) of 0.1 ml of compound, applied by intracutaneous injection into the untreated flank. The skin reactions were evaluated both 24 and 48 hours after the challenge procedure. After a 5-day induction period a weakly inflammatory skin reddening was observed. None of the treated animals showed allergic reactions within 24 hours. The compound resulted non-sensitizing for guinea pigs.

Photosensitization: The compound was applied, 30 µl in 30 % injectable water, on the shoulder region of 15 female Pirbright white guinea pigs. Positive control guinea pigs were treated with Hexachlorophene. Afterwards, animals were irradiated with UV-A and UV-B light

for 105 min. Such treatments were repeated 10 times. 2 weeks later the challenge was performed with 5, 1, 0.5, 0.1 % of compound applied on the shaved back of animals. The left side of the back was irradiated with UV-A for 105 min, and the right side remained unirradiated. The allergic reaction was evaluated 24 and 48 hours after. The compound resulted non-photosensitizing in guinea pigs.

6. Teratogenicity

The compound was orally administered by gastric intubation to mated female Sprague-Dawley rats (23-28 for each group) from days 6 to 15 of gestation at doses of 0, 150, 350 mg/kg day (10 ml/kg in distilled water with few drops of 23 % ammonia). The analyses were performed on day 19 of gestation. At 350 mg/kg the body weight gain of dams was below mean values during treatment period, and the rate of skeletal variation increased compared with the control group. Such differences were due to retarded ossifications of the osseous occipitale and parietale. No other adverse effects were observed in dams and foetuses. The dose of 150 mg/kg b.w. day represents the NOAEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: Two hair dye formulations (I and II) containing the compound (C-ring labelled), as dihydrochloride (I =1.05 % and II = 2.1 %) were epicutaneously applied for 30 min on the clipped back (3 cm^2) of HIM:OFA-Sprague-Dawley rats. For each experimental group 3 males and 3 females were considered. The rats were treated either with 1 g of the formulation I or 0.5 g of formulation II, mixed with 0.5 g of 9% H_2O_2, in both cases corresponding to 38 mg/kg b.w. of compound. Similarly, another group of rats was treated with 0.3 ml of the 3.5 % aqueous solution of compound at dose of 37 mg/kg b.w. for 30 min. 0.13 %, 0.033 % and 0.24 % of the applied dose were absorbed, after treatment with formulation I, formulation II and aqueous solution, respectively. 0.57 % , 1.51 %, 0.75 % of the applied dose were revealed in treated skin area 3 days after treatment with formulation I, formulation II and aqueous solution, respectively. After 72 of treatment low radioactivity was found in organs. The resorbed activity was quickly discharged with urine.

Human skin absorption: A hair dye formulation “Koleston 2000 (1/0)” containing the compound (2.2 %) was epicutaneously applied (70.64 g, i.e. 1554 mg of compound) on five healthy female volunteers by a professional hairdresser for 15 min. Blood samples were taken at 0, 10, 20, 30, 40, 50 and 60 min and 2, 3, and 24 h. after applications. The results showed within the range of the sensitivity of method (16 ng/ml, HPLC technique and fluorescence photometer) that neither the hair dye nor the metabolises could be detected in the serum; therefore the volunteers (57.86 kg mean b.w.) — presuming a whole body distribution and absorption of at least 925.67 mg (on the basis of the method sensitivity) per volunteers — absorbed none or less than 0.06 % of the applied dose of test compound.

8. Mutagenicity

The compound tested as sulphate was able to induce gene mutation *in vitro* on TK+/- mouse lymphoma assay, both in the presence and in the absence of rat liver metabolic activation, with a clear dose-related effect. The increases in mutation frequency over the control were up to

2.26 (19.6 µg/ml, -S9mix) and 3.18 (147.1 µg/ml, +S9mix)*;* sister chromatid exchanges *in vivo* on bone marrow cells of Sprague-Dawley rats SIV 50 treated i.p. (250 mg/kg: x 1.74, $P < 0.001$ one-side test; 300 mg/kg (2/10 animals survived): x 1.80).

The compound, tested also as sulphate, was found negative for:
- gene mutation *in vitro* on:

Salmonella
E. coli

mouse lymphoma L5178Y cells (Na^+/K^+ ATP-ase and HPRT loci) after reevaluation of data

- chromosome aberrations in cultured of human lymphocytes *in vitro*;
- UDS (autoradiographic method) in primary culture of rat hepatocytes *in vitro*;
- UDS *in vivo* on male Wistar rats (750 mg/kg b.w. for 4 h and 75 and 750 mg/kg b.w. for 16 h);
- sister chromatid exchanges *in vivo* on bone marrow cells of Sprague-Dawley rats SIV 50 treated both orally (50, 100, 200, 300, 400, 500 mg/kg) and dermally (topical applications: 100, 200, 5 x 200, 1000, 2000 mg/kg).

The compound tested as dihydrochloride was unable to induce gene mutations *in vitro* in five strains of *Salmonella.*

The compound tested as free base does not induce micronuclei *in vivo* on bone marrow cells of mice treated by oral gavage at doses up to 2 x 500 mg/kg. The treatment was performed twice in two equal doses separated by a 24-hour interval, and an analysis 6 hours after the last dose.

10. Special investigations

Phototoxicity: The compound was applied on the back (2 cm^2) of 10 female Pirbright white guinea pigs on two test areas at doses of 5 % and 1 % in injectable water. Another area was treated with positive control (8-Methoxypsoralen) and one area remained untreated. The animals were then irradiated with UV-B light for 80 sec and UV-A light for 80 min. The compound resulted non-phototoxic 24 and 48 hours after the last irradiation.

11. Conclusions

In the absence of carcinogenicity data, the SCC requires *in vitro* cytogenetic and *in vivo* UDS studies.

Classification: B

Approved by the SSC on February 19th, 1991.

Revised June 29, 1993.
Additional information were provided on *in vitro* cytogenctic and *in vivo* UDS studies.

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

(1-methoxy-2-amino-4-ß-hydroxyethyl amino-benzene)
(A 84)

oxidation or permanent

Based on a usage volume of 100 ml, containing at maximum 1.5 %

Maximum amount of ingredient applied:	**I (mg)= 1500 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 0.06 % (human)**
Dermal absorption per treatment:	**I (mg) x A (%)= 1500 x 0.06/100 =0.9 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg b.w. = 0.9 mg/ 60 kg b.w. =0.015 mg/kg b.w.**
No observed adverse effect level (mg/kg): (rat oral, 13 weeks)	**NOAEL = 2 mg/kg b.w.**
Margin of Safety	**NOAEL / SED = 2 mg/kg b.w./0.015 mg/kg b.w. = 130**

B 37: *N1,N4,N4-TRIS-(2-HYDROXYETHYL)-1,4-DIAMINO-2-NITROBENZENE*

1. General

1.1 Primary name

N1,N4,N4-tris-(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene

1.2 Chemical names

N1,N4,N4-tris-(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene
2,2'-((4-(2-hydroxyethyl)-amino)-3-nitrophenyl)-imino-bis-(ethanol)
1-ß-hydroxyethylamino-2-nitro-4-bis-(ß-hydroxyethyl)-aminobenzene

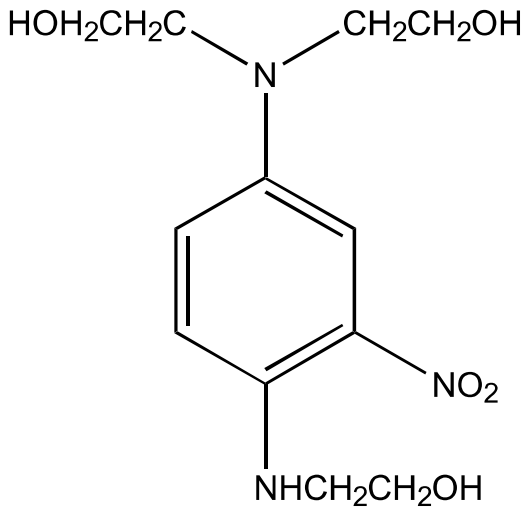
1.3 Trade names and abbreviations

Imexine FAF
HC Blue N°2

1.4 CAS no.

33229-34-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}H_{19}N_3O_5$
Mol weight: 285

1.7. Purity, composition and substance codes

Purity sample: The acute oral toxicity, the 14-days oral toxicity and the 3-weeks diet studies on rats and mice: 75% (lot. no.513077); the 14-days oral toxicity, the 2-years carcinogenicity on rats and mice, and the NTP mutagenicity studies: 98% (lot no. 9233); the metabolism and dermal absorption studies on mice and rats: >98% (TLC).

2. Function and uses

Semipermanent hair dye (nitrophenylenediamine derivative); max. use 2.8 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rat, oral > 5000 mg/kg

Acute toxicity: The compound (1% carboxymethyl cellulose ether sodium salt saline) was administered by gavage to F344/N rats (5 animal/sex/group) at a single dose of 31, 62, 125, 250 or 500 mg/kg, and to $B6C3F_1$ mice (5 animal/sex/ group) at doses of 62, 125, 250, 500 or 1000 mg/kg. No animals died at the end of observation period (14 days).

3.4 Repeated dose oral toxicity

Two NTP 14-days repeated-exposure studies were conducted with two different samples of B37 (75 % and 98 %) on male and female F344/N rats and male and female $B6C3F_1$ mice. Groups of 5 males and 3 or 5 females received in the diet 0, 3, 100, 6200, 12500, 25000, or 50000 ppm of test compound for 14 days (max dose: rats = 1.95 g/kg; mice = 11 g/kg). The second study was conducted as the NTP 13 wks study. No compound related toxic effects were observed at necropsy in both studies.

3.7 Subchronic oral toxicity

Male and female F344/N rats and $B6C3F_1$ mice received in the diet 0-3100-6200-12500-25000-50000 ppm of B37 (75% pure) for 13 weeks to evaluate the cumulative toxic effects and to determine the concentration to be used in the 2-year NTP carcinogenicity assay. After necropsy the thyroid glands were dark in rats (40-80 % in each dose) and the incidence was dose-related. Purple urine and dark feces were observed after day 9. No compound-related histopathologic effects were observed.

3.8 Subchronic dermal toxicity

The compound containing formulation (1.7 %) was topically applied twice weekly for 13 wks. on abraded and intact skin to 12 adult New Zealand white rabbits: no evidence of systemic toxicity was observed analyzed as gross abnormalities in several organs, microscopic lesions and hematologic and clinical chemistry examens. No dye discoloration of the urines was observed at any time during the test.

3.10 Chronic toxicity

B37 contained in a commercial dye/base composite (1.63 %) administered in the diet (19.5 and 97.5 mg/kg/day) to 6 males and 6 females beagle dogs for 2 years (7 day/wk) showed no adverse toxic effects. (Necropsy was performed on one male and one female of each group at 6, 12 and 18 months, and on all survivors at the end).

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 3% (w/w) extemporaneous solution in polyethylene glycol 300, applied on intact and abraded rabbit's skin (0.5 ml per 6.5 cm^2 per animal), resulted non irritant under patch-test for 24 h.

4.2 Irritation (mucous membranes)

The compound as a 3 % (w/w) extemporaneous solution in polyethylene glycol 300 instilled into one rabbit's eye resulted only very slightly irritant.

5. Sensitization

It was induced in guinea pigs by two simultaneously intradermal injections of 5 % test compound in distilled water, Freund's complete adjuvant and a 1:1 mixture of the above solution in a shaved intrascapular area (4 x 6 cm^2) on day 0, 3. One week later 5 % of test substance in petrolatum was topically applied, under occlusion, on the same area for 48 h. 14 days later the guinea pigs were challenged by a single topical application of 5 % of test compound in distilled water under occlusion for 24 h on the right flank (2x2 cm^2). The results evaluated after 24 and 48 hours showed a slight positive reaction on 4/20 test animals 24 hours after challenge. The compound resulted a weak sensitizer.

6. Teratogenicity

A formulation containing the compound (1.7 %) was topically applied (2 ml/kg/day = 34 mg/kg/days) to the shaven skin on 20 rats on day 1-4-7-10-13-16-19 of gestation. No embryotoxic or teratogenic effects were observed, except only a significant reduction of the mean live fetal weight.

B37 contained in a commercial dye/base composite (1.63 %) was administered in the diet to rats from day 6 through day 15 of gestation at levels of 0, 1950 and 7800 ppm (ca. 616 mg/kg/day): no evidence of teratogenicity or embryotoxic effects were observed(5): NOAEL = >600 mg/kg.

B37 contained in a commercial dye/base composite (1.63%) was administered daily by gavage (19.5 or 97 mg/kg/day with composite and 0 and 97.5 mg/kg/day without dyes in 0.5 % aqueous methyl cellulose) to 12 rabbits/dose on days 6-18 of gestation: no evidence of a teratogenic effect was observed.

6.1 One-generation reproduction toxicity

Reproduction: B37 contained in a commercial dye/base composite (1.63 %) was administered in the diet to rats (1950 e 7800 ppm) for fertility and reproduction study divided into two parts: Part I): females: 8 wk prior to mating through the weaning of their litters; males: 8 wk prior to, and during mating period; Part II): males: 8 wk prior to, and during mating; females: 8 wk prior to mating, during gestation and 21 days lactation. Mating 1 male with two females. No abnormal pups were seen upon dissection of embryos after 13 days of gestation or upon gross examination at weaning after 21 days. The study is considered inadequate for the evaluation of the potential effects of the chemical on the reproductive activity of rats.

7. Toxicokinetics (incl. Percutaneous Absorption)

Metabolism: [^{14}C]-B37 at 4th day after administration to rats by different route (oral, i.p. or s.c.) showed that ca. 5% of the applied dose (73.8 mg in 0.1 ml ethanol and 0.5 ml Tween 80) was retained in the body (tissue and carcasses ^{14}C-level). In mice a dosing s.c. with [^{14}C]-B37 up to 2.2 % of the applied dose was recovered in the carcasses (^{14}C-level) after 4 days. Urine (6 or 24 hs. after treatments) and faecal analysis revealed acetylated and conjugated products of parent HC Blue No.2, Violet A isomers and HC Red 3 dyes.

Dermal absorption: A formulation containing B37 radiolabelled (1.77 %) applied on human hairs under conditions of use (35-38 min.) showed a cumulative dose absorption evaluated by means of urine radioactivity assay (1-10-20-30 days) less than 0.1 % and a time required for 50 % excretion (T½) of 52 h.

[^{14}C]-B37 (1076 µg) in ethanol solution showed, under occlusive protective patch on skin of rats (200 µl, 10 cm^2) and mice (40 µl, 2 cm^2), that 0.31 % (males) and 0.27 % (females) of the applied dose penetrated in the rats skin during the 48 h after topical treatment; while 6.5 % (females) and 3.4 % (males) penetrated in the mice skin.

[^{14}C]-B37 (0.5 %, 50 % aqueous shampoo solution of a semi-permanent hair dye) showed that penetration trebled from 0.03 µg/cm^2 after 5 min. application to 0.10 µg/cm^2 after 30 min. application in rat; in mice after 10 min. contact penetration was less than 0.04 µg/cm^2 (0.07 µg/cm^2 in rat).

When different levels of [^{14}C]-B37 (1.5 %, 0.75 %, 0.4 %, 0.2 %) in a semi-permanent hair dye (200 µl of 50 % aqueous shampoo solution for 5 min.) were used, the skin penetration in female rats increased in proportion with the increased concentration of the test compound (from 0.01 µg/cm^2 to 0.12 µg/cm^2).

[^{14}C]-B37 (1070 µg in 50 % aqueous shampoo solution of a semi-permanent hair dye) showed that multiple application to female rats (5 min., 200 µl application) resulted in increased penetration: 0.03 µg/cm(single), 0.23 µg/cm^2 (2 appl.), 0.60 µg/cm^2 (3 appl.)

Female rats treated topically with a 50 % shampoo base (200 µl on 10 cm^2 for 5 min.) containing 0.65 % (w/v) of [^{14}C]-B37 (1295 µg) showed a skin penetration of 0.04 µg/cm when skin was clipped and 0.03 µg/cm^2 in the presence of hair.

8. Mutagenicity

The studies presented have shown that B37 is able to induce UDS on rats hepatocytes and sister chromatid exchange in the presence of metabolic activation system on chinese hamster ovary cells *in vitro*. Two NTP studies (*Salmonella* with and without activation from rat and hamster liver, and Mouse lymphoma with rat liver activation) have shown positive results. In reevaluation of NTP *Salmonella* studies using more stringent criteria the compound was classified as negative. Negative results were obtained in the induction of chromosome aberration on CHO cells *in vitro*; in this study the induction of SCE resulted positive.

In another reverse mutation study on *Salmonella* the compound resulted negative. The compound did not induce chromosome aberrations *in vivo* by micronucleus test on mice (2 x 750 and 1000 mg/kg i.p.). Unscheduled DNA Synthesis study on male and female rat hepatocytes and male and female mice hepatocytes following *in vivo* treatment up to 1000 mg/kg b.w., and cell proliferation in rats and mice studies, were found negative.

Additional *in vitro* studies, requested by the SCC, performed with a sample of 99.5 % of purity, have shown that B37 is negative in the Ames test, in mouse lymphoma L5178Y (6-TG^R) assay and in human lymphocytes chromosome aberrations test.

Literature studies with a sample of 99.77 % of purity showed that the compound was positive in *Salmonella* assay, in mouse lymphoma L5178Y (TFT^R) and in rodents UDS *in vitro* test. This sample of compound did not induce forward mutation on *E.coli,* micronuclea on ICR and CD-1 mice bone marrow and UDS *in vitro* on monkey primary hepatocytes.

9. Carcinogenicity

Long term studies were carried out on mice and rats (NTP bioassay): the compound (98% pure) fed in the diet for 103 weeks to 50 F344/N rats/sex/group and 104 weeks to 50 $B6C3F_1$ mice sex/group at dietary concentrations of 0-5000-10000-20000 ppm to male rats (195 and 390 mg/kg/day) and mice (465-1000 mg/kg/day) and 0-10000-20000 ppm to female rats (1320-2240 mg/kg/day) and mice (2330-5600 mg/kg/day). B37 caused a dose related increase in the incidence of hyperostosis of the skull in male and female rats. A uncommon tumour (mixed mesenchymal neoplasms of the kidney) was noted for female F344/N (2/50 at high dose) and a marginal positive trend in the incidence of lymphomas in male mice (1/50; 5/48; 8/49) not significant when survival were taken into account.

Under the conditions of these studies there was "*no evidence"* of carcinogenicity in F344/N rats and $B6C3F_1$ mice receiving B37 in the diet.

11. Conclusions

The SCC do not see any possible health risk connected with the use of this dye.

Classification: A

P 8: *HEXAMIDINE*

1. General

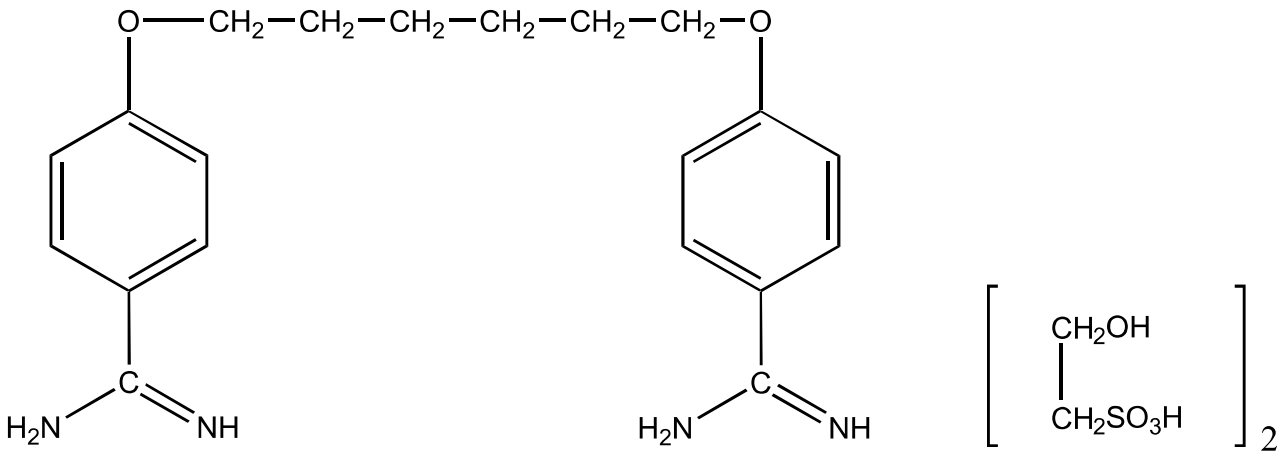
1.1 Primary name

Hexamidine

1.2 Chemical names

1,6-di(4-amidino phenoxy)-n-hexane and its salts including di-isethionate and di (phydroxybenzoate)

1.5 Structural formula



1.9 Solubility

Hexamidine is soluble in water and insoluble in organic solvents.

2. Function and uses

Hexamidine is used in cosmetics as a preservative at a maximum dose level of 0.1 %, and for other uses at concentrations up to 0.3 % in non-rinsed skin products.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The acute toxicity of hexamidine is considerable. Oral LD_{50} values (in mg/kg b.w.) are 710-2500 in mice, 750 in rats, 500 in rabbits. Intraperitoneal values of 17-51, and 57 were reported for mice and rats respectively. Intravenous values are 17 for mice and 8 for rabbits. A dermal value for rats was > 4000.

3.4 Repeated dose oral toxicity

In a 90-day oral study in male rats, daily doses of 400 and 800 mg/kg by gavage induced mortality, growth depression, signs of anaemia, increased liver weight and decreased liver- and kidney function. The lower dose of 200 mg/kg was not a clear No Effect Level.

3.5 Repeated dose dermal toxicity

A subacute (28-day) dermal toxicity study in rabbits showed that solutions of up to 2 % were only slightly irritant. Daily application of 4 ml/kg b.w. of a 0.05, 0.1 and 2.0 % solution revealed no systemic toxicity. A 90-day dermal study in rabbits with the very low dose level of 16 mg/kg b.w. revealed no systemic toxicity.

3.7 Subchronic oral toxicity

A recent short-term (4-wk) oral study was conducted by gavage administration of 50, 100 and 200 mg/kg b.w./day to groups of 5 rats/sex. All test animals showed post-treatment symptoms (salivation, wet fur, brown oral staining). The top-dose rats also showed abnormal position and locomotion, and increased counts of white blood cells and lymphocytes. In the two higher dose groups there were increases in the values for GPT, GOT and calcium in blood plasma. All treated rats showed caecal enlargement. The lungs, heart, liver, kidneys and caecum did not reveal treatment-related microscopical changes. Other organs (including spleen and adrenals) were not examined. The clinical signs and the caecum enlargement were not considered to be of toxicological significance. The No-toxic Effect Level was established at 50 mg/kg, but the study showed several deficiencies.

4. Irritation & corrosivity

4.1 Irritation (skin)

A concentration of 0.1 % was slightly irritating to the skin of rabbits.

4.2 Irritation (mucous membranes)

A 0.1 % solution was slightly irritating to the eye of rabbits.

5. Sensitization

Hexamidine did not produce any evidence of sensitization in guinea pigs, nor of photosensitization using a rabbit model. However there is some evidence for sensitization reactions occurring in man following its use as a topical bacteriocide.

7. Toxicokietics (incl. Percutaneous Absorption)

Studies using radiolabelled material to investigate skin absorption in the rat indicated very poor absorption. When the compound was applied as a 0.1 % formulation in cold cream under an occlusive dressing for 96 hours a mean of ca 0.6 % was absorbed (maximum value 1.4 %). Very little absorption is thus likely to occur in use.

8. Mutagenicity

An Ames test using *S. typhimurium* strains TA 1535, 1537, 98 and 100 and concentrations up to 500 μg/plate was negative. It was reported that no clastogenic activity was observed in a limited *in vitro* test for chromosomal aberrations in CHO cells exposed to up to 34 μ/ml in the absence of metabolic activation and 420 μ/ml in its presence. The negative result was however not convincing, because of an increase in aberrations at the low dose that was not seen at higher doses. Furthermore these equivocal results were not followed up in a repeat experiment. This finding cannot be disregarded.

11. Conclusions

Hexamide has moderate acute toxicity by the oral route, but is highly toxic by injection. It is poorly absorbed through the skin and has low toxicity by this route. A 0.1 % solution was slightly irritating to the skin and eyes of rabbits, and there is no evidence of any sensitization potential. The No Effect Level in a 28-day repeated dose oral study was 50 mg/kg. In a 90-day repeated dose study marked toxicity occurred in various organs (especially liver, kidney, haematopoietic system) at 400 mg/kg with marginal effects at 200 mg/kg. Negative results were obtained when the compound was tested for mutagenic potential using the Salmonella assay, but equivocal results were obtained in an *in vitro* assay for chromosome damage in mammalian cells. In view of the very low levels of compound likely to be absorbed through the skin in use studies to specifically investigate effects on the reproductive system are not required. However in view of the equivocal findings in the chromosome aberration study, a further study (metaphase analysis using mammalian cells) is needed.

Classification: B

P 21: *BENZYLFORMAL*

1. General

1.1 Primary name

Benzylformal

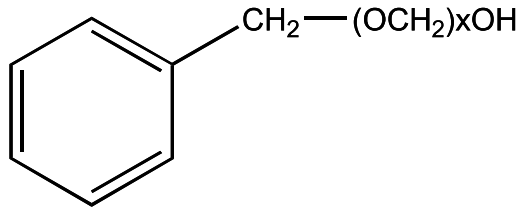
1.2 Chemical names

Benzylformal

1.3 Trade names and abbreviations

Preventol D_2

1.5 Structural formula



x = ~ 1.5

1.7 Purity, composition and substance codes

Benzylformal is a mixture of benzyloxymethanol and benzyloxymethoxymethanol.

1.9 Solubility

Soluble in organic solvents; solubility in water 25 g/l.

2. Function and uses

The substance is used up to 0.2 % in all types of cosmetics.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The oral LD_{50} in rats was 1700 mg/kg; the i.v. LD_{50} in rats was 153 mg/kg. The animals showed sedation, loss of consciousness, paralysis.

3.2 Acute dermal toxicity

The dermal LD_{50} in rats was > 1000 mg/kg. In rabbits, dermal LD_{50}-values of 1429 and 2000 mg/kg for males and females respectively were obtained.

3.8 Subchronic dermal toxicity

A subchronic dermal study has been carried out in the rabbit. Doses of 1, 4 and 16 mg/kg body weight were given to groups of 10 male and 10 female rats. The only sign of toxicity noted was a slight reduction in body weight gain at 300 mg/kg in the male animals. Haematological examination revealed increased leucocyte count in the males at 300 mg/kg but no other effects. At autopsy increased adrenal weight was seen in the females at the top dose level only; minor changes were reported in other organs but there were no dose releated trends and these were not significant. Histopathology revealed inflammatory changes in the mucosa of the glandular stomach but no other adverse effects. The No Effect Level in this study was 100 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

A skin irritation test in rabbits with 500 mg undiluted substance applied to the intact skin of the ear for 8 hours induced redness and oedema; when applied for only two hours, slight redness was observed. A 0.2 % aqueous solution applied for 24 hours did not induce any changes.

4.2 Irritation (mucous membranes)

In an eye irritation test in rabbits 50 mg undiluted substance caused erythema and oedema and an opaque cornea. A 0.2 % aqueous dilution only produced erythema.

5. Sensitization

A sensitization test by the Landsteiner-Draize method with 0.1 % of the test substance in saline both for the induction and for the challenge treatment did not reveal signs of sensitization.

11. Conclusions

The substance liberates formaldehyde (at a maximum of 0.004 % under test conditions). Although studies on dermal absorption are not available, appreciable uptake through the skin is suggested by a comparison of the oral and the dermal LD_{50} values and the dermal toxicity study in rabbits. The No Effect Level in a 29-day oral study in the rat was 100 mg/kg. A much lower value was however obtained in a 90-day dermal study in rabbits, namely 1 mg/day. The maximum dermal exposure to humans in use may be calculated to be about 1 mg/kg body weight per day, which allows no safety factor at all when compared to the No Effect Level in the subchronic study in rabbits. Unless convincing arguments are provided to explain the effects on the pituitary in the dermal study in rabbits, this preservative should not be used in cosmetics. If such reassurance can be provided, additional studies to investigate clastogenicity in a mammalian cell assay *in vitro* and, since the compound has appreciable absorption through the skin, from a teratogenicity study, will be needed.

Classification: D

P 91: *3-IODO-2-PROPYNYL BUTYL CARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butyl carbamate

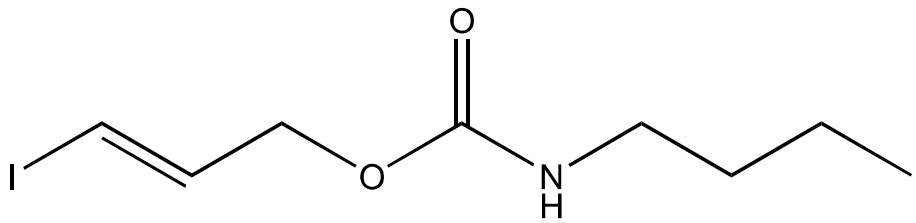
1.2 Chemical names

3-iodo-2-propynyl butyl carbamate
iodo propynyl butyl carbamate

1.4 CAS no.

55406-53-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}NO_2I$

Mol weight: 281

1.9 Solubility

It has low solubility in water (156 ppm at 20°C) and is soluble in organic solvents.

2. Function and uses

It is proposed for use as a preservative in all types of cosmetic products at up to 0.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal

signs of toxicity were seen at 500 mg/kg or below. In a percutaneous toxicity study in rabbits a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing resulted in no deaths. The only signs of toxicity seen were slight irritant effects at the site of application.

3.7 Subchronic oral toxicity

In a subchronic study, rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The No Effect Level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritancy study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe oedema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours.

4.2 Irritation (mucous membranes)

Severe effects were noted in an eye irritation study in rabbits. The substance (0.1 g) produced moderate to severe hyperaemia, chemosis and discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. If the compound was washed out of the eye 20-30 seconds post instillation only transient irritant effects were seen.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximisation test. Induction concentration were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson Kligman maximisation tests have been carried out on formulations containing 0.5 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical) were used. In the second case the concentrations were 0.1 % and 0.5 % respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation. There was no evidence of sensitization in either test. These studies suggest that the compound does not have any significant potential for skin sensitization.

6. Teratogenicity

Teratogenicity studies have been carried out in both the rat and the mouse. In the study in rats the compound was given on day 6-15 of gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The No Effect Level was 50 mg/kg. A similar dosing regime was used in the study in mice. No compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The No Effect Level was 50 mg/kg.

6.2 Two-generation reproduction toxicity

A two-generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week pre-mating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound related effects were seen at any dose level on clinical chemistry or at necropsy. Reduced weight gain was seen in the males at 300 ppm and above in the initial generation during the pre-mating period and at 750 ppm at the females. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index at 750 ppm, with a marginal effect at 300 ppm; postnatal growth of the offspring however was not affected. No effects were seen on the development of the offspring. The No Effect Level in this study was 120 ppm test compound in the diet. (This dietary level is roughly equivalent to a dose of the order of 10 mg/kg body weight). No marked effects were seen on fertility or general reproductive performance at any dose level.

7. Toxicokinetics (incl. Percutaneous Absorption)

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C radiolabelled material. Following i.v. administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the level and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicate that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

8. Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies. Negative results were obtained in the Salmonella assay versus strains TA 1535, 1537, 1538, 98 and 100 but this study was limited by investigating only 3 concentrations (6.2-55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537,

98 and 100 and concentrations of 1-1000 µg/plate against TA 1535. In all cases the top concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 %-25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

The compound is a carbamate and studies have been carried out to investigate whether significant blood cholinesterase inhibition occurs in the rat following intravenous administration. The compound was given in PEG/400: water vehicle at 2-16 mg/kg and blood samples taken and analysed for erythrocyte cholinesterase activity at 15, 30, 60 minutes and 2 and 5 hours post dose. No effects on blood cholinesterase levels were observed.

11. Conclusions

The substance has moderate acute toxicity by the oral route and low toxicity following dermal exposure. It is a mild to moderate skin irritant, but it is a severe (corrosive) eye irritant. No data are available on the irritancy at in use concentrations. Negative results were obtained in 3 Magnusson Kligman maximisation tests for skin sensitization. In a sub-chronic (90 day) oral study in the rat the No Effect Level was 50 mg/kg.

Mutagenic potential has been investigated in Salmonella assays for gene mutation and in a study to investigate Unscheduled DNA Synthesis (UDS) in hepatocytes. Negative results were consistently obtained. There was no evidence for any teratogenic potential in studies in 2 species (rat and mice) nor for any significant effects on reproductive performance in a two generation fertility study in rats. The compound is well absorbed orally but is rapidly metabolised and excreted.

The severe eye irritancy of the compound is of concern, and data on the eye irritancy of in use formulations is needed.

Classification: B

OPINIONS ADOPTED DURING THE 47TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 24 September 1991

S 8: *2-ETHYLHEXYL-P-DIMETHYLAMINOBENZOATE*

1. General

1.1 Primary name

2-ethylhexyl-p-dimethylaminobenzoate

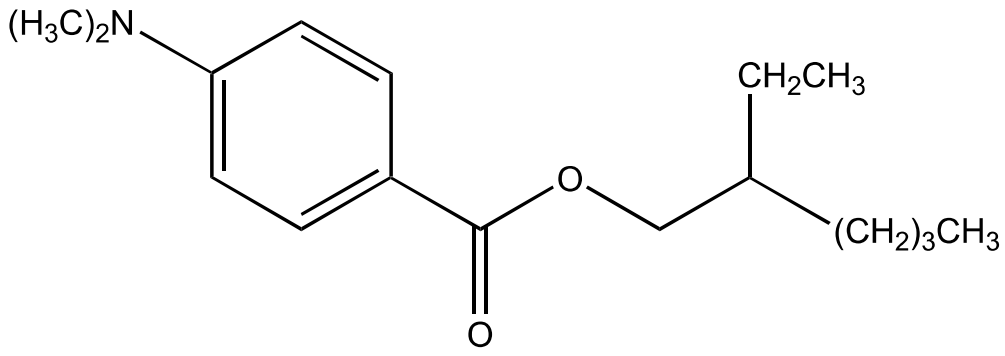
1.2 Chemical names

2-ethylhexyl-p-dimethylaminobenzoate
2-octyl-4-dimethylaminobenzoate

1.3 Trade names and abbreviations

Padimate O
Escalol 507

1.5 Structural formula



1.7 Purity, composition and substance codes

The substance is stated by the manufacturer to contain not less than 98.5 % of active ingredient.

1.8 Physical properties

Appearance: Yellow fluid.
Maximum absorption: 310 nm.
Not known to polymerise.

1.9 Solubility

Soluble in isopropyl alcohol, mineral oil, and ethanol. Insoluble in water.

2. Function and uses

Use level: up to 8 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Values for oral toxicity in the rat varied from 3 to 15 g/kg b.w.

3.8 Subchronic dermal toxicity

A 13-week dermal toxicity study was carried out in groups of 20 rabbits at dose levels of 140 and 280 mg/kg b.w. No significant abnormality was detected.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Solutions of 5 % a.i. were applied to both intact and abraded skin for 24 hours under occlusion. The test was negative.

Man. Occlusive patch tests with 5 % a.i. in yellow soft paraffin were applied for 48 hours. The test was negative.

4.2 Irritation (mucous membranes)

A Draize test in the rabbit at concentrations of 2 % and 5 % in mineral oil showed slight transient irritation.

5. Sensitization

Guinea pig. Ten male animals had an initial intracutaneous injection of 0.05 ml of a 0.1 % solution of a.i. in saline, followed by 9 injections of 0.1 ml 3 days a week. After a 12-week rest period, a challenge dose of 0.05 ml was given. There were no adverse effects.

Man. (a) Fifteen applications of a 4 % solution of a.i. in soft paraffin were made under occlusion over 3 weeks. A challenge application was made after a 2 week rest. There was no adverse reaction.
(b) A mixture of 7 % a.i. with 3 % oxybenzone was used in 150 subjects in a repeated insult patch procedure. No abnormality was found.
(c) Ninety subjects were similarly tested using 8 % a.i. and 8 % benzophenone. The test was negative, although there were occasional slight irritant responses during the induction.
(d) A panel of 156 subjects was similarly tested with 7 % a.i. in soft paraffin. The test was negative.

6. Teratogenicity

Rat. Dermal applications of 2 ml/kg b.w. of a preparation (concentration of a.i. not specified) were made daily from days 6 to 16 of pregnancy. In the test group 7/56 foetuses had bilateral wavy ribs and 2/56 had unilateral wavy ribs. There were no such findings in the control group.

This effect is not regarded by the authors as indicating teratogenic activity, as they consider it a common finding in rats of this strain, but the reason for its appearance in foetuses of the test group only is unexplained.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man. An 8 % ethanolic solution of ^{14}C a.i. was applied over 100 cm^2 of forearm skin in 4 male and 4 female subjects. After the ethanol had dried, the areas were covered with a gauze pad for 24 hrs. No radioactivity was found in the blood; the urine contained between 1.2 % and 2.5 % of the applied radioactivity.

8. Mutagenicity

A standard Ames test was negative. A second similar test is also reported negative, but figures are given for plates with activation only.

A micronucleus test was carried out in the mouse, using a dose which caused disorders of gait and hypotonicity. The a.i. was given intraperitoneally in a dose of 5000 mg/kg b.w. to 3 groups of 10 animals. Positive and negative control groups were included. Sacrifice was at 30, 48 and 72 hours. The test was negative.

10. Special investigations

Phototoxicity
Guinea pig. The ears of 10 animals were stripped and a formulation containing 7 % a.i. and 3 % oxybenzone was applied several times to one ear with vigorous rubbing. The untreated ear served as a control; 2 of the animals had 8-methoxypsoralen applied as a positive control. Thereafter the animals were exposed to UV radiation (wavelength not stated) for 2 hrs. The test was negative; the positive controls showed marked effects.

In another test, a similar preparation was applied to the nuchal area with occlusion for 2 hrs. This was followed by irradiation with 3 J/cm^2 at 320-400 nm. Suitable positive and negative controls were used. The test was negative.

Man. In a poorly reported test, a mixture of 7 % a.i. and 3 % oxybenzone was tested in 26 human subjects. No adverse effects were seen. In another similar test, a 5 % ethanolic solution was used. At 30 J, the control area showed more damage than the test area.

Ten fair-skinned subjects were treated with a mixture of 7 % a.i. and 2 % oxybenzone under occlusion for 24 hrs. A control was similarly applied. After removal of the patches, a further application was made to the skin and irradiation was carried out using 1 m.e.d. of UVB followed by 12 minutes of UVA. The test was negative.

11. Conclusions

The tests for sensitization were carried out at less than the proposed use level. It would have been preferable to have carried them out at irritant levels, to reveal any sensitising potential. In one of the tests for phototoxicity in the guinea pig, the dose of radiation and its wavelength are not given and the application tested contained oxybenzone as well as the a.i. In the second test,

the dose of radiation (3 J cm^{-2}) was small, and the wavelength used was 320-400 nm, which is inappropriate for a UVB blocker. In the tests in man, no figure is given for the amount of UVA irradiation. On the whole, the tests presented for phototoxicity and photosensitivity are poor, but seem to be negative. Tests for photomutagenic activity have not been carried out. The experimental procedure used in the test for teratogenic activity were unsatisfactory, and the results are anomalous. Tests for percutaneous absorption suggest that about 1 mg/kg b.w./day may be absorbed.

A chromosomal aberration test *in vitro* and a 28 or 90 day oral toxicity test should be carried out. It is believed that numerous further investigations have been carried out with this compound; these should be submitted.

Classification: C

S 28: *2-ETHYLHEXYL-4-METHOXYCINNAMATE*

1. General

1.1 Primary name

2-ethylhexyl-4-methoxycinnamate

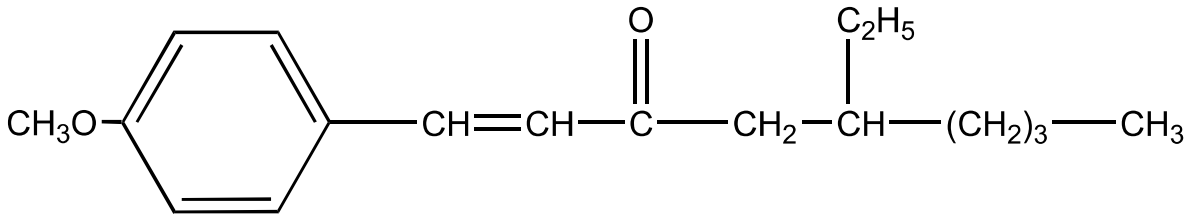
1.2 Chemical names

2-ethylhexyl-4-methoxycinnamate

1.3 Trade names and abbreviations

Parsol MCX

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{18}H_{26}O_2$
Mol weight: 290

1.8 Physical properties

Appearance: Colourless pale yellow slightly oily liquid.

1.9 Solubility

Miscible with alcohols, propylene glycol, etc.
Immiscible with water.

2. Function and uses

Use level up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}: Mouse, greater than 8 g/kg b.w. Rat, greater than 20 ml/kg b.w.

3.4 Repeated dose oral toxicity

Rat. Three week oral study. Groups of 5 male and 5 female animals were given 0, 0.3, 0.9 and 2.7 mg/kg b.w./day by gavage for 3 weeks. All animals of the top dose groups exhibited loss of body weight and a reduced relative and absolute weight of the thymus. Male rats showed a decrease in absolute weight of the left kidney and female rats showed a decrease in the absolute weight of the heart. At the two lower doses, the only significant alteration observed was an increased absolute weight of the pituitary gland in male rats receiving the lowest dose. As the number of animals was small, the investigators considered this not to be biologically significant. The NOAEL was put at 0.9 ml/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Four groups of 12 male and 12 female SPF rats received the compound in the diet at levels of 0, 200, 450 and 1000 mg/kg b.w./day. During the experiment the usual clinical observations were carried out, as well as extensive haematological and biochemical studies. Full gross necropsy was carried out on all survivors. Histological investigations were carried out in half the animals of the control and top dose groups. The organs studied included the heart, lungs, liver, stomach, kidneys, spleen, thyroid and retina. In the remaining animals histological examination of the liver only was carried out. Six control animals and 6 top dose animals were allowed to recover over 5 weeks, and then examined.

The results of the experiment showed no dose related mortality. The kidney weights of top dose animals were increased, but were normal in the recovery animals; the increase was attributed to a physiological response to an increased excretion load. There was a diminution of glycogen in the liver, and a slight increase in iron in the Kupfer cells in the high dose animals. Two of these also showed minimal centrilobular necrosis of the liver with some infiltration; similar less marked findings were made in 2 of the control animals as well. These findings were attributed to infection. High dose females had increased GLDH which reversed during the recovery period. The NOAEL was put at 450 mg/kg b.w./day.

3.8 Subchronic dermal toxicity

Rat. Thirteen week dermal study. Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55.5, 277 and 555 mg/kg b.w./day applied to shaved skin 5 days a week for 13 weeks. (The top dose is believed to be about 135 times the amount which would be used daily by the average consumer). Various laboratory and clinical tests were carried out during the experiment.

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination. The authors put the NOAEL at 555 mg/kg b.w./day, but in view of the liver findings this may be 227 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Guinea pig. The a.i. was applied undiluted twice daily to 20 animals for 16 days. There were no signs of irritation.

Man. Occlusive applications of undiluted a.i. were made to 60 subjects, of whom 20 had sensitive skin. The applications were made for 24 hours. Observations at removal of the patches, and 24 and 48 hours later, showed no evidence of a reaction. In 51 male and female subjects, similar patch tests were carried out. The dilution of the a.i. (if any) was not stated. There was no irritation. A formulation (concentration not stated) tested on the skin of 50 subjects caused no adverse effect. In 53 subjects, a Draize repeated insult patch test at a concentration of 2 % caused no irritation. In 54 subjects, a Draize repeated insult patch test of a 7.5 % dilution of a.i. in petrolatum caused no irritation.

4.2 Irritation (mucous membranes)

Rabbit. Groups of 4 animals had 0.1 ml of a test preparation instilled into the conjunctival sac (concentration not stated). No further treatment in one group; in the other, the instillation was followed by washing out. There were no signs of irritation.

A Draize test carried out with undiluted a.i. was found to be practically non-irritant.

5. Sensitization

Guinea pig. Twenty animals received applications of undiluted a.i. twice daily for 16 days. After a 3 day interval without treatment, a daily challenge application was made for 3 days. There was no evidence of sensitization.

Two groups of 4 animals were used. Animals of one group were exposed to 0.05 ml injections of undiluted a.i. daily for 5 days. In the other group, 0.025 ml of a 50 % acetone solution of a.i. was applied to 2 cm^2 areas of shaved skin on either side. There was no evidence of sensitization.

Man. A Draize repeated insult patch test was carried out at a concentration of 2 % in 53 subjects. There was no sensitization. In 54 subjects, a formulation of 7.5 % a.i. in petrolatum was applied for 48 hours under occlusion for 11 applications. After a 14 day rest, a challenge application of a single dose was made. There was no adverse reaction. In an extensive series of patch tests carried out in man, the a.i. was found to be very rarely responsible for allergic contact effects.

A 10 % solution of a.i. in dimethylphthalate was used. A total of 58 subjects was recruited, 12 males and 46 females, aged 18-63. Of these, 6 subjects failed to complete the test for reasons unconnected with the experimental procedure. Induction applications were made on the skin of the back, for 24 hours with occlusion, 3 times a week for 9 applications. Following a rest period of 2 weeks, a further patch was now applied to a new site on the back for 24 hours with occlusion. The area was inspected at 0, 24 and 48 hours after removal of the patch. No adverse reaction was noted at any stage of the experiment.

6. Teratogenicity

Rabbit. Groups of 20 female animals were mated and given a.i. in doses of 0, 80, 200 and 500 mg/kg b.w./day by gavage during the period of organogenesis. Except for a slight reduction of maternal and foetal weight in the top dose animals, no abnormality was found.

Rat. Following a pilot study, groups of 36 rats were mated and treated with 0, 250, 500 and 1000 mg/kg b.w./day of a.i. (probably by gavage) during days 6-14 of pregnancy. Owing to an error, the preparation of the control foetuses led to their destruction, so this part of the test was repeated under identical conditions. Subgroups of each dose group were allowed to litter normally and rear the offspring. The percentage of resorptions in the high dose group was elevated by comparison with the other groups. The investigator records, however, that this relatively high rate is the usual one with this strain of rat in this laboratory, and he attributes the difference to an unusually low level of resorption in the other groups. No other abnormality was found.

7. Toxicokinetics (incl. Percutaneous Absorption)

Tests for percutaneous absorption.

(a) *In vitro* tests. Rat. Naked rat skin. This was studied in a chamber experiment. Most of the material was found in the stripped skin; there was less in the stratum corneum, and least in the chamber. The approximate amounts found in the chamber were: after 6 hrs, 1.13 %; after 16 hrs, 11.4 %; and at 24 hrs 17,9 %. The figures for the horny layer and the strippings combined were, respectively, 31.4 %, 44.4 % and 45.7 % (percentages of applied doses). Solutions of 3 % and 20 % of a.i. gave similar results. In another set of experiments, various amounts of "Parsol 1789" (4-tert-butyl-4'-methoxydibenzoylmethane) were added to the a.i. in the formulation. There seemed to be no effect on the absorption of the a.i.

Pig. A similar experiment using mini-pig skin was carried out in which "Parsol 1789" was used as well as the a.i. Using 3 sorts of formulation, about 3 % of a.i. was found in the chamber in 6 hrs. Using the concentrations proposed for a particular commercial use (i.e., 7.5 % of "Parsol 1789" and 2 % of a.i.) about 2.2 % was found in the chamber. It is calculated by the authors that the total absorption for a 75 kg consumer would be about 70 mg, or 0.9 mg/kg b.w. (Note however that the maximum proposed use level of a.i. is 10 %).

Man. A test on human abdominal skin in a chamber was carried out. With 7.5 % a.i., about 0.03 % is found in the camber in 2 hours, 0.26 % in 6 hours, and 2.0 % in 18 hours. Various combinations of a.i. and "Parsol 1789" were investigated.

(b) *In vivo* tests. Man. Eight healthy volunteers had small amounts of radioactive a.i. applied to the interscapular region. One group of 4 had the material applied under a watch glass; the other 4 had it applied on gauze, whith occlusion in one case. Tests for absorption of a.i. were negative except for about 0.2 % in urine. The concentrations used were not stated.

In a preliminary experiment, a capsule containing 100 mg of a.i. was taken orally. As a lipophilic substance, the a.i. is very likely to be metabolised; it is known in any case to be hydrolysed by plasma esterases, although slowly. The cumulative excretion of 4methoxycinnamate in the urine over 24 hours was studied by GC/MS of the methyl ester

derivative. (This method would also detect 4-hydroxycinnamic acid). Over 24 hours, 13.2 % of the amount ingested was recovered, equivalent to 21.5 % of the amount that would be expected if the a.i. were completely absorbed. In the main part of the experiment, an o/w cream containing 10 % a.i. was used. Applications of 2 grams of this material (= 200 mg a.i.) were made to the interscapular area of each of 5 male subjects, aged 29 to 46. The area of skin covered was 25x30 cm. After application, the area was covered with 3 layers of gauze, left in place for 12 hours. Blood was taken at times 0, 0.5, 1, 2, 3, 5, 7, and 24 hours. Urine was collected at 0, 1, 2, 3, 4, 5, 6, 7, 12, 24, 48, 72 and 96 hours.

The control plasma samples showed a level equivalent to about 10 ng/ml before any application had been made. There was no evidence of any rise in plasma levels during the experiment. The urine showed a "physiological" level of 100 to 300 ng/ml. No significant increase in this amount was found in any sample. The authors conclude that very little, if any, of the compound was absorbed under the conditions of the experiment.

8. Mutagenicity

Salmonella mutagenesis assays were performed on the usual strains. There was a positive result with TA 1538 without metabolic activation. This was thought to have been a batch effect. From another laboratory, a very weak positive was found with TA 1538 without activation, at 10 µl/plate; it was not found in 2 replicates, nor in a second Ames test. A test for mutagenesis and crossing over in *S. cerevisiae* was negative. A test using Chinese hamster V 79 cells showed a very slight increase in mutant colonies with dose. A test in human lymphocytes *in vitro* was negative.

A test for cell transformation in Balb/c 3T3 cells was negative. A test for unscheduled DNA synthesis was negative.

Tests in *Drosophila:* There was an increase in the frequency of sex-linked recessive lethals. There was no evidence of mutagenicity in feeding tests (adults and larvae). Somatic mutation and combination tests using wing structure were negative. Mouse. Micronucleus test. No effect was found up to 5000 mg.

Test for photomutagenic activity. These were carried out in cells of *S. cerevisiae,* which had previously been shown not to be affected by a.i. *(supra)*. Doses of a.i., dissolved in DMSO, ranged from 0.06 to 625 µg/ml, and radiation up to 500000 J m^{-2} UVA and up to 12000 UVB (50 and 1.2 J cm^{-2}). Chlorpromazine was used as the positive control. Suitable negative controls were also employed. The experiment appears to have been well carried out. The results show that UVA and (more markedly) UVB are mutagenic; and that the a.i. protects against this effect in a dose dependent manner.

10. Special investigations

Test for capacity to produce phototoxicity. Man. In 10 subjects, patches were applied for 24 hours and the areas then exposed to a suberythematous dose of UV irradiation. There was no evidence of phototoxicity.

Test for capacity to produce photosensitization. Tests which "showed that the product did not provoke photosensitization." No details supplied.

Test for inhibition of UV-induced tumors. Hairless mouse. The animals were exposed to repeated doses of UV simulating the solar energy spectrum. After a rest period, 3 applications a week were made to an area of skin of 12-o-tetradecanoyl phorbol-13-acetate (at first at 10 g/ml, but later at 2 g/ml, as the higher concentration was found to be irritant). Suitable controls were used. The test group was completely protected by 50 % a.i., and 7.5 % gave an effect equivalent to reducing the insolation four-fold. It had been suggested that the a.i. could itself have been a promoter, but there was no evidence of this.

11. Conclusions

The compound appears to have low acute and subchronic toxicity, orally and dermally; it does not irritate the mucous membranes in conventional animal tests. The data presented suggest that the compound is not an irritant or sensitizer in animals and man; however, tests for sensitization were carried out at levels below the proposed maximum use level. Clinical investigation shows that this compound is very rarely responsible for allergic contact dermatitis in man. There is no carcinogenicity study, but an extensive range of mutagenicity studies were nearly all negative. A test for photomutagenicity was negative, although the dose of UVB used was rather low. Animal studies for teratogenic activity were negative. Percutaneous absorption in man appears to be very low.

Classification: A

OPINIONS ADOPTED DURING THE 48TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 4 October 1991

A 1: *1,7-NAPHTHALENEDIOL*

1. General

1.1 Primary name

1,7-dihydroxynaphthalene

1.2 Chemical names

1,7-dihydroxynaphthalene
1,7-naphthalenediol

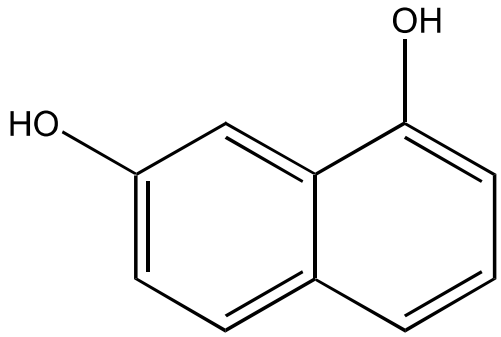
1.3 Trade names and abbreviations

Ro 577

1.4 CAS no.

575-38-2

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10} H_8 O_2$
Mol weight: 160.18

2. Function and uses

Oxidative hair dye; max. use 1 %; 0.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Male mice, oral: 1700 (1570-1840) mg/kg body weight.

3.7 Subchronic oral toxicity

The compound was administered to 20 male and 20 female rats by oral gavage 5 times a week for 12 weeks at a single dose of 0 and 50 mg/kg in water suspension. The histological examination showed that the compound was able to induce mild alterations to isolated liver cells. No other adverse toxicity effects were seen.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound when applied at a concentration of 5 % (0.5 ml of 10 % water suspension) in 2 % carboxymethylcellulose solution (pH=9), to clipped intact rabbit's skin under occlusion for 4 hours, did not produce any signs of irritation after 4, 24, 48 and 72 hours.

The compound as a 10 % (w/v) in olive oil suspension, applied (2 droplets) on adult male hairless mice (strain *hr hr*) twice daily for 10 days to the same skin area, produced a mild dermal irritation after 12 or 18 applications and until the end of the study.

4.2 Irritation (mucous membranes)

The compound applied as a 5 % solution in 2 % carboxymethylcellulose solution (pH=9), instilled into one eye of each sex of albino rabbits at doses of 0.1 ml without rinsing off, produced no signs of irritation after 2, 6, 24, 48 and 72 hours.

5. Sensitization

Female guinea pigs were used with induction by simultaneously intradermal injections of 5 % (w/v) of the test compound suspended in water, 0.1 ml of Freund's complete adjuvant (FCA) and a 1:1 mixture of FCA and 5 % water suspension of the test substance dermally applied under occlusion on day 0. Seven days later, on the same area for 48 hours. On day 21 the guinea pigs were challenged by dermal application at a new skin side of a 25 % (w/w in vaseline), under occlusion for 24 hours. The results evaluated after 24 and 48 hours of challenge showed that the compound was not a sensitizer in guinea pigs.

7. Toxicokinetics (incl. Percutaneous Absorption)

Cutaneous absorption: The ^{14}C-1,7-dihydroxynaphthalene (labelled at the C-1 atom of the naphthalene ring) applied on 10 cm^2 intact and clipped skin of 5 male and 5 female Wistar rats for 48 hours (1 % in ca. 200 mg of cream without developer; the formulation saturated the exposed air of the skin) showed these values of cutaneous resorption: 13.8 % (=29.19 µg, for males), and 17.0 % (=33.25 µg, for females) of the applied compound equivalents. The radioactivity was eliminated within 24 hours after treatment. In the expired air practically no radioactivity has been observed (0.012 % of applied dose in males; 0.060 % in females). The same study with radiolabelled compound formulated in a cream (2 %) with developer and hydrogen peroxide when applied on the intact clipped skin (10 cm^2, ca. 200 µg of compound/cm^2) for 30 min., gave the following results for cutaneous absorption after 48 hours:

1.32 % (2.61 µg/cm^2, males) and 1.2 % (2.37 µg/cm^2, females). The radioactivity was excreted mostly in the urine in the first 24 hours after application.

Organ distribution: ^{14}C-1,7-dihydroxynaphthalene has been orally administered to 5 male Wistar rats at a single dose of 10 mg/kg b.w. for evaluating the organ distribution and retention of the test compound 30 min, 1, 2, 6, and 24 hours after treatment by whole body autoradiography. The results after 30 min revealed that the stomach, the small intestine, the bladder and the kidney were labelled intensively, while the other organ showed lower radioactivity values. The radioactivity decreased rapidly and after 6 hours only the eyes and the caecum were marked. At the end of the study practically no retention of radioactivity (as test compound or metabolites) has been found in any organ.

Excretion: ^{14}C-1,7-dihydroxynaphthalene has been subcutaneously applied to 5 male Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expired air and in the carcass has been evaluated after 144 hours observation period. These results have been obtained as percent of the administered radioactivity: 79.6 % (24 h, urine); 84.2 % (144 h, urine), 6.4 % (144 h, feaces); 0.50 % (144 h, carcass); 0.29 % (expired air). Examination of the urine by Thin Layer Chromatography for radioactivity showed that the parent compound was nearly completely metabolized. At the end of the study 92.2 % of the administered radioactivity has been recovered.

^{14}C-1,7-dihydroxynaphthalene has been orally administered to 5 male Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expired air, carcass and gastrointestinal tract, has been evaluated after 96 hours observation period. A value of ca. 79.1 % of the administered dose was observed with the following excretion values being obtained (percent of the applied dose): 54.2 % (8 h, urine); 72.9 % (24 h, urine); 18.3 % (96 h, faeces); 0.25 % (carcass); 0.03 % (gastrointestinal tract); negligible (expired air). Investigation of the urine by Thin Layer Chromatography and examination for radioactivity showed that the parent compound was nearly completely metabolized. No information on metabolites was available.

8. Mutagenicity

The compound has been tested and found negative for gene mutation *in vitro* on *Salmonella typhimurium* 5 strains with and without metabolic activation and, for chromosome aberrations *in vivo* on mouse by the micronucleus assay (total dosages: 100-1000-2000 mg/kg by oral gavage; 2 equal doses separated by an interval of 24 h, analysis 6 h after the last dose).

11. Conclusions

The present subchronic oral toxicity study is not adequate for defining the No Effect Level. The SCC requires a 90 days repeated administration study.

Classification: B

A 7: *1,4-DIAMINOBENZENE*

1. General

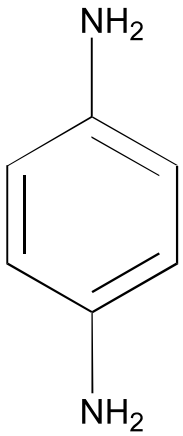
1.1 Primary name

1,4-diaminobenzene (para-Phenylenediamine)

1.2 Chemical names

1,4-diaminobenzene (para-Phenylenediamine)

1.5 Structural formula



1.7 Purity, composition and substance codes

No purity data were available.

1.8 Physical properties

Appearance: The compound is a white crystalline powder.

1.9 Solubility

It is slightly soluble in water and is soluble in various organic solvent; ethanol, ether, benzene, chloroform and acetone.

2. Function and uses

The compound is supplied as an oxidative hair dye at concentrations up to 4 % and used at a concentration of 50 % of that supplied after dilution with hydrogen peroxide.

The compound has been used since 1883 for dyeing hair and furs and there is a considerable body of literature on the toxicity dating back to the early years of this century. The compound was considered by the SCC in 1980 and found acceptable for use in cosmetic products. It is

currently in Annex III part 1 number 8 and is restricted to a maximum concentration of 6 % with certain warning on the label. The entry was last modified by 83/341/EEC.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

Acute toxicity has been investigated following oral, subcutaneous, intraperitoneal and topical application in a variety of species. The LD_{50} following oral administration was 80-100 mg/kg in the rat, 290 mg/kg in mice, 250 mg/kg in rabbit and 100 mg/kg in cats. The values following subcutaneous application were 170, 200 and 100 mg/kg for rat, rabbit and dog respectively.

The intraperitoneal and topical LD_{50} values have each only been determined in the rabbits respectively. A variety of toxic effects have been reported with some variation between species.

There are several reports of deliberate or accidental para-phenylenediamine poisoning in humans but no details of the amount ingested were available. The symptoms reported include oedema of the glottis and acute renal failure.

3.8 Subchronic dermal toxicity

A 90 day study has been carried out in the rabbit with the compound administered dermally twice weekly. Four hair-dye formulation containing 1, 2, 3 or 4 % of para-phenylenediamine and other hair-dye constituents were mixed with an equal volume of 6 % hydrogen peroxide. A dose of 1 ml/kg of this mixture was applied for 1 hour without occlusion to three application sites on six animals of each sex. The application sites were abraded prior to the first dose each week. No dose-related changes were observed on weight gain, clinical chemistry, haematology, urinalysis or on examination of the tissues at necropsy.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was mildly irritating when applied in a 2.5 % aqueous solution containing 0.05 % sodium sulphite to abraded rabbit skin under gauze for 24 hours. There was no reaction at intact skin sites in the same rabbits under identical conditions. There are several reports in the literature from the 1930s of oedema and dermatitis after the use of para-phenylenediamine containing shampoos.

4.2 Irritation (mucous membranes)

Eye irritation has been studied in the rabbit. A 2.5 % aqueous solution containing 0.05 % sodium sulphite was instilled into one eye of a group of albino rabbits and rinsed out after 10 seconds with destilled water. The result was considered negative with minimal conjunctival irritation, being seen in one animal at the one hour time point only.

5. Sensitization

The ability of p-phenylenediamine to induce skin sensitization has been investigated in an animal study. Twenty guinea-pigs had a 3 % formulation of para-phenylenediamine applied on six days per week for three weeks using the open epicutaneous method. Two weeks later a challenge dose was applied to the opposite flank. The challenged produced an inflammatory response in 17 of the 20 animals challenged. There have been many reports of sensitization to para-phenylenediamine in humans. A number of studies in patients have taken place. A study in Stockholm of 2903 eczema patients patch tested between 1958 and 1960 showed a total of 10.6 % reacted positively. This was somewhat higher than the 4.3 % recorded in 3287 patients from 1948-51 by the same workers although they noted a change in the test method might be responsible. The other two studies reported rates of 6 % in 543 patients and 5.8 % in 378 patients suffering from eczema. This was significantly larger in a group of 100 leg ulcer patients where the rate was 27 % although the author's suggest that topical treatment with a related substance may be responsible for the sensitization. The only data on the general population is from patch tests prior to hair-dyeing at the Clairol test room in New York over two periods January 1974 to August 1975 and October 1973 to July 1978, where 5/21597 (0.023 %) and 42/67268 (0.0624 %) subjects respectively where positive when challenged with para-phenylenediamine.

6.1 One-generation reproduction toxicity

Three studies on the toxicity to reproduction of para-phenylenediamine alone or in hair-dye formulations have been reported. In one study groups of 25 pregnant mice received a subcutaneous dose of 28 mg/kg para-phenylenediamine in aqueous solution on days 5 to 7, 8 to 10 or 11 to 14 of gestation. No treatment related differences were reported in either the dams or the fetuses. In a second study twelve hair-dye formulation, four of which contained para-phenylenediamine at 1, 2, 3 or 4 % were mixed with hydrogen peroxide prior to topical application at 2 ml/kg to 20 mated female rats on days 1, 4, 7, 10, 13, 16 and 19 of gestation. There was no statistically significant difference found between control and para-phenylenediamine treated dams and fetuses. The third study included application of 0.05 ml of a hair-dye formulation containing 3 % para-phenylenediamine mixed with an equivalent volume of hydrogen peroxide dermally twice per week to female mice from 4 weeks prior to mating and throughout mating and gestation. The initial group size was 50 and mating proceeded until at least 30 mice had vaginal plugs. There was no evidence of maternal toxicity nor of a teratogenic effect, however, there was a suggestion of a possible retarding effect on the ossification process.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption of para-phenylenediamine has been investigated in dogs *in vivo,* 1.5 g of the compound was applied to shaven skin and subsequently either occluded, left exposed to air or mixed with hydrogen peroxide and applied to an open site. An analysis for free para-phenylenediamine in blood produced absorption values of 110, 16 and < 2 mg respectively. This correspondends to 7, 1 and < 0.1 % of the dosed material respectively. A less specific assay applied to the latter, which also detected Bandrowski's base, indicated material was absorbed in the presence of hydrogen peroxide but did not quantify this material.

A significantly lower value was however obtained in studies in human volunteers using hair dye formulations under in use conditions. ^{14}C-radiolabelled para-phenylenediamine was used in this study and urinary excretion measured for 30 days after treatment with the hair dye. Absorbed compound was known to be eliminated mainly in the urine. A total of 0.19 % of the dosed material was eliminated in the first 24 hours, increasing to 0.31 % at 10 days and 0.34 % after 20 days; no significant elimination was seen over the next 20 days.

Additional information on the toxicokinetics of para-phenylenediamine, following oral and intravenous administration to rats and mice indicates extensive absorption of the compound from the gastrointestinal tract. The compound is excreted predominantly in urine mainly as metabolites. Biliary excretion shows a decrease with increasing dose from around 60 % at 6 µmol/kg to 20 % at 600 µmol/kg. There is a species variation in the metabolite profile and an apparent sex difference in mice but not in rats.

8. Mutagenicity

The data on mutagenicity is summarised in Table 1. The compound has produced positive results in studies to investigate the ability of para-phenylenediamine to produce gene mutation in *Salmonella typhimurium*. The positive reactions have been observed with strains TA98 and TA1538 in the presence of an exogenous metabolic activation system. Negative results were however reported in studies to investigate gene mutation in *Escherichia coli* and bacteriophage T4D. The compound has been shown to cause gene mutation in studies using the TK locus of mouse lymphoma L5178Y cells and human lymphoblast TK6 cells. There are also reports of the compound producing chromosomal aberrations and sister chromatid exchange in CHO cells. Negative results have been reported in studies using the mouse embryo C3H/10T1/2 cell transformation assay and in studies using to investigate unscheduled DNA synthesis in hepatocytes. The compound has produced both positive and negative results in the sex-linked recessive lethal test in *Drosophila melanogaster*; the positive result appeared to be associated with impurity or oxidation of an old sample whilst a second test with a new batch of compound was negative. Para-phenylenediamine has been investigated in both the micronucleous test in rats and the dominant lethal test in mice. The latter test was carried out twice with doses up to 20 mg/kg i.p. thrice weekly for 8 or 10 weeks to 20 male mice. The micronucleous test was performed in 5 CFY rats of each sex with a total oral dose of 300 mg/kg given in 2 equal doses 24 hours apart. Bone marrow cells were harvested 6 hours after the second dose. Negative results were obtained in both these tests. There was no covalent binding of para-phenylenediamine derived radioactivity to hepatic DNA after a single dose of 600 µmol/kg i.v. The limit of detection was 1 pmol equivalent/mg DNA.

9. Carcinogenicity

A number of studies to investigate the carcinogenicity of para-phenylenediamine have been performed. The early studies in the 1930s and 1940s were negative, however, all suffered deficiencies by current standards in both the number of animals used and duration of treatment. A study in mice involving weekly dermal administration of 1.5 % para-phenylenediamine in a hair-dye formulation to groups of 28 male and female rats produced no significant differences between treated and control animals. A study involving dermal application either weekly or fortnightly of three hair dye formulations containing 1.5 % para-phenylenediamine after

mixing with an equal volume of hydrogen peroxide to 50 mice of each sex for 18 months has been reported. There were no significant differences between dosed and control animals. A second study involving hair dye formulations mixed with hydrogen peroxide prior to dermal application also produced no significant differences between control and treated animals. The formulation contained 1, 2, 3 or 4 % para-phenylenediamine and was administered weekly to 50 mice per sex for two years. Apart from some discolouration of the skin at the application site no significant differences were observed between dosed and control animals. A two year oral study in rats and mice (50 per sex) with administration of 625 or 1250 ppm in the diet has been reported. A decrease in body weight at the higher dose was noted in all groups except the male mice. There were no significant differences observed in tumour incidence between treated and control animals. There are two recent reports from Thailand of carcinogenicity assays with 4 hair-dye formulations containing para-phenylenediamine and with 5 % oxidised para-phenylenediamine following subcutaneous or topical application. These are inadequate for assessment due to deficiencies in group size and duration.

11. Conclusions

Para-phenylenediamine has moderate acute toxicity by the oral route and low toxicity by the dermal route. A 2.5 % solution has no significant skin or eye irritant properties. There was evidence of skin sensitization in both animal and human studies. The results of studies in patients indicate 6-11 % are sensitised to para-phenylenediamine whilst patch testing on the general population prior to hair dyeing over a 5 year period indicated less than 0.1 % give positive results. In a 90 day dermal study no effects were reported with hair dye formulations containing up to 5 % of the compound. Para-phenylenediamine has clearly been shown to have mutagenic potential *in vitro*, with positive results in assays for gene mutation in Salmonella and mammalian cells (mouse lymphoma assay) and also for clastogenicity in mammalian cells and UDS in hepatocytes. This activity does not appear to be expressed *in vivo* with negative results in a bone marrow assay for clastogenicity (micronucleus test), binding to DNA in liver and two dominant lethal assays. The *in vitro* activity may be related to formation of the Bandrowski's base which is very unstable. There have been no compound related effects reported during well conducted chronic studies of hair-dye formulations containing the compound by the dermal route or following oral administration of para-phenylenediamine. No compound related effects were reported in reproduction toxicity studies with either the compound alone or in hair-dye formulations. The compound is absorbed through the skin to a significant extent under occlussive dressing in the absence of oxidation (7 %); oxidation with hydrogen peroxide decreases the absorption of the compound to almost negligible levels (< 0.1 %). This is supported by studies on absorption in humans using in use conditions and radiolabelled material, only 0.34 % of the applied dose was absorbed over a 30 day period. The compound is predominantly excreted in urine and is extensively metabolised.

Classification: A.

Subject to restrictions on concentration and labelling already in force namely 6 % as the free base and 'can cause an allergic reaction.
Do not use to dye eyelashes or eyebrows.'

ANNEX

TABLE 1.

MUTAGENICITY DATA PUBLISHED FOR para-PHENYLENEDIAMINE

Test system	Dose/concentration	Activation	Result	Notes
TA 1538	Not given	+/-	+?	In DMSO
TA 98	Not given	+/-	Weak +	
TA 1538,	Hair-dye	+/-	+	Only with H_2O_2
TA 1535, 1537	formulations	-		
TA 1538	Not given	+/-	+	Increase with
TA 1535			-	H_2O_2
TA 1538, 98	Urine and aqueous solution	+/-	-	$\pm H_2O_2$ ±non mutagenic couplers
	Aged DMSO solution		+	
	Bandrowski's base		+	
TA 100, 1535 1537	5-1000 µg	+/-	-	No dose response
TA 1538, 98			+	
TA 98, 1538	25-250 µg/plate	+	-	Water or fresh DMSO solution
			+	Aged DMSO solution
TA 98	200 µg/ml	+	-	No light exposure
			+	Light exposure
TA 1535, 98, 100, 1538	5-250 µg/plate	+	-	PB induced S9
				Aroclor induced S9
TA 98, 100	0.1-10,000 µg/plate	+/-	-	
TA 98	0.25-2 mg/plate	+/-	-	Pure
			+	Commercial grade
	0-1 mg/plate	+/-	+	+Resorcinol
	0-200 µl urine/plate	+/-	+	From 300 mg topical
*E. coli*WP2 WP 100				
TA 98	DMSO sol of components	+/-	+	
TA 98	0-1000 µg/plate	+/-	+	Top dose only
	0-25 µg/plate			
	30 min with H_2O_2		+	

Test system	Dose/concentration	Activation	Result	Notes
TA 98	10 µg/plate after H_2O_2	+/-	-	
	10 µg of precipiate		+	
	10 µg of AcOEt extract		+	
TA 97, 98, 100	a) aqueous mixture with resorcinol and H_2O_2	+/-	-	
	b) Same aqueous mixture without resorcinol 0-5.5µmol/plate	+		
TA 98	Brandowski's base 0-0.0621 µmoles/plate	+		
Mouse lymphoma	a) 0-0.552 µmole/plate	+		
L5178Y cells	b)	-		
Human	a)	+		
lymphocytes	b)	-		
CHO-K cell SCE	a and b		+	Only at cytotoxic concentration no
Chromosome aberrations	a and b		+	dose response
E.coli 343/113	0-1000 µg/ml	+/-	-	
E.coli 343/120	-1000 µg/ml	+/-	-	
Bacteriophage T40	4.7-190.4 µg/ml	-	-	
L5178Y	0-6.5 µg/ml	-	+	Weakly positive
mouse lymphoma cells	0-300 µg/ml	+	+	Initial studies equivocal
C3H/10T1/2	0.8-20 µg/ml	-	-	
mouse embryo cells	0.5-5 µg/ml	-	-	
Hepatocyte UDS	0.5-1000mol/ml			
TA 1537, 1537 TA 100, G46 C3976, D3952 *E.coli* WP2 (P2 A-	-1000 µg/ml	+/-	-	
TA 1538, 98			+	

Test system	**Dose/concentration**	**Activation**	**Result**	**Notes**
Drosophila sex-linked	2.5-15.5 mM (feeding)		-	
Recessive lethal	2.5-10 mM (injection)		+	Old compound discoloured
Hepatocytes	0.01-0.1 mg/ml		-	Top dose toxic
UDS	0.001-0.05 mg/ml		-	
Dominant lethal in mice	20 mg/kg i.p.		-	3 weeks, 3 x/week 20 males
	2, 6, 20 mg/kg			10 weeks
Micronucleous test in rats	300 mg/kg p.o. in doses 24 h 5 males and females		-	harvest 6th after final dose

CALCULATION OF SAFETY MARGIN

para-PHENYLENEDIAMINE
A7

Oxidation or Permanent

Based on a usage volume of 100 ml, containing at maximum 2 %

Maximum amount of ingredient applied:	**I (mg)= 2000 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 0.34 % (human)**
Dermal absorption per treatment:	**I (mg) x A (%)= 2000 x 0.34/100 = 6.8 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg = 6.8 mg/60 kg b.w. = 0.113 mg/kg b.w.**
No observed adverse effect level (mg/kg):	**NOAEL = 30 mg/kg b.w. (rats: long-term toxicity dermal application) 28 mg/kg b.w.s.c.(mice: teratogenicity)**
Margin of Safety:	**NOAEL / SED = 28 mg/kg b.w./0.133 = 210**

A 8: *2-CHLORO-P-PHENYLENEDIAMINE*

1. General

1.1 Primary name

2-chloro-p-phenylenediamine

1.2 Chemical names

2-chloro-p-phenylenediamine
1,4-diamino-2-chlorobenzene
2-chloro-p-phenylenediamine
ortho-chloro-phenylenediamine

1.3 Trade names and abbreviations

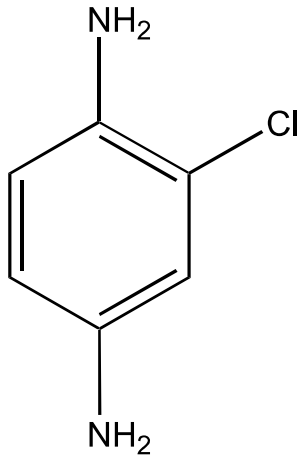
Ursol Brown O

1.4 CAS no.

615-66-7

CI 76065

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6 H_7 N_2 Cl$
Mol weight: 240.7

1.7 Purity, composition and substance codes

No purity data were provided.

2. Function and uses

Hair dye; max. use 2.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rats, oral: 1190 (1070-1320) mg/kg b.w.
Rats, oral: 729 mg/kg b.w.

3.7 Subchronic oral toxicity

2-chloro-p-phenylenediamine sulphate was administered in the diet at two concentrations to six groups of F344 rats and B6C3F1 mice, 5 female and 5 male each to determine the concentration to be used in chronic studies.

The dietary concentrations used were 0.03, 0.1, 0.3, 1.0 and 3.0 percent; the sixth group of each species served as control and the dosed dietary preparations were administered for 8 weeks.

At dose of 1 % all the male and 1 female rat died; at the dose of 0.3 % no mortality occurred in rats and mean body weight depression were 10.4 % and 6.4 % in male and female rats. At the dietary concentration of 1.0 % 1 male mouse died, body weight depression was 3.8 % and 14.4 % for male and female respectively. The dose of 0.3 % induced no mortality and produced body weight depression of 16.1 % in female mice and no changes in male mice. No data are reported for 3.0 % dosed animals. The NOAEL for rats was 0.3 % in the diet; the NOAEL for mice was 0.1 % in the diet.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound (2.5 % aqueous solution in distilled water with 0.05 % sodium sulphite, pH=7) did not produce any signs of irritation when applied to intact and abraded site on the clipped skin of 3 rabbits after 72 h of observation period.

4.2 Irritation (mucous membranes)

The compound (2.5 % aqueous solution in distilled water with 0.05 % sodium sulphite, pH=7) instilled into one eye of 3 rabbits produced no signs of irritation after 1, 2, 3, 4 and 7 days of observation period; in this study the treated eye was irrigated with distilled water 10 sec. after treatment.

NIOSH reported that the compound (24 mg/24 h) was a ”severe eye irritant“ (species not indicated).

5. Sensitization

The compound (3 % in aqueous solution) intradermally applied for the induction on 15 ”Pirbright white“ female guinea pigs showed moderate reaction in 10/15 animals (Classification: Grade III). The test was performed according to Magnusson & Kligman’s method.

6. Teratogenicity

Embryotoxicity: o-chloro-p-phenylenediamine was administered by gavage to Sprague-Dawley rats on days 6-15 of gestation at dose levels of 100, 200 and 400 mg/kg: a statistically significant difference between control and high dose treated rats was found in the number of resorptions; the weights of both male and female foetuses from high dosed treated rats were significantly less than those in the control. The dose of 200 mg/kg was the No Effect Level for the embryotoxcity.

8. Mutagenicity

The compound has been able to induce reverse mutations in *Salmonella* in the presence of metabolic activation without and with 6 % hydrogen peroxide (ratio S9 mix/H_2O_2 = 1:1). The compound did not induce gene mutation in 5 loci on *E. coli* (343-313 strain) by fluid test. The compound did not induce micronuclei in mice treated orally with a total dose of 1800 mg/kg in two equal doses separated by an interval of 24 hours (analysis 6 hours after the second dose).

9. Carcinogenicity

Long term studies were carried out on mice and rats: the compound (as sulphate) was administered at two doses, 0.15 % and 0.30 % respectively, in the diet for 105-107 weeks to 50 Fischer 344 rats/sex/group and 87 weeks; the compound was administered at the two doses 0.6 % and 0.3 % in the diet for 104-105 weeks low dose to 50 B6C3F1 mice/sex group; 20 animals species/sex were used as control group. After the end of treatment the high dosed mice group was observed for further 18 weeks. Survival rates for male rats were 80 % (40/50) at high dose, 94 % (47/50) at low dose and 90 % (18/20) in the control group; 94 % (47/50) of the female rats at high dose, 86 % (43/50) at low dose, and 80 % (16/20) of the control survived until the end of the study. Survival rates for male mice were 74 % (37/50) high dose, 90 % (45/50) low dose and 75 % (15/20) in the control group; in the female mice, 50 % (25/50) of the high dosed group, 80 % (40/50) of the low dose, and 80 % (16/20) of the control group survived until the end of the study.

In female and male rats an increase in the incidence of transitional-cell hyperplasia of the renal pelvic epithelium (males: 17/49, low dose; 30/50, high dose; females: 14/48, low dose; 8/49, high dose; control 0/20 for both sexes) and transitional-cell tumours of the urinary bladder in 3 rats (in 2 rats at low dose: 1/47 carcinoma and 1/48 papilloma, and in 1/48 rats (carcinoma) at high dose) were observed; historical control for bladder tumour: 0/250 males; 1/249 females.

Three different statistical methods of analysis of the results (Tarone test, Cochram-Armitage test, Fisher exact test) produced contrasting conclusions.

There is no clear evidence of carcinogenicity in rats and mice. It was noted that the control group size was only 20 animals/sex.

11. Conclusions

The SCC requires a percutaneous absorption study, a 90 days repeated oral administration study and a study to determine the induction of UDS or DNA damage in the liver of rats treated *in vivo*.

Classification: C

A 10: *CATECHOL*

1. General

1.1 Primary name

Catechol

1.2 Chemical names

ortho-benzenediol
1,2-benzenediol
Catechin
Catechol
1,2-dihydroxybenzene
ortho-dihydroxybenzene
ortho-dioxybenzene
ortho- hydroquinone
2-hydroxyphenol
ortho-hydroxyphenol
Oxyphenic acid
ortho-phenylenediol
Pyrocatechin
Pyrocatechol

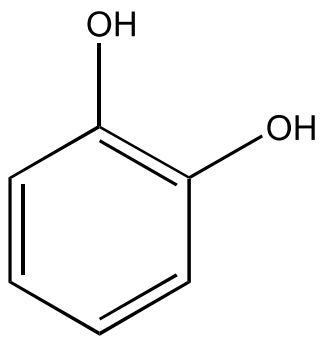
1.3 Trade names and abbreviations

CI Oxidation Base 26

1.4 CAS no.

120-80-9
CI: 76500

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6 H_6 O_2$
Mol weight: 110.11

2. Function and uses

Oxidative hair dye: max. use 3 %; 1.5 % in combination with hydrogen peroxide.

Eyebrow and eyelash tinting with silver nitrate solution (3.8 %): 2 % (ca. 1 ml of the solution for one application).

TOXICOLOGICAL CHARACTERIZATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:

Rats, oral	200 (170-240) mg/kg b.w.
Rats, oral	320 mg/kg b.w. (Kärber's method)
	330 mg/kg b.w. (Litchfield and Wilcoxon's method)
	280 mg/kg b.w. (Weil's method)
Rats, oral	3900 mg/kg b.w. (IARC)
Rats, oral	3890 mg/kg b.w.
Rats, inhalation	2800 mg/m^3
(8 h. exposure: several toxic signs;	1500 mg/m^3 : no toxic effects)
Mice, oral	260 mg/kg
Mice, intraperitoneal	190 mg/kg
Mice, subcutaneous	250 mg/kg b.w
Mice, subcutaneous	179 mg/kg b.w.(IARC)
Mice, subcutaneous	247 mg/kg
Rabbit, epicutaneous	800 (500-1400) mg/kg b.w

LD_0:

Dogs, oral	130 mg/kg
Dogs, intravenous	40 mg/kg
Cats, oral	100 mg/kg
Rabbits, oral	1000 mg/kg
Rabbits, cutaneous	800 mg/kg
Guinea pigs, intraperitoneal	150 mg/kg
Guinea pigs, subcutaneous	200 mg/kg
Frog, subcutaneous	160 mg/kg

LC_{50}:

Rainbow trout: *Salmo gairdneri*	8,9 mg/l
Fathead minnow: *Pimephales promelas*	3,5 mg/l

3.7 Subchronic oral toxicity

The compound has been given orally (per os) to 20 male and 20 female SPF-Wistar rats/group at the doses of 40 - 80 - 150 (reduced 100 mg/kg at 5th wk) - 200 (reduced at 150 mg/kg at 5th wk) mg/kg (1 ml/100 g, in distilled water) for three months. The control group (30 males and 30 females) received only the vehicle. The following results have been obtained: 80 mg/kg: significant increase in SGPT and slight reduction (within normal values) in haemoglobin in females; 150 mg/kg: slight but significant increase in SGPT in females (6th wk); significant reduction in haemoglobin (females, 13th wk, indication for a trend of hypocromic anaemia) and body weight gain (males, 1-6 wks); some clinical effects; 200 mg: significant increase in MCV (mean cell volume of the erythrocytes, indicative of hypochromic anaemia) and in SGPT (serum glutamate pyruvate transaminase) in females (6th wk); some clinical effects and reduction (1-6 wk) and increase (7-13) in body weight gain in males. All the adverse effects observed at the highest test dose (200 mg/kg/day x 3 mo.) were reversible 5 weeks after the end of the treatment. No significant histopatological alterations were observed at all doses. The no effect level in this study was 40 mg/kg/ b.w. day.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound (2.5 % aqueous solution in distilled water with 0.05 % sodium sulphite, pH=7.0) applied on intact and abraded clipped skin (dorsum) of 3 rabbits produced minor, transient signs of irritation namely grade: 1 oedema after 24 h. at the abraded site of one rabbit that disappeared after 72 h. (primary irritation index= 0.1).

The compound (0.5 g) has been applied to intact and abraded skin (belly) of albino rabbits for 24 h. The following results have been obtained; at 24 hours: slight to moderate erythema and slight edema (intact areas); necrosis (abraded skin); at 72 h the irritation to the intact skin was reduced. No irritation was observed at 14 days, except for slight epidermal flaking and that the necrotic areas were incrusted and beginning to slough. A value of 5.5 has been calculated for the primary irritation index.

Human skin irritation: Catechol causes dermatitis when in contact with the skin. Its absorption through the skin may give rise to symptoms similar to those seen in phenol poisoning.

4.2 Irritation (mucous membranes)

The compound (2.5% aqueous solution in distilled water with 0.05 % sodium sulphite) instilled into one rabbit's eye which was irrigated with water after 10 sec. of instillation, gave a negative results after 1-2-3-4-5-6-7 days observation period.

The catechol (0.1 g) instilled into one eye of male albino rabbits produced severe irritation after 24-48-72 h. with following scores being obtained: 103 (24 h), 85 (48h) and 78 (72 h). A score of 110 (extremely irritating) was the maximum obtainable for each time.

5. Sensitization

Guinea pigs (14 females) were treated with a solution of 3 % in distilled water with 10 % isopropanol, 0.05 % sodium sulphite, 2 % Tween 80 and 2 % Natrosol 250 H2: the method used was open epicutaneous test (OET) without Freund's Adjuvant (2 weeks, 5 days/week, treatment and 2 weeks challenge reaction). The result indicated a moderate sensitizing effect.

7. Toxicokinetics (incl. Percutaneous Absorption)

Metabolic studies: The compound was absorbed from the gastrointestinal tract and possibly through the skin. Chickens and dogs treated with ^{3}H-catechol into the renal artery showed in the urine free catechol and its glucuronide and sulphate conjugates.

8. Mutagenicity

The compound has been tested for gene mutations and found negative in the *E.coli* (5 loci, fluid test) and *Salmonella* (adequate tests performed : TA100, TA1535, TA1537, TA98 ± metabolic activation from rat and hamster livers).

Negative results have been obtained for the *Salmonella* assay performed with and without S9mix.

Negative results were also obtained for 3 unpublished studies using *Salmonella* namely spot test and plate test: TA1535, TA1537; TA1538 ±S9mix; plate test: TA 1535, TA1538 ±S9mix; plate test: TA1535, TA1538 ± S9mix, with inadequate overall evaluation, because these assays did not include TA98 and TA100 strains.

In a micronucleus tests performed by oral administrations (2 equal doses separated by an interval of 24h) on rats (total dose = 360 mg/kg) and mice (total doses = 25-50-100 mg/kg, analysis 6 h. after the second dose), negative results were obtained.

Negative results were also obtained in mice treated by subcutaneous injections 6 days with 5-42 mg/kg. However catechol when administered p.o. or i.p. to Swiss CD-1 mice at a single dose of 40 mg/kg b.w. induced a significant increase in micronuclei ($P<0.05$-0.01 respectively) in bone marrow cells only when the analyses are performed 24 h after the treatment, but not after 18 h, 48 h and 72 h of treatment.

Further experiments have confirmed these *in vivo* mutagenic effects of catechol.

A study to investigate the mechanism of induction of micronuclei has demonstrated that they are the results of aneuploidy.

Catechol induced chromosome aberrations or karyotypic effects in *Allium cepa*, in CHO cells grown *in vitro,* and SCE in human lymphocytes treated *in vitro.*

9. Carcinogenicity

Carcinogenicity: ***Tumour promoting action*** of the compound has been tested in albino mice. 30 mice received a single application of a 0.3 % solution (25 µ) of 9,10-dimethyl-1,2-benzanthracene (DMBA, initiator agent) in benzene and 20 mice the solvent only. A 15 % solution (ca. 25 µ) of 1,2-dihydroxybenzene in benzene (potential promoter agent) was then

applied (single drop, 2 times/week for 15 wks) 1 week after the application of DMBA to the back of each mice. Negative results were obtained: 14 % survivors with typical papillomas (>1 mm of diameter) vs. 13 % in the control and 0 % survivors with epithelial carcinoma vs. 0% in the control.

Cocarcinogenic action. The compound has been tested in mice for its cocarcinogenic effect in combination with benzo(a)pyrene. The mice (50 females ICR/Ha Swiss per group) received, on the back skin, the test compound (2 mg/0.1 ml acetone per application) alone, benzo(a)pyrene alone (5 µg/0.1 ml acetone per application) or test compound with benzo(a)pyrene (5 µg/0.1 ml acetone per application) 3 times a week for 368 days. A group of 100 mice remained untreated. The results showed that the compound enhanced the carcinogenicity of benzo(a)pyrene: 90 papillomas on 36 mice and 31 mice with squamous carcinoma (catechol + B[a]P); 16 papilloma on 14 mice and 10 mice with squamous carcinoma (benzo(a)pyrene alone); 1 papilloma on 1 mice and 1 mice with squamous carcinoma (catechol alone); no tumours (untreated group). (11). IARC reported that a previous (1973) similar study (2 mg catechol/0.1 ml acetone and 5 µg benzo(a)pyrene/0.1 ml acetone or 5 µg benzo(a)pyrene alone, skin painting on ICR/Ha Swiss mice, 3 times a week for 52 weeks) performed by the same authors gave the same results: 86 skin papilloma in 35 mice and squamous-cell carcinoma in 31 mice (namely - catechol +B[a]P); 14 papillomas/13 mice and squamous-cell carcinoma of the skin in 10 mice; controls (untreated and acetone alone): no tumours.

The comparison between tumour-promoting activity (no promoter agent) and cocarcinogenic activity (strong cocarcinogen agent) of catechol did not show direct correlation for these type of carcinogenic effects.

No information is available to assess the carcinogenic effects of catechol itself.

In bladder implantation studies (20% catechol in 10 mg cholesterol pellets) on mice (19 survivors), catechol did not increase the incidence of bladder carcinomas in mice after 25 wks.: 1 mouse with papilloma and 3 mice with carcinoma (P=0.03) vs. 4/77 mice with adenomas or papillomas and 5/77 mice with carcinomas of the bladder (cholesterol alone).

11. Conclusions

The compound has been demonstrated to induce micronuclei *in vivo,* due to a chromosomal non disjunction process and to be a co-carcinogen.

The compound is a known *in vivo* metabolite of the human carcinogen benzene.

The SCC considers that this compound may pose a health risk in the common use.

Classification: D

A 13: *PYROGALLIC ACID*

1. General

1.1 Primary name

Pyrogallic acid

1.2 Chemical names

1,2,3-trihydroxybenzene
1,2,3-benzenetriol
Pyrogallol
Pyrogallic acid

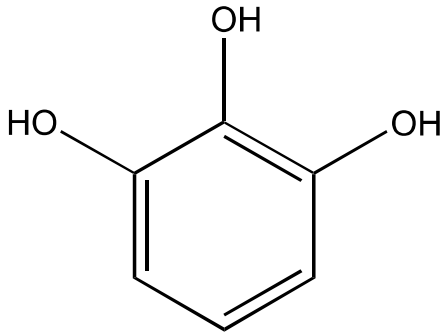
1.3 Trade names and abbreviations

Oxidation base 32

1.4 CAS no.

87-66-1
CI: 76515

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6 H_6 O_3$
Mol weight: 126

2. Function and uses

Oxidative hair dye; max. use: 2 %; 1.0 % upon application.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rats, oral 1800 (1420-2290) mg/kg

3.8 Subchronic dermal toxicity

The compound was applied as formulation (0.4 %), mixed 1:1 with hydrogen peroxide (6 %) by topical application for 13 weeks (twice weekly) on abraded and intact skin of rabbits. No signs of toxicity were reported.

3.10 Chronic toxicity

Chronic toxicity and carcinogenicity: (Dermal application). An oxidative formulation (P-21, mixed 1:1 with 6 % hydrogen peroxide) containing 0.4 % Pyrogallol tested on Swiss Webster mouse by dermal application (0.05 ml/cm^2, once weekly for 21-23 months). No signs of toxicity were reported.

The compound containing formulation (P-21, 0.4 %, 1:1 with 6 % hydrogen peroxide) was tested on Charles River rats (F0 generation) from the time of weaning to the weaning of their young (840 rats from the first mating F1a generation from multigeneration study) by dermal topical applications (0.2 ml increased by 0.1 ml to 0.5 ml, 2 times/week x 2 years). These observations were made: signs of toxicity and mortality (daily); detailed observation (weekly); individual body weights (weekly for the first 14 weeks and monthly thereafter); sex group food consumptions (weekly); and hematological, biochemical and urine analysis (3, 12, 18 and 24 months). Slight to marked deviations were observed for single rats at 21 and 24 months in several haematological parameters, and during the study in one or more biochemical parameters. The incidences of hyperkeratosis and dermatitis seen at variety of sites was considerably higher in the skin of treated group than in control. Further, at necropsy, the treated rats had slightly higher incidence of several different skin lesions from various location than the controls. These microscopic and gross skin changes were considered to be possibly compound related. No differences have been observed between treated and control groups for tumours incidences. (Not available the tables with the results and the statistical analysis of the data).

Pyrogallol topically applied on the dorsal skin, between the flank, of the female Swiss mice (0.02 ml, 5%-25%-50%, 50 mice/dose), twice a week for the life span (up to 120 weeks) showed negative results.

The compound applied to the interior left ear of the New Zealand rabbits (0.02 ml, 5-25-50% in acetone), twice a week for 180 weeks, did not show any skin tumours.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was applied to abraded and intact skin of six albino rabbits (0.5 g) for 24 hours using the patch-test. The compound produced mild irritation. The primary irritation index was 0.5.

4.2 Irritation (mucous membranes)

The compound has been tested as powder (100 mg) and as a solution in propylene glycol (1.0 % w/v) in 6 male New Zealand white rabbits. The compound was instilled (100 mg of dye

or 0.1 ml 1.0 % w/v solution) into one rabbit's eye and the reaction evaluated at 0, 30, 60 min and 1 and 2 days or as long as the damage lasted, after treatment. These reactions, according to Draize, were recorded: density, area of corneal opacity, iris irritation, conjuctivae redness, lid swelling and discharge. The compound alone resulted in a positive reaction in 6/6 rabbits after 4 days (positive scores were observed for all types of reactions). The dye as 1.0 % solution in propylene glycol showed negative results. No signs of irritation were seen with the 1 % solution.

5. Sensitization

The compound has been examined using the Magnusson Kligman in guinea pigs method. Induction was made by three simultaneously pairs of intradermal injection (0.05 ml) of 1 % test compound in propylene glycol, Freund's complete adjuvant 1:1 in water, and 1:1 mixture of the above solution in a shaved interscapular area (4 x 6 cm^2). One week later 25 % (w/v) in propylene glycol of the sample was topically applied, under an occlusive patch, on the same area for 48 hours. Two weeks later the animals were challenged by topical applications of the compound (25.0 %) under occlusion for 24 hours on the shaved flank. A re-challenge was made at 5 % doses of the sample. Reaction were evaluated at 24, 48 and 72 hours after treatments. There was no evidence of skin sensitization.

6. Teratogenicity

The compound (0.4 %) as a formulation (1:1 with hydrogen peroxide) was applied (2 mg/kg/day) to the shaven skin at the dorso-scapular area, on 20 mated Charles River CD female rats on days 1-4-7-10-13-16-19 of gestation. The pregnant rats were killed on day 20 of gestation and the uteri were examined, corpora lutea of pregnancy counted, and the number, distribution, and location of live, dead, and resorbed fetuses recorded. All fetuses were examined for gross anomalies, sexed and weighed; one-third of fetuses from each litter were examined for visceral anomalies and the remaining fetuses for skeletal anomalies. No evidence of toxicity was obtained.

6.2 Two-generation reproduction toxicity

In a multigeneration study in rats a formulation containing 0.4 % of compound was applied dermally (0.5 ml/rat) twice a week during growth, gestation and lactation to weaning at the F1b, F2b and F3c litters of respective generations: negative results were obtained. The initial dosage level was 0.2 ml per application, which was increased by increments from 0.1 ml appl./week to 0.5 ml appl. No significant difference between treated (parental rats or pups) and control groups for general behaviour and appearance, body weight gain, survival and the fertility, gestation and viability were observed. The test group (F0 and F1 generation) showed skin reaction as mild scabbing, fissuring, atonia and leathery texture intermittently throughout the treatment period, while no skin effects were observed in control group. No treatment related gross or microscopic pathologic lesions were observed in any F1b parental rats or F3b weaning rats at necropsy; and no treatment related gross pathologic lesion in rats died during the study.

8. Mutagenicity

The compound showed these results for gene mutations on *Salmonella*: in one study on TA 98 strain (±S9mix; ±H_2O_2, doses up to 150 µg/plate) and in another study on TA1538 and TA98 (+S9mix, up to 100 µg/pl., 2 different samples on TA1538; 500 and 1000 µg/plate showed inhibition of bacteria) was found negative; in an abstract it has been reported that pyrogallol (0.1-15 (moles) was found mutagenic in TA100 (-S9mix), TA98 and on TA1537 (spot test).

Pyrogallol was found mutagenic on *Salmonella* in strain TA100 and TA98 (±S9mix) and in strain TA1537 without S9mix, on *Drosophila* (sex-linked recessive lethals) and on mice (induction of micronuclei) treated with 2x126 mg/kg, 2 x252 mg/kg, 2x504 mg/kg, and 2x757 mg/kg. Pyrogallol induced chromatid breaks and exchanges in CHO cells grown *in vitro,* with and without S9mix. Pyrogallol induced mitotic gene-conversion in D7 strain of *S.cerevisiae*.

9. Carcinogenicity

The compound has been tested in mice for its cocarcinogenic effect in combination with benzo(a)pyrene. The mice (50 females ICR/Ha Swiss per group) received, on the back skin, the test compound (5 mg/0.1 ml acetone per application) alone, benzo(a)pyrene alone 5 µg/0.1 ml acetone per application) or test compound with benzo(a)pyrene (5 µg/0.1 ml acetone per application) 3 times a week for 440 days. A group of 100 mice remained untreated. The results showed that the compound enhanced markedly the carcinogenicity of benzo(a)pyrene: 95 papillomas on 40 mice and 33 mice with squamous carcinoma (Pyrogallol + B[a]P); 26 papillomas on 16 mice and 12 mice with squamous carcinoma (benzo(a)pyrene alone); no tumours with Pyrogallol alone and for untreated groups.

11. Conclusions

The compound is mutagenic *in vitro* and *in vivo*, and has been shown to be a co-carcinogen.

The compound is a known *in vivo* metabolite of the human carcinogen benzene.

The SCC considers that this compound may pose a health risk in the common use.

Classification: D

A 17: *1-NAPHTHOL*

1. General

1.1 Primary name

1-naphthol

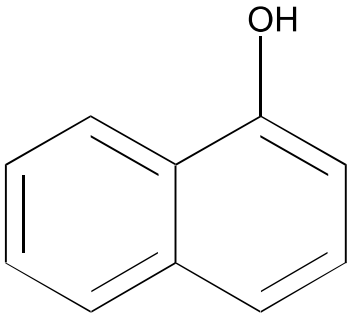
1.2 Chemical names

1-hydroxynaphthalene
1-naphathalenediol
1-naphthol
a-naphthol

1.4 CAS no.

90-15-3.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_{10} H_8 O
Mol weight:144.16

2. Function and uses

Oxidative hair dye; max. use 1.0 %; 0.5 % in combination with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	Rats, oral	2300 (1700-3300) mg/kg b.w.
	Rats, oral	2590 mg/kg

3.4 Repeated dose oral toxicity

In a 30 day repeated dose study in mice treated with 200, 100 and 50 mg/kg b.w. (five animals/sex/group; controls included undosed and solvent groups) gastric lesions related to the treatment were observed only at the dose of 200 mg/kg in male mice. No other signs of toxicity were observed.

3.7 Subchronic oral toxicity

1-naphthol orally administered to rats (20 males and 20 females) for 12 weeks (5 times a week) showed that the dose of 20 mg/kg b.w./day (10 ml/kg) does not represent a toxic cumulative dose.

3.8 Subchronic dermal toxicity

1-naphthol containing formulation (0.5 %), mixed 1:1 with hydrogen peroxide, by topical application for 13 weeks (twice weekly) on abraded and intact skin of rabbit showed no evidence from any toxic effect.

3.10 Chronic toxicity

One oxidative formulation (7403, mixed 1:1 with 6 % hydrogen peroxide) containing 0.5 % 1-naphthol was tested on Swiss Webster mouse by dermal application (0.05 ml/cm^2 x 21 months). No adverse effects were reported.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was applied to intact and abraded skin of rabbit at doses of 2.5 % (0.5 % aqueous gum tragacanth solution with 0.05 % sodium sulphite, pH=7) resulted not irritating after reading at 24 and 72 hours (primary irritation index=0). No signs of irritancy were noted.

4.2 Irritation (mucous membranes)

The compound has been instilled into one eye of 12 rabbits at concentrations of 0.5 % - 1.5 % - 2.0 % - 2.5 % w/v (0.5 % in aqueous gum tragacanth with 0.05 % sodium sulphite, 3 animal/doses) and the eyes were washed out 10 sec after treatment. The results (ocular reaction evaluated at 1 h and 1-2-3-4-7 days) showed the minimum irritant level between 1.5 % and 2.0 %: positive reactions were observed in 2/3 rabbits at 2.0 % w/v and 1/3 rabbits at 2.5 % w/v.

5. Sensitization

1-naphthol (3 % in water with 2.0 % Natrosol, 2 % Tween 80, 0.05 % sodium sulfite and 10 % isopropanole) showed no allergic reaction in guinea pig by open epicutaneous method.

Sensitization has been induced in 20 guinea pigs by simultaneously intradermal injections in the shoulder region of 0.1 ml of Freund´s Complete Adjuvant (FCA), 0.1 ml 1-naphthol (0.1 %

in water) and a 1:1 mixture of test compound and 0.05 ml Adjuvant at day 0. 7 days later the test compound was dermally applied (0.1 % in water) under occlusion on the injection site for 48 hours. 14 days later the guinea pigs were challenged by dermal application on the flank with 0.1 % and 0.05 % of 1-naphthol (aqueous solutions), under occlusion for 24 hours. The results evaluated after 24 and 48 hours of challenge showed that 1-naphthol was not a sensitizer in guinea pigs.

6. Teratogenicity

A formulation containing 1-naphthol (0.5 %, 1:1 with hydrogen peroxide) was topically applied (2 mg/kg/day) to the shaven skin of rats on day 1-4-7-10-13-16-19 of gestation. Only a significant reduction for the mean no. of corpora lutea has been observed between treated and two control groups (12.85 vs. 15.35 or 13.55). There was no evidence for any teratogenic or other adverse effects in the developing embryo/fetus.

7. Toxicokinetics (incl. Percutaneous Absorption)

Metabolism: 1-naphthol has been administered to 6 male and 6 female white rats (20 % w/v in corn oil, 0.67 ml/rat, total amount of the compound = 6.4 g) by injection under the skin of the back for 4 days after the feeding period. The urine analysis, after extraction and using chemical methods, showed the following data (in brackets the percent of 1-naphthol administered): p-toluidine 1-naphthylglucuronidate: 2.8 g (14.7 %), 2.0 g (15.2 %) and 3.2 g (16.8 %); p-bromoaniline 1-naphthylsulphate: 0.063 g (0.4 %), 0.087 g (0.5 %), 0.008 g (0.6 %).

These results showed that 1-naphtohl has been excreted in urine as 1-naphthylglucuronidate and 1-naphthylsulphate after subcutaneous injections. The study was performed in 1950.

Human absorption: 1-naphthol-(1-^{14}C) containing ointment (3 g, 50 % soft soap and 50 % white soft paraffin) has been applied in the inter-scapular region (10 cm, circular area) of the skin of 3 subjects, under occlusion for 8 hours. The percutaneous study showed a rapid and efficient absorption of the compound (3 days): 65.0-23.8-48.1 % (mean = 45.6 %) of the applied dose not recovered from the skin. The estimation of total urinary radioactivity has been only made in one subject: 88.55 % (day 1), 5.2 % (day 2) and 2.8 % (day 3) of the dose not recovered from the skin (ca. 97 %). The analysis of the major metabolites showed these results (percent of the dose not recovered from the skin): Subject 1: glucuronide fraction (day 1: 31.0 %; day 2: 1.0 %; day 3: 0.8 %), sulphate fraction (day 1: 1.3 %; day 2: 1.0 %; day 3: 1.2 %); acid hydrolysable fraction (day 1: 2.6 %; day 2: 0.2 %; day 3: 0.9 %); Subject 2: glucuronide fraction (day 1: 1.3 %; day 2:1.0 %; day 3: 1.2 %), sulphate fraction (day 1: 0.8 %; day 2: 0.0 %; day 3: 0.03 %); acid hydrolysable fraction (day 1: 0.26 %; day 2: 0.04%; day 3: 0.04 %); Subject 3: glucuronide fraction (day 1: 2.6 %; day 2: 0.3 %; day 3: 0.9 %), sulphate fraction (day 1: 0.0.8 %; day 2: 0.03%; day 3: 0.0 %); acid hydrolysable fraction (unmeasurable). In conclusion, the radiolabelled compound, when applied topically, under occlusion for 8 hours shows an absorption value of 45.6 %; ca. 97 % of the absorbed dose is found in the urine during 3 days analysis.

8. Mutagenicity

Mutagenicity/ Genotoxicity studies have demonstrated that 1-naphthol does not induce gene mutation *in vitro* in *Salmonella* and in mouse lymphoma L5178Y cells and *in vivo* on Drosophila (recessive lethals, Basc test); chromosome aberrations *in vivo* on bone marrow cells by micronucleus test on mice (2 x 144-288 mg/kg i.p. = 2 x 1-2 mmoles/kg; 2 doses with an interval of 24 h; analysis 30 h after second dose) and rats (2 x 3000 mg/kg intragastric intubation, 2 doses separated by an interval of 24 h, analysis 6 h after second dose); genotoxicity effects *in vitro* by DNA repair test on *E.coli* (3 strain) and *B.subtilis* (2 strain). Positive results were obtained for DNA repair test in one strain of *E.coli* (JC5547) using the spot test technique.

9. Carcinogenicity

See 3.10.

11. Conclusions

The SCC considers that the use of 1-naphthol in the oxidative hair dyes does not appear to present any health risk.

Classification: A

A 18: *1,5-DIHYDROXYNAPHTHALENE*

1. General

1.1 Primary name

1,5-dihydroxynaphthalene

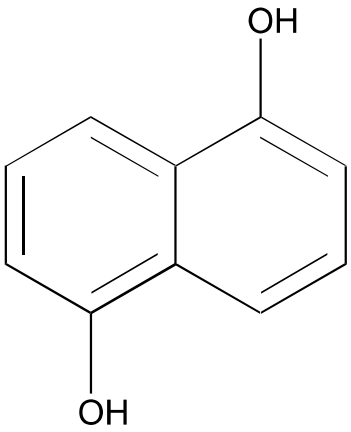
1.2 Chemical names

1,5-dihydroxynaphthalene
1,5-naphthalenediol
Ro 576

1.4 CAS no.

83-56-7

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_{10} H_8 O_2
Mol weight: 160.18

2. Function and uses

Oxidative hair dye; max. use: 1 %; 0.5 % with H_2O_2.

TOXICOLOGICAL CHARACTERIZATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Male mice, oral 680 (543-851) mg/kg.

3.7 Subchronic oral toxicity

The compound was administered to 20 male and 20 female rats (Wistar strain, MuRa Han 67 SPF) by oral gavage 5 times a week for 12 weeks at a single dose of 50 mg/kg b.w./day in water suspension: no adverse effects were reported. The dose of 50 mg/kg represents the NOAEL for 1,5-dihydroxynaphthalene after oral treatment of rats.

4. Irritation & corrosivity

4.1 Irritation (skin)

Application of 0.5 ml of a 10 % aqueous suspension (in 2 % carboxymethylcellulose, pH 9.0) to a clipped intact rabbit´s skin under occlusion for 4 hours. No signs of irritation were observed after 4, 24, 48 and 72 hours.

The compound as a 10 % (w/v) olive oil suspension, applied (2 droplets) to adult male hairless mice (strain hr hr) twice daily for 5 days to the same skin area showed no skin irritation.

4.2 Irritation (mucous membranes)

The compound as 5 % in carboxymethylcellulose solution (2 %, pH 9), instilled into one eye of each of six albino rabbits at doses of 0.1 ml (aqueous suspension) without rinsing off, resulted not irritating after 2, 6, 24, 48 and 72 hours.

5. Sensitization

In a study in female guinea pigs (20 females) induction doses consisted of simultaneous intradermal injections of 5 % (w/v) aqueous suspension of the test compound, 0.1 ml of Freund´s complete adjuvant (FCA) and a 1:1 (v/v) mixture of FCA and 5 % water suspension of the test substance on day 0. Seven days later 5 % (w/w in vaseline) of test substance was dermally applied, under occlusion, on the same area for 48 hours. On day 21 the guinea pigs were challenged by dermal application at a new skin side of a 25 % (w/w in vaseline), under occlusion for 24 hours. The results were evaluated after 24 and 48 hours. There was no evidence for any sensitization.

7. Toxicokinetics (incl. Percutaneous Absorption)

Cutaneous absorption: The ^{14}C-1,5-dihydroxynaphthalene (1,5-DHN, labelled at the C-1 of the naphthalene molecule, in a cream formulation) applied on 8 cm^2 intact and clipped skin of 7 male and 7 female Wistar rats (SPF-TNO) for 48 hours (1 % in formulation: 6.0 mg 14C-1,5-DHN, 54.7 mg 1,5-DHN, 422,1 mg distilled water and 173.0 mg ammonia conc., creme: 5.34 g; the formulation saturated the exposed air of the skin) showed these values of cutaneous resorption: 7.73 % (= 28.6 µg/cm^2, for males), and 9.49 % (= 25.7 µg/cm^2, for females) of the applied compound equivalents. The radioactivity was eliminated within 24 hours after application. In the expired air practically no radioactivity has been observed (0.026 % males; 0.065-0.072 % females).

The same study with radiolabelled compound contained in a cream (ca. 1 %) with developer and hydrogen peroxide when applied on 8 cm^2 of the intact clipped skin for 30 min., showed

after 48 hours, these results of cutaneous resorption: 0.486 % (1.02 µg/cm^2, males) and 0.981 % (2.09 µg/cm^2, females). The radioactivity was excreted mostly with the urine in the first 24 hours after application. In the expired air these values have been revealed: 0.293 µg/cm^2 (males) and 0.358 µg/cm^2 (females).

Organ distribution and placental transfer: ^{14}C-1,5-dihydroxynaphthalene has been administered to 5 pregnant and 1 non-pregnant Wistar rats by tail vein injection at a single dose of 15 mg/kg b.w. (at 19 days of gestation) for evaluating by whole body autoradiography, the organ distribution and placental transfer of test compound 30 min, 1, 2, 6 and 24 hours after treatment. Significant amounts of radioactivity have been revealed in the small intestine and kidney 30 min after application. The blood, the lungs and the placenta resulted distinctly labelled. The placenta barrier protected the fetal tissues as confirmed by the autoradiographic analysis in punched out portion. The radioactivity in the placenta and in the fetuses decreased in the further course of the study. A temporary labelling of the bones and the eyes in the maternal body has been observed 6 hours after application. No selective retention in the fetal organs has been observed. Low retention of radioactivity have been revealed in mammary tissue 24 hours after treatment. No difference in the distribution of radioactivity has been observed between pregnant and non pregnant rats (1 hour after treatment). The excretion was very rapidly with the urine (1 hour: 46.6 %; 24 h: 81 %); in the feces 12.1 % of the dose has been excreted after 24 hours.

Excretion: ^{14}C-1,5-dihydroxynaphthalene has been subcutaneously applied to 6 male and 6 female Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expiered air and in the carcass has been evaluated after 8, 24, 48 and 72 hours observation period. These results have been obtained as percent of the administered radioactivity after 3 days (main values): 84.1 % (72 h. males, urine); 78 % (72 h, female, urine); 8.42 % (72 h, males, faeces); 8.07 % (72 h, females, faeces); 0.292 % (expired air, male); 0.123 % (expired air, female); >1 % (carcass). Radio-Thin layer chromatography study of the urine showed that the parent compound was completely metabolized. At the end of the study 95.8 % of the administered radioactivity has been recovered.

^{14}C-1,5-dihydroxynaphthalene has been orally administered to male and female Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expired air, carcass and gastrointestinal tract, has been evaluated after 72 hours observation period. A value of ca. 94.6 % of the administered dose has been found for the intestinal absorption. Within 8 hours in the urine have been excreted 59.5 % (males) and 65.1 % (females) of the applied dose. These results for excretion have been obtained (percent of the applied dose, 72 h): 86.5 % (urine, males); 83 % (urine, females); 5.57 % (faeces, males); 6.83 % (faeces, females); 0.061 % (carcass, males); 0.106 % (carcass, females); 0.027 % (gastrointestinal tract, males); 0.036 % (gastrointestinal tract, females); 0.025 % (liver, males); 0.016 % (kidney, males); 0.0086 % (blood, males); 0.0069 % (plasma, males); 0.021 % (liver, females); 0.010 % (kidney, females); 0.006 % (blood, females); 0.005 % (plasma, females); negligible (expired air).

8. Mutagenicity

The compound has been tested for gene mutation and found negative in the *Salmonella* assay. In the micronucleus test performed by oral gavage on mice (2 equal doses separated by an

interval of 24 h, 10 ml/kg) at doses of 2x75-150-300 mg/kg b.w. negative results have been obtained.

11. Conclusions

The SCC requires a study on the chromosome aberration on mammalian cells grown *in vitro*.

Classification: B

A 25: *6-HYDROXYBENZOMORPHOLINE*

1. General

1.1 Primary name

6-hydroxybenzomorpholine

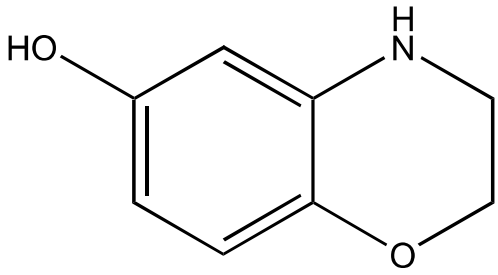
1.2 Chemical names

6-hydroxybenzomorpholine
Hydroxy-6-phenomorpholine
Imexine OV (Chimex)
N°2164 E
Compound n°2164
IFG 58-78

1.4 CAS no.

977067-94-9.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8 H_9 NO_2$

Mol weight: 151.

2. Function and uses

Oxidative hair dye; max. use 2.0 %; 1.0 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERIZATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Mice, oral (gastric intubation): 860 (720-1020) mg/kg b.w.

3.7 Subchronic oral toxicity

The compound has been administered orally to groups of 10 male and 10 female Sprague Dawley rats at doses of 40 mg/kg b.w. (2 % in propylene glycol, 5 ml/kg as water suspension) for 3 months. One rat died on the 40th day without correlation with the treatment. The treated male rats showed a slight decrease in the mean body weight gain at the end of treatment when compared with the control male rats (174 g vs. 222 g; mean absolute weight gains: 377 vs. 430 g). Only 1/20 rats died after 40 days. The hematological, biochemical and urine analysis, and anatomopathologic exams (macroscopic or histological) did not show significant differences between treated and control group. The compound produced very slight toxic effects (hepatocytes vacuolisation in one rat) such as isolated lesions revealed after histopathological examinations.

The compound (as suspension in hydrogel with 2 g polysorbate 80 per 100 ml sterile water for injectable preparation) was administered by oral intubation to groups of 10 male and 10 female Sprague-Dawley OFA rats at doses of 0, 10, 100 or 1000 mg/kg day for 30 days (males) or 31 days (females). No-treatment-related abnormalities were observed at gross necropsy. The microscopic examination of the cortical tubules of the kidney in the males (100 or 1000 mg/kg/day) revealed the following changes: epithelial necrosis, anhistic acidophilic substance deposits, cytoplasmic basophilia and dilatation. The severity of the changes was dose dependent. No histopathological lesions were observed in the low-dose (10 mg/kg/day) group.

3.8 Subchronic dermal toxicity

The compound containing formulation (codes as P-25) at dose level of 1.1 % (1:1 with 6 % hydrogen peroxide), has been topically applied (1 ml/kg) on abraded and intact skin of rabbits for 13 weeks (twice weekly). Haematologic and clinical chemistry have been performed at 0-3-7-13 weeks. In females a statistical significant decrease in the mean haemoglobin values (11.87 ± 0.59 vs. 12.54 ± 0.68 g, $P<0.05$), has been observed between treated and combined control groups, at the end of treatment. These differences were not considered to be of toxicological significance (in the range of historical control values). No evidence of systemic toxicity has been observed.

3.10 Chronic toxicity

Dermal topical application. One oxidative formulation (codes as P-25, 1:1 with 6 % hydrogen peroxide), containing 1.1 % of the compound has been tested on Swiss Webster mouse by dermal topical application (0.05 ml/cm^2 on interscapular area) once weekly for 23 months. These observation have been made: mortality, behaviour and dermal changes (daily); skin lesions (weekly) and gross appearance (continuously). Gross and microscopic examinations have been performed in mice found dead or sacrificed during the study, and in all surviving animals at the end of study. Negative results have been obtained.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound has been applied, under an occlusive patch, on the abraded and intact skin of 3 male and 3 female albino Bouscat rabbits as 1 % solution in propylene glycol for 24 hours. The compound resulted "slightly irritating" (primary irritation index = 0.45).

4.2 Irritation (mucous membranes)

The compound has been instilled into one eye of 3 male and 3 female albino rabbits as 1 % solution in propylene glycol (0.1 ml) without rinsing off after instillation. The compound resulted "practically not irritating" to the rabbit eye at reading 48-72 hours and 4-7 days after treatment.

5. Sensitization

Sensitization has been induced in 20 guinea pigs by topical occlusive applications of 0.5 g of the compound (3 times a week, with 2 days interval, for 3 weeks and one at the start of the 4th week; 10 applications, patch test for 48 hours, right shoulder blade) and an intradermal injection of Freund's complete Adjuvant (0.1 ml diluted to 50 % in sterile isotonic solution) on days 1 and 10. The treatment has been suspended for 12 days (from day 24 to 35 of the experiments). On day 36 the guinea pigs have been challenged by topical application (0.5 g) under occlusion for 48 hours on the left untreated flanks. Evaluation of sensitizing reaction has been made at 1, 6, 24 and 48 hours after removal of the occlusive patches. The compound showed no skin reaction.

6. Teratogenicity

The compound containing formulation (1.1 %), coded as P-25 (1:1 with 6 % of hydrogen peroxide), has been applied topically to the shaven skin on Charles River rats at the dose of 2 mg/kg on days 1-4-7-10-13-16-19 of gestation. The results did not show embryotoxic or teratogenic effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human dermal absorption *in vitro:* The penetration of the compound through human epidermis placed on Franz type diffusion cells was studied in four separate assays. The section of epidermis of human mammary skin (lower layer) was in contact with 0.625 % of the dye solution of the compound (9 % sodium chloride, 0.01 % sodium ascorbate) for 30 min and then the skin was rinsed off by an aqueous solution (2 % sodium lauryl sulfate and 10 ml distilled water). The amount of the compound that penetrated the epidermis (evaluated after 4 hours) averaged 0.05, 0.048 and 0.06 % of the applied dose in each of the for assay, respectively.

8. Mutagenicity

Mutagenicity / Genotoxicity studies have demonstrated that the compound has been found negative *in vitro* for: gene mutations by the reserve system analysis on *Salmonella* by plate and

spot test (with 2 % NH_4OH and 1:1 H_2O_2) and forward mutation on *Schizosaccharomyces pombe* P_1; chromosome aberration *in vivo* by micronucleus test on mice (400 mg/kg i.p., analysis at 24, 48, 72 and 96 hours); genotoxicity by the UDS assay on Hela human cells line using two different methodologies (scintillation count and autoradiography).

9. Carcinogenicity

See 3.10.

10. Special investigations

Photoallergenicity: The test was performed at a concentration of 0.4 % (w/w) of the compound in propylene glycol, using 25 albino Hartley guinea pigs. The compound was applied to the shaved skin on day 2 and then 20 animals (group 2) were immediately exposed to UVA (1.32 mW/cm^2 at 360 nm) and UVB (1.32 mW/cm^2 at 310 nm) radiation (2 lamps at 5 cm from the back of the animal) for 20 min. Five animals received no irradiation (group 1). The test sites were scored at 1 and 6 h, and on day 3. On day 4 and 9 the procedure of day 2 was repeated. The test sites were scored on day 5 and 10 and shaved on day 3 and 8. The induction phase was performed 13 weeks after the third application by application of the compound on previously untreated area with the substance or with irradiation. The animals (group 2) were irradiated only with the UVA lamp for 5 min. (20 cm from the back) and then for 15 min. (5 cm from the back). Photoallergic reactions were evaluated 1, 6, 24 and 48 hours after the treatment of the compound. No edema were observed in both groups of guinea pigs. No evidence of allergic reaction (group 1) or photoallergic reactions (group 2) were seen at the microscopic examination. The compound was no photoallergen in guinea pigs in this study.

11. Conclusions

The possibility of nitrosamine formation with this compound should be considered. The SCC requires a chromosomal aberration test in mammalian cells grown *in vitro.*

Classification: B

A 31: *2-METHYL-5-N-ß-HYDROXYETHYLAMINOPHENOL*

1. General

1.1 Primary name

2-methyl-5-N-ß-hydroxyethylaminophenol

1.2 Chemical names

1-methyl-2-hydroxy-4(ß-hydroxyethyl)aminobenzene
2-hydroxy-4-(ß-hydroxyethyl)aminotoluene
2-methyl-5-N-ß-hydroxy-ethylaminophenol
6-methyl-3-ß-hydroxyethylamino-phenol

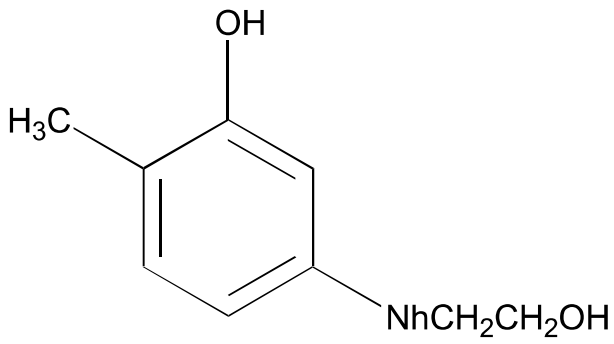
1.3 Trade names and abbreviations

Imexin OAG (Chimex)
N° C 3267
3267 PAN
Orex 119

1.4 CAS no.

55302-96-0

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9 H_{13} NO_2$
Mol weight: 167

2. Function and uses

Oxidative hair dye; max. use: 2.0 %, 1.0 % in combination with H_2O_2 upon application.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Mice, oral: 3100 (2500-3480) mg/kg

3.7 Subchronic oral toxicity

The compound has been administered orally to Sprague Dawley rats (10 male and 10 female) at the dose of 150 mg/kg b.w. (in propylene glycol, 1 ml/100 g b.w.) for 90 days. The results showed a low incidence of toxic effects (isolated injuries at anatomopathological examinations; atrophy of the hypophysis in one male; and gastric ulceration in one female).

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was applied on the intact and abraded skin of six albino Bouscat Rabbits at the dose of 2 % w/v in propylene glycol for the evaluation of the primary irritation index after 24 and 72 h of treatment. The compound produced only mildly irritant effects. The primary irritation index was equal to 0.04. In this study the maximum value proposed for the classification of the primary irritation index was 8 (Draize scale).

4.2 Irritation (mucous membranes)

3 male and 3 female Bouscat Rabbits were treated with a dose of 2 % w/v of test compound in propylene glycol. Treated eyes were not rinsed-off after instillation. The ocular irritation was evaluated 1, 2, 4 and 7 days after applications. The compound gave no signs of irritation.

5. Sensitization

The pure compound, mixed with 50 % Freund' s Adjuvant, was applied to the abraded skin of 10 guinea pigs (5 males and 5 females) under occlusion for 48 hours. A second application was carried out 8 days later with a solution of 25 % of the compound in vaseline (48 hours of treatment). The last treatment with a 25 % vaseline solution of the compound (24 hours under occlusive condition) was carried out 15 days after the 2nd application. The skin reactions were evaluated 48 and 72 hours after last application. The test of Magnusson gave an „allergenicity Index" = 0.6 (6 out of 10 animals showed positive reaction) estimated to be corresponding to a medium „experimental potential of allergenicity".

6. Teratogenicity

A preliminary toxicity study was carried out at doses of 500-1000-2000 mg/kg/day b.w. with the following results: decrease in body weight gain at 2000 mg/kg/day. The compound was therefore administered orally to Charles River rats (10 males and 10 females per group) at doses of 50-300-1800 mg/kg/day (0.5 % w/v in CMC, 1 ml/100 g b.w.) from day 6 to 15 of

pregnancy. No death and sign of toxicity was noted to be due to the treatment. At the doses of 300 and 1800 mg/kg there was a production of brownish coloured saliva after treatment and a brown staining of the fur. At the dose of 1800 mg/kg: brown staining of body extremities; brown/orange discolouration of the urines. In the dams no apparent changes of internal organs due to the treatment have been observed at post mortem exams, although staining of the fur was still evident. Litter parameters: 300 mg/kg/day: post-implantation loss slightly higher than control; mean litter weight (NS) and mean foetal weight ($P < 0.05$) lower than control; 1800 mg/kg/day: mean pre-implantation loss slightly higher than control (associated with the higher mean number of corpora lutea). Only one major malformation reported at 300 mg/kg/day, was considered to be spontaneous and not compound related.

Embryonic and foetal development (evaluated by incidence of minor internal organ changes and skeletal malformations) were not affected by the treatment. There is no evidence for teratogenicity, embryotoxicity nor foetotoxicity.

The No Effect Level in this study was 50 mg/kg b.w./day, based on embryotoxicity. There was no evidence of compound related teratogenicity at any dose level.

7. Toxicokinetics (incl. Percutaneous Absorption)

Rat: A formulation (60 mg) containing 24 mg of the compound and p-Phenylendiamine (1:1.5 ratio), mixed with 36 mg of 20 volumes of H_2O_2, in the presence of 18 mg of bleached hair, was applied *in vitro* on 3 cm^2 of the abdominal skin of Hairless rats for 30 min. The skin was rinsed off after 4 h and 30 min and the amount of the compound which passed through the skin was determined to be 0.04 $\mu g/cm^2$ (mean value corresponding to a fraction of 0.025 % of the applied dose).

Human: A formulation (60 mg) containing 24 mg of the compound and p-Phenylendiamine (1:1.5 ratio), mixed with 36 mg of 20 volumes of H_2O_2, in the presence of 18 mg bleached hair, was applied *in vitro* on 1.5 cm^2 of Human epidermis for 30 min. The skin was rinsed off after 4 h and 30 min. and the amount of the compound which passed through the skin was determined to be 0.07 mg/cm^2 corresponding to a fraction of 0.044 % of the applied dose.

8. Mutagenicity

The compound was tested for gene mutation and found negative in several studies on *Salmonella typhimurium* (1 study ±hydrogen peroxide), in 2 studies on *E.coli* (1 study ±hydrogen peroxide), *S.pombe* and on mouse lymphoma L5178Y TK +/- assay. Negative results were obtained for the induction of SLRL in *Drosophila* (25 and 100 mM). The compound did not induce chromosome aberrations and sister chromatid exchanges on CHO cells treated *in vitro*. The compound was negative for the ability to induce UDS in HeLa human cells line (scintillation count and autoradiography methods) and gene conversion on *Saccharomyces cerevisiae* D4 strain.

The compound was evaluated for its ability to induce micronuclei in five different studies, on mouse and found positive in the following study:

1) Swiss male mice CD1: 1600, 2000, 2400 mg/kg b.w. i.p. (10 ml/kg bw, 20 % aqueous DMSO, 2 equal doses separated by an interval of 24 hrs, sampling time at 30 hr); the dose

of 2400 mg/kg showed a significant increase in the no. of MN per 2000 PCE: 16.2 vs. 3.8 (x 4.26) (Schmid method). It has been reported (data not shown) that this result is associated with a toxic effect of test compound (LD_{50}= 1350 mg/kg (route of exposure not indicated).

and negative in these other four studies :

2) Swiss male mice CD1: 1000 mg/kg bw. i.p. (single dose, 20 % aqueous DMSO, sampling time at 30-48-72-96 hrs) (salmone method).

3) CD1 mice: 2,500-5,000-10,000 mg/kg b.w. i.p. (0.1 ml/100 g b.w. suspension in sterile water, two equal doses separated by an interval of 24 hrs, analysis 6 hours after the last dose);

4) Swiss male mice: 12.5-25.0-50.0 mg/kg i.p. in water (single doses, analysis at 24, 48 hrs);

5) Swiss mice: a) 12.5-25.0-50.0-100-200-400 mg/kg and b) 100-200-400 mg/kg bw. i.p. in saline solution (single doses, 0.4 ml/mouse, analysis at 24 and 48 hrs).

Summary of mutagenicity data:

The compound was extensively studied for its ability to produce gene mutation, chromosome aberration and DNA damage *in vitro.* Negative results were obtained. Similarly 5 *in vivo* bone marrow assays were carried out: one study gave indications of a positive effect, which however was not confirmed in the other four studies, which were negative.

11. Conclusions

The short-term oral study on rats (one dose: 150 mg/kg b.w. and only 10 male + 10 female animals) is not adequate for defining the No Effect Level, due also to the presence of some toxic effects.

The SCC requires a subchronic toxicity study (90 days) on rats to define the No Effect Level.

Classification: B

CALCULATION OF SAFETY MARGIN

2-METHYL-5-N-ß-HYDROXYETHYLAMINOPHENOL (A 31)

Oxidation or Permanent

Based on a usage volume of 100 ml, containing at maximum 1 %

Maximum amount of ingredient applied:	**I (mg)= 1000 mg/kg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 0.044 % (human epidermis in vitro)**
Dermal absorption per treatment:	**I (mg) x A (%)= 1000 x 0.044/100 = 0.44 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg b.w. = 0.44 mg/60 kg b.w. = 0.0073 mg/kg b.w.**
No observed adverse effect level (mg/kg):	**NOAEL = could not be identified**

A 48: *2,6-DIHYDROXY-4-METHYL-PYRIDINE*

1. General

1.1 Primary name

2,6-dihydroxy-4-methyl-pyridine

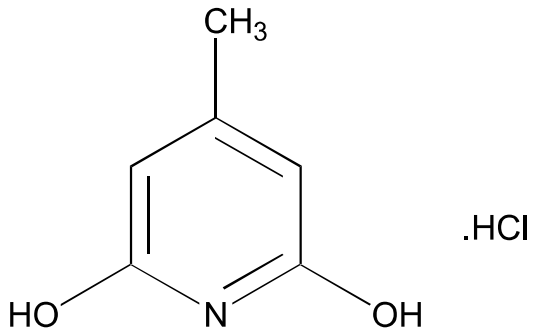
1.2 Chemical names

2,6-dihydroxy-4-methyl-pyridine
2,6-pyridindiol-4-methyl
2,6-dihydroxy-4-methyl-pyridine, hydrochloride
2,6-pyridindiol-4-methyl, hydrochloride

1.3 Trade names and abbreviations

Ro 271

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6 H_7 NO_2 \cdot HCl$
Mol weight: 140

2. Function and uses

Oxidative hair dye; max. use 1.0 %; 0.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rat, oral (stomach tube) 1420 (1240-1620) mg/kg b.w.
(as hydrochloride).

3.7 Subchronic oral toxicity

The compound has been administered by oral intubation to 20 male and 20 female rats (Wistar Han 67 SPF) at doses of 75 mg/kg b.w./day (aqueous solution) for 12 weeks (5 times a week). After 4 administrations the urine showed a red coloration and the hairless skin animals a blue-black coloration. In some rats a slight increase in erythrocytes has been noted. The compound did not show any cumulative toxic effects.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound has been applied on abraded and intact skin of six male albino "New Zealand white" rabbits, under occlusion, for 24 hours at dose of 10 % (500 µl/animal). The compound resulted not irritating after removal of the patches and 24-48-72 hours after treatment.

The compound (as hydrochloride) applied twice daily (2 droplets of 10 % w/v, aqueous suspension, pH = 8) for 10 days to the same skin area of 5 male hairless mice hr strain did not show dermal irritation at reading during the treatment period.

4.2 Irritation (mucous membranes)

The compound has been instilled into one eye of six male albino "New Zealand white" rabbits, at doses of 5 % (w/v) aqueous suspension (100 µl, pH = 9) without rinsing. The reading made at 2-6-24-48-72 hours after treatment showed negative results.

5. Sensitization

It has been induced in 20 female Pirbright White guinea pigs by three pairs simultaneously intradermal injections of Freunds complete adjuvant 0.1 ml, 0.1 ml solution (5 % in distilled water) of the compound, and a 1:1 mixture (v/v) of these two solutions (0.1 ml/animal) in a shaved interscapular area (4 x 6 cm). One week later 5.0 % w/v solution of test compound in vaseline was topically applied, under occlusion, on the same area for 48 hours. Two weeks later the guinea pigs were challenged by a topical application of 5 % in vaseline under occlusion on the shaved flank. The compound showed negative after reading at 24, 48 hours.

8. Mutagenicity

The compound tested for gene mutations on five strains of *Salmonella typhimurium* (± S9mix, Aroclor or Phenobarbital induced rat liver) showed negative results.

The compound (1 % suspension (w/v) in methylcellulose) did not induce micronuclea in CD-1 mice treated by oral gavage at doses of 100-2000-4000 mg/kg b.w. (in two equal doses separated by an interval of 24 hours; analysis 6 hours after the second dose).

11. Conclusions

The present available short term toxicity studies is not adequate for the definition of a No Effect Level.

The SCC requires a percutaneous absorption study, a 90 days repeated toxicity study and an *in vitro s*tudy for the induction of chromosome aberrations.

Classification: C

A 70: *1-METHYL-2,6-DIAMINOBENZENE*

1. General

1.1 Primary name

1-methyl-2,6-diaminobenzene

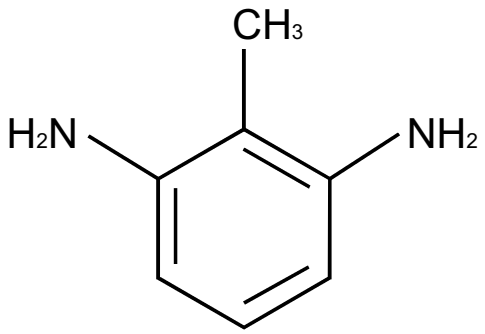
1.2 Chemical names

1-methyl-2,6-diaminobenzene
2,6-diaminotoluene
1,3-benzenediamino-2-methyl
1,3-diamino-2-methylbenzene
toluene-2,6-diamino

1.4 CAS no.

823-40-5.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_7 H_{10} N_2$
Mol weight: 122.

2. Function and uses

Oxidative hair dye; max. use 1 %; 0.25 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} rat, oral gavage: The results have been evaluated according to three different methods:

a) 230 mg/kg b.w. (Kärber method)
b) 190 mg/kg b.w. (Litchfield/Wilcoxon method)
c) 190 (140-260) mg/kg b.w. (graphical method)

3.7 Subchronic oral toxicity

Groups of 5 males and 5 females of rats (Wistar Bor: Wissw SPF/TNO) have been treated by oral gavage with 0-0.3-3-30 mg/kg b.w./day of test compound (1 ml/100 g, in deionized water) for 91 days. The dose of 0.3 and 3 mg/kg did not show significant biological effects. Only a slight reduction in body weight gain in males (7-13 wks., statistically significant differences: 80.4 % vs 100.0 % control, $P < 0.05$) was seen in rats treated with 3.0 mg/kg. This body weight gain reduction was not biologically significant because at the end of the 13 wk study, no difference between treated (3.0 mg/kg) and control group has been obtained for the mean body weights in male rats: 289.2 ± 29.7 g (treated at 3.0 mg/kg) vs. 308 ± 37.2 g (control). The dose of 30 mg/kg gave the following toxicological effects: reduction in activity and hyporeflexia (10-120 min. post treatment); decrease in body weight gains (1-13 wks) in males; reduction in food consumption in males (1-13 wks) and females (1-6 wks); significant increase in glucose levels (males and females; presence in the urine of erythrocytes and proteins (3/5 males); significant increase in liver and kidney weights. No significant differences for the histological changes have been observed between control and treated group at 30 mg/kg (10 males and 10 females/group). The "No Effect Level" (NEL) in this study was at 3.0 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Dermal irritation: The compound has been applied to intact and to abraded skin of 6 White New Zealand rabbits at doses of 1 % in water (0.5 ml, pH = 7.3) under occlusive conditions for 4 hours. The skin reactions have been evaluated 30 and 60 min. after the end of treatment and after 24-48-72 hrs. The results showed that the test compound did not produce any skin reaction.

4.2 Irritation (mucous membranes)

Eye irritation: The compound has been instilled into the conjunctival sac of one eye of 9 white New Zealand rabbits at doses of 1 % in water (0.1 ml, pH = 7.3). The eyes of 6 rabbits were rinsed out (4 sec. or 30 sec. after applications in two equal groups) and the other treated 3 animals remained as such. Observations were made at 1-2-8-24 h and daily up to 7 days after treatment. The rabbits whose eyes were not rinsed-off showed slight reddened of the conjunctiva up to 8 h after treatment. 1 % solution of the compound in this study did not produce any significant irritant effects.

5. Sensitization

20 guinea pigs have been treated topically (once a week for 3 weeks) with a 0.1 % water solution of test compound under occlusive conditions. The method of Buehler, occlusive patches without Freund´s Adjuvant, has been applied. The patches were removed after 6 hrs.

and the animals challenged (0.5 ml of test compound solution on the shaven untreated skin) after an interval of 2 weeks. The reaction, evaluated 24 and 48 hours later, showed no skin sensitization. The low concentration used for the induction in this non-adjuvant technique was noted.

Human sensitization: data not available.

8. Mutagenicity

Mutagenicity and Genotoxicity studies have shown that 1-methyl-2,6-diaminobenzene is mutagenic in *Salmonella* and is able to induce cell transformation in secondary hamster embryo cells (HEC); it enhances the transformation of primary HEC by Simian adenovirus 7 (SA 7) when given after virus.

Negative results were obtained for micronucleus test on mice treated orally by gavage (25-50-100 mg/kg in 1 % methylcellulose), 2 equal doses separated by an interval of 24 hrs., analysis 6 hours after the second dose.

The compound does not induce UDS *in vivo* on male rats (Fisher-344) treated orally by gavage with a dose of 150 mg/kg in corn oil (analysis at 2 and 12 hours).

9. Carcinogenicity

Long term studies have been carried out on B6C3F1 mice and F344 rats in a NCI bioassay, the compound (as dihydrochloride) being fed in the diet at 250 and 500 ppm for rats for 103 weeks (observed for 1 additional week) and at 50 or 100 ppm for mice for 103 weeks (observed for 1 additional week). The compound was considered not to be carcinogen in both sex and in both species.

In male rats, islet cells adenomas of the pancreas (P = 0.025) and neoplastic nodules or carcinomas of the liver (P = 0.037; 4/50 (8 %) vs. 2/334 (0.6 %)) showed a significant dose-related trend using Cochran-Armitage test, but not with Fisher exact test. The incidence of the neoplastic nodules or hepatocellular carcinomas in male rats in the highest treated group is 4/50 (8 %) vs. 2/344 (0.6 %) of the historical control of NCI laboratory and the 36/2,230 (1.6 %) across all laboratories. The incidence of islet-cell adenoma of the pancreas in the male of highest dose group is 4/45 (ca. 9 %) in comparison with 2/35 (5.7 %) observed in one group of vehicle control male rats or 0/344 of historical control (NCI laboratory).

11. Conclusions

The SCC requires a percutaneous absorption study and, depending on the results, possibly a teratogenicity study.

Classification: B

A 82: *1-HYDROXY-3,4-METHYLENEDIOXYBENZENE*

1. General

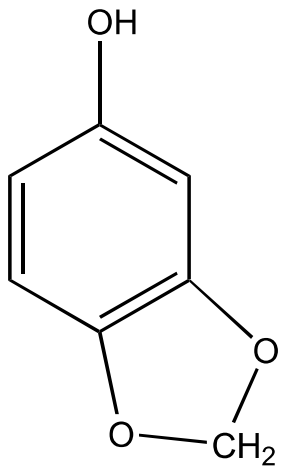
1.1 Primary name

1-hydroxy-3,4-methylenedioxybenzene

1.2 Chemical names

1-hydroxy-3,4-methylenedioxybenzene
Sesamol

1.5 Structural formula



1.7 Purity, composition and substance codes

Purity: 99 %

1.8 Physical properties

Appearance: Colourless crystalline powder

1.9 Solubility

Soluble in water and various organic solvents; ethanol, isopropanol, acetone, chloroform and ethyl acetate. No quantitative solubility data were available.

2. Function and uses

The compound is supplied as an oxidative hair dye at concentrations up to 3 % and used at a concentration of 1.5 % after dilution with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats and mice following administration in 10 % gum arabic. The following LD_{50} values were obtained, female mice 415 mg/kg, male rats 430 mg/kg and female rats 300 mg/kg. The observed signs of toxicity were decrease in activity, staggering and exitus.

3.7 Subchronic oral toxicity

Two 90 day studies have been carried out in the rat with the compound administered by gavage. Dose levels of 5, 10 and 15 mg/kg (5 days/week) and 10, 30 and 60 mg/kg (7 days/week) were used. No compound related effects were noted on weight gain, clinical chemistry, haematology or on examination of tissues at autopsy.

4.1 Irritation (skin)

No signs of skin irritation were observed in guinea pigs following application of a 3 % solution for 4 hours under occlusion. Similarly no signs of skin irritation were observed in rabbit exposed to 50 mg/kg under occlusion for 4 days or rats given the same dose daily for 30 days.

4.2 Irritation (mucous membranes)

Eye irritation has been studied in both the rabbit and the guinea pig. The compound was added to one eye of groups of albino rabbits (1.2, 2.3 or 4.6 mg); this resulted in signs of irritancy being seen in all groups at 4 hours (slight swelling of palpebral membrane, conjunctivitis and oedema of nictating membrane). No effects were seen after 24 hours in animals given 1.2 mg of sesamol nor in the animals given 2.3 mg after 48 hours. The only effect seen at this time in the group given 4.6 mg was a slightly inflamed nictating membrane. In the guinea pig study no significant effects were seen in studies using a 1 % solution.

5. Sensitization

The ability of the compound to induce skin sensitization has been investigated in one study in guinea-pigs using small numbers of animals (4) given i.c. or topical application ten time over 20 days, followed by challenge 15 days after final application. No evidence of sensitization was observed; however in the light of the small number of animals and the use of a non-standard method no conclusions can be drawn. Limited studies in humans have been reported. In one of these, no reactions were seen when 5 subjects were treated with 1.25 mg of compound in alcohol for nine daily doses and challenged 12 days after the final dose. Although no reactions were reported the number of subjects was far too small to allow any definite conclusions to be drawn. Sensitization was seen in a high proportion (8/13) of patients sensitized to sesame oil (from therapeutic treatment of leg ulcers) and in one of 15 subjects sensitized to p-aminoaryl compounds. In the first of these cases the relevance to individuals with normal skin is unclear in view of the frequency of contact allergies in individuals with

stasis eczema and in the second case was thought to be a consequence of the polyvalent allergy in this subject. The limitations of the animal and human data preclude any conclusions being drawn regarding the sensitization potential of sesamol.

8. Mutagenicity

Negative results were obtained in studies to investigate the ability of sesamol to produce gene mutation in *Salmonella typhimurium*. Strains TA1535, TA1537, TA1538, TA98 and TA100 were investigated both in the presence and absence of an exogenous metabolic activation system at concentrations up to 30 μmol. Similar results were obtained in an investigation of gene.

11. Conclusions

Sesamol has moderate acute toxicity by the oral route, however studies suggest dermal penetration is low from hair dye formulation. The compound produced eye irritation but a 1 % solution was found to be practically non-irritating. There was no evidence of skin irritation with sesamol. There was no evidence of skin sensitization in the animal or human repeated insult study, but sensitization was seen in 2 studies on patients with allergies. In a 90 day oral study no effects were reported at doses up to 60 mg/kg. Mutagenicity data comprised negative results in both a *Salmonella* and a CHO assay for gene mutation and an *in vivo* study on sister chromatid exchange in bone marrow. No adverse effects were reported in an oral teratogenicity study at 10 mg/kg, the only dose level studied. No compound related effects were observed in a chronic study, however no conclusions can be drawn from this study due to the inadequate study design.

A further study is needed to investigate whether sesamol can induce sensitization using a more rigorous protocol to current standards. There is also a need for an *in vitro* study to investigate the clastogenicity of sesamol in mammalian cells.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-HYDROXY-3,4-METHYLENEDIOXYBENZENE

OXIDATION OR PERMANENT

Based on a usage volume of 100 ml, containing at maximum 1.5 %

Maximum amount of ingredient applied:	**I (mg)= 1,500 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 1 % (rat)**
Dermal absorption per treatment:	**I (mg) x A (%)= 15 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg** **15 mg/60 mg b.w. = 0.25 mg/kg b.w.**
No observed adverse effect level (mg/kg): **(rat: 90 days oral study)**	**NOAEL = 60 mg/kg b.w.**
Margin of Safety:	**NOAEL / SED = 60 mg/kg b.w./0.25 mg/kg b.w. = 240**

A 98: *1-(ß-HYDROXYETHYLAMINO)-3,4-METHYLENEDIOXY-BENZENE*

1. General

1.1 Primary name

1-(ß-hydroxyethylamino)-3,4-methylenedioxy-benzene

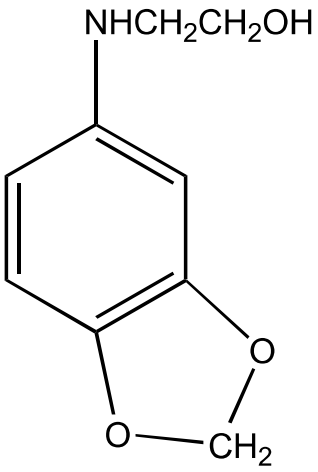
1.2. Chemical names

1-(ß-hydroxyethylamino)-3,4-methylenedioxy-benzene

1.3 Trade names and abbreviations

Aminol

1.5 Structural formula



1.7 Purity, composition and substance codes

Purity: 99 %

The compound is normally supplied as the hydrochloride salt owing to the limited stability of the free base.

1.8 Physical properties

Appearance: Beige crystalline powder

1.9 Solubility

The substance is soluble in water and ethanol but no quantitative solubility data were available.

2. Function and uses

The compound is supplied as an oxidative hair dye at concentrations up to 3 % and used at concentrations of 1.5 % after dilution with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats and mice following administration in 10 % gum arabic. The following LD_{50} values were obtained: female mice 0.85 g/kg, male rats 1.65 g/kg and female rats 1.55 g/kg. The observed signs of toxicity were a decrease in activity, abdominal position and exitus.

3.7 Subchronic oral toxicity

Two 90 day studies have been carried out in the rat with the compound administered by gavage. Dose levels of 5, 275 and 550 mg/kg in the first study and 20 mg/kg in the second study were used. No compound related effects were seen on weight gain, clinical chemistry, haematology or on examination of tissues at autopsy at either 5 or 20 mg/kg. There were significant differences in body weight gain in males, haematological (decrease in red blood cells and thrombocytes) and clinical chemistry parameters at 275 mg/kg. The liver weight in both sexes and spleen weight in males were slightly increased. The effects were more pronounced at 550 mg/kg and included mortalities in 12 of the 35 animals. A subsidiary group dosed at 550 mg/kg and observed for an additional 4 weeks to study the reversibility of the effects showed no significant differences from controls at the end of this period.

4. Irritation & corrosivity

4.1 Irritation (skin)

No signs of skin irritation were observed in guinea-pigs in a method described as analogous to that of Draize following daily application of a 5 % solution for 5 days. In a human study involving application of a hair dye formulation diluted 1:1 with hydrogen peroxide (1.5 % aminol) and subsequently with water (0.75 % aminol) under occlusion for 24 hours to 40 volunteers, mild erythema was observed in 7 volunteers.

4.2 Irritation (mucous membranes)

Eye irritation has been studied in the guinea-pig. A 0.1 ml aliquot of a 2 % aqueous solution was instilled into the right eye. Slight transient irritation was seen at 30 minutes (mean primary irritation index 0.6) which had disappeared by 4 hours.

5. Sensitization

The ability of the compound to induce skin sensitization has been studied in the guinea-pig using the maximisation method. The methodology and results are inadequately described. The compound was described as a moderate sensitizer.

6. Teratogenicity

Two studies on reproductive toxicity were available, one in the rat and the other in the rabbit. Groups of 23 mated female rats received 500 or 1000 mg/kg orally on days 6 to 15 of gestation. No treatment related differences were reported in either the dams or the fetuses, except for a slight difference in weight gain over the dosing period at the lower dose. Groups of up to 21 artificially inseminated female rabbits were orally dosed 50, 250 or 1000 mg/kg on days 6 to 18 of gestation. All the animals dosed at 1000 mg/kg died after dosing between days 6 and 10 and findings at necroscopy were consistent with emphysema. No treatment related differences were observed between controls and those animals dosed at 50 mg/kg. The results were similar at 250 mg/kg but there was a slight increase in pre- and post-implantation loss, an unusual sex distribution and two abortions, none of which could definitely be ascribed to treatment. There was no evidence of any teratogenic effects in rats or rabbits.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption has been investigated *in vivo* both in rats and humans. The rat study involved application of radiolabelled aminol (10 mg) in a test solution and a hair dye formulation either with or without hydrogen peroxide for 30 minutes. The presence of hydrogen peroxide resulted in a decrease in elimination over 72 hours to 0.05 % compared to 0.35 % from the other formulations. The application site skin contained 1.56, 0.56 and 0.34 % of the dose for the test solution and hair dye formulation without and with hydrogen peroxide respectively (96 % or greater was recovered on washing the application site). No accumulation of radioactivity was seen in any of the organs. The human study involved application of 0.7 % aminol in a hair dye to 5 healthy female volunteers under normal conditions of use. Levels in the serum of 3 volunteers were below the limit of detection (20 mg/ml) with an implied absorption of less than 0.25 %. In the remaining 2 volunteers levels of serum suggested absorption of 0.7 and 2.1 % respectively and an apparently biphasic elimination.

8. Mutagenicity

Negative results were obtained in two studies to investigate the ability of aminol to produce gene mutation in *Salmonella typhimurium*. Strains TA1535, TA1537, TA1538, TA98 and TA100 were investigated both in the presence and absence of an exogenous metabolic activation system at concentrations up to 25 µmol (4525 µg) and in strains TA1537, TA98 and TA100 at concentrations up to 55 µmol (10000 µg) under similar conditions. Negative results were also obtained in an investigation of gene mutation in L5178Y mouse lymphoma cells (thymidine kinase locus) at concentrations up to those causing considerable toxicity both in the presence (up to 73 µg/ml) and absence (up to 539 µg/ml) of an exogenous metabolic activation system. No significant effect of aminol on chromosomal structure in cultured human peripheral lymphocytes was observed in either the presence or absence of an exogenous metabolic activation system at concentrations up to 80 µg/ml (this resulted in some inhibition of mitotic index, 60 and 48 % in the absence and presence of an exogenous activating system). Negative results were obtained in an *in vivo* study to investigate induction of sister chromatid exchange in bone marrow following oral, i.p. or dermal administration and using dose levels up to those producing toxicity (1500 mg/kg oral, 600 mg/kg i.p., 2000 mg/kg dermal). Aminol was not mutagenic in the micronucleus test at total doses of 500, 1000 and 2000 mg/kg given orally in

two equal doses 24 hours apart; a dose-related increase in bone marrow toxicity was observed at the two higher doses.

9. Carcinogenicity

No data on carcinogenicity studies on aminol was available.

11. Conclusions

Aminol has moderate acute toxicity by the oral route. Limited studies suggest that dermal absorption from hair dye formulations can be up to 2 %. There was no evidence of skin irritancy in animals using a 5 % solution of aminol, and only a mild effect in some humans using a hair dye formulation containing hydrogen peroxide and 2 % aminol and using an occlusive dressing for 24 hours. A 2 % solution produced no significant eye irritation in animals. In a 90 day oral study the No Effect Level was 20 mg/kg with evidence of bone marrow toxicity at 275 mg/kg, and lethality at 550 mg/kg. Aminol has been examined in a range of mutagenicity studies *in vitro* (gene mutation in Salmonella and mouse lymphoma cells, metaphase analysis of lymphocytes for clastogenicity) with negative results. Negative results were also obtained in *in vivo* assays for sister chromatid exchange and micronucleus induction in bone marrow. No adverse effects were reported in oral teratogenicity studies in rats at up to 1000 mg/kg or rabbits up to 250 mg/kg.

An adequate test for the sensitization potential of aminol is required.

Classification: B

B 24: *1,2-DIAMINO-4-NITROBENZENE*

1. General

1.1 Primary name

1,2-diamino-4-nitrobenzene

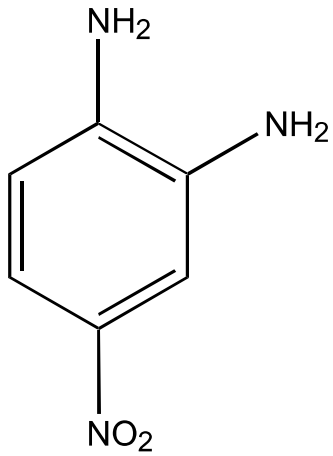
1.2 Chemical names

1,2-diamino-4-nitrobenzene
4-nitro-o-phenylenediamine

1.3 Trade names and abbreviations

4-NOPD

1.5 Structural formula



TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.7 Subchronic oral toxicity

4-NODP, tested for subchronic oral toxicity in Wistar rats for 90 days by gavage, showed toxic effects (i.e., changes in body weights, organ weights and haematological and biochemical parameters) at the only tested dose level of 20 mg/kg body weight. A No Effect Level could, therefore, not be determined.

8. Mutagenicity

4-NOPD induces gene mutations in bacteria, mammalian cells and Drosophila as well as chromosome aberrations in Chinese hamster lung and prostate cells and SCE in Chinese

hamster ovary cells. Moreover, morphological transformations were observed in Syrian hamster embryo cells *in vitro.*

On the other hand, several *in vivo* studies, such as micronucleus assays in mice and rats, a dominant lethal test in rats and an SCE test in Chinese hamster bone marrow gave negative results. UDS tests in HeLa cells and rat hepatocytes *in vitro* were also negative as did *in vivo* studies for chromosome mutations. The possibility that gene-mutations demonstrated in bacteria and in cultured mammalian cells as well as in Drosophila, could also occur in the mammalian organism, cannot be ruled out.

9. Carcinogenicity

Carcinogenicity studies with 0, 3750 and 7500 ppm 4-NOPD in the diet of mice for 102 weeks and 0, 375 and 750 ppm in the diet of rats for 103 weeks (groups of 50 male and female animals in each treatment group, 20 animals of each sex in the control groups) did not reveal a statistically significant increase of tumor incidence. Rats exposed to the compound showed neither a distinct depression of body weight nor any other sign of toxicity. Hence, the substance was not tested at the maximum tolerated dose, a prerequisite for adequate bioassays, especially to rule out the carcinogenicity of chemicals. This shortcoming in experimental design gains particular importance since an increased incidence, albeit statistically not significant, of hepatocellular adenomas was observed in female mice. In addition, the control group of rats and mice consisted only of 20 animals. For these reasons, it was concluded, that 4-NOPD has not been definitely shown to be devoid of carcinogenic properties.

11. Conclusions

The short-term oral toxicity study requested by SCC in 1987 has still not been submitted. Clear evidence for gene mutations in different test systems, including mammalian cells *in vitro* and Drosophila *in vivo*, suggests a potential for such mutations also in mammals. Appropriate tests (i.e., mouse spot test) to exclude this possibility has not been carried out.

In light of the
— lack of information on a No Effect Level and on gene mutation *in vivo,*
— inadequate carcinogenicity studies,
— its cell-transforming properties,

4-NOPD should be classified as C.

Classification: C

B 25: *1,4-DIAMINO-2-NITROBENZENE*

1. General

1.1 Primary name

1,4-diamino-2-nitrobenzene

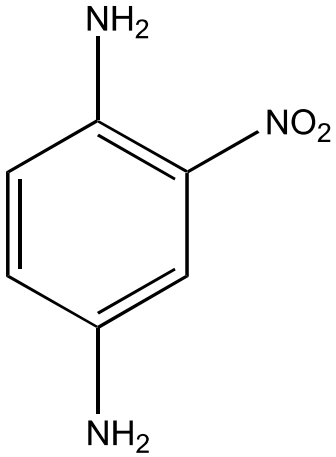
1.2 Chemical names

1,4-diamino-2-nitrobenzene
2-nitro-p-phenylendiamine

1.3 Trade names and abbreviations

2-NPPD

1.5 Structural formula



TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.4 Repeated dose oral toxicity

2-NPPD was tested for subacute oral toxicity in Sprague-Dawley rats for 28 consecutive days by gavage. 20 male and 20 female rats were dosed with 0, 3, 30 or 100 mg 2-NPPD/kg/day. Although red staining of the urine was observed in animals treated with 3 mg/kg/day, this dose can be regarded as No Effect Level.

7. Toxicokinetics (incl. Percutaneous Absorption)

Under hair dying conditions in man (hair dye formulations were applied to dry hair, worked into the hair for 5-8 min, left on the hair for 30 min, rinsed off) 0.75 % of the dose applied was absorbed by scalp in a period of 30 days (cumulative urine sample).

8. Mutagenicity

2-NPPD induces gene mutations in bacteria and mammalian cells in culture. *In vivo* assays for gene mutation are not available. In mammalian cell cultures, chromosomal aberrations and SCE were induced. However, *in vivo* two micronucleus assays (rats) and a SCE-test (Chinese hamster bone marrow) gave negative results. *In vitro* assays for UDS in primary cultures of rat hepatocytes were also negative or marginally positive. *In vivo,* no UDS could be induced in rat livers. Two different cell transformation assays using Syrian hamster embryo cells and the permanent mouse cell line C3H/10T1/2 were clearly positive.

9. Carcinogenicity

NCI bioassays for carcinogenicity were carried out in mice and rats. Dietary administration of 2200 and 4400 ppm 2-NPPD to B6C3F1 mice for 78 weeks caused a dose-dependent, statistically significant increase of hepatocellular neoplasms, primarily adenomas, in the females. There was, however, no conclusive evidence for carcinogenicity, either in male mice or in male (550 and 1100 ppm) and female (1100 and 2200 ppm) Fischer 344 rats under the conditions of the bioassay.

Independent blind histological evaluation of slides of the mouse hepatic neoplasma resulted in different conclusions. One pathologist concluded that the induction of only benign neoplasms indicated a proliferative stimulus that might be suggestive of a potential carcinogenic effect. The other pathologist found an enhancement of parenchymal cell proliferation in treated female mice. Both stated that a carcinogenic effect was not demonstrated. Nevertheless, the induction of benign hepatomas in mice must, at least, be regarded as limited evidence of carcinogenicity according to the criteria applied by IARC.

11. Conclusions

The ability of 2-NPPD to produce hepatocellular adenomas in female mice must be regarded, at least, as limited evidence for carcinogenicity. This view is supported by clear evidence for its mutagenic potential: 2-NPPD has been found to be mutagenic and genotoxic in several *in vitro* assays aiming at different genetic endpoints. *In vivo* studies to detect chromosome mutations and DNA damage have produced negative results. However, *in vivo* assays for gene mutations are not available and the ability of 2-NPPD to morphologically transform mammalian cells in culture must be regarded as an indication for its possible carcinogenicity.

Based on a maximum in use concentration of 1 % 2-NPPD and an average use of 100 ml of hair dye formulation (most products are not mixed with an oxidant), a maximum amount of 1 g of 2-NPPD comes into contact with hair and scalp. Considering a rate of penetration of 0.75 % under use conditions this results in a resorption of 7.5 mg per treatment which corresponds to a systemic dose of 0.15 mg/kg assuming an average bodyweight of 50 kg. Based on a No Effect Level of 3 mg/kg, as determined in the subacute toxicity study on rats, the calculated safety margin of 20 is clearly not acceptable. For this reason alone, any further testing of this possible carcinogenic compound does not appear to be justified.

Classification: D

OPINIONS ADOPTED DURING THE 49TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 10 February 1992

CI 15585: *1-(4-CHLORO-O-SULPHO-5-TOLYLAZO)-2-NAPHTHOL*

1. General

1.1 Primary name

1-(4-chloro-o-sulpho-5-tolylazo)-2-naphthol

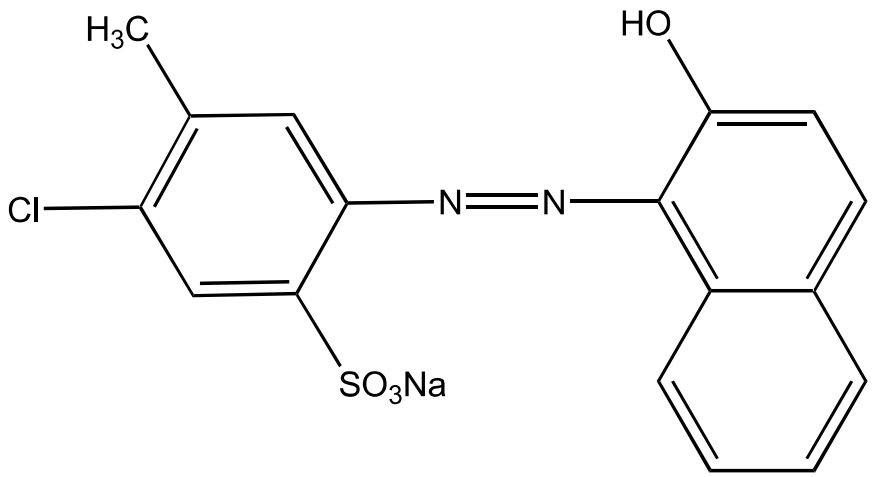
1.2 Chemical names

1-(4-chloro-o-sulpho-5-tolylazo)-2-naphthol

1.3 Trade names and abbreviations

Pigment Red 53
C-Rot 55

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{17}H_{13}O_4N_2ClS$ (free acid)
Mol weight: 376.7

1.7 Purity, composition and substance codes

CI 15585:1 is the barium salt of 1-(4-chloro-o-sulpho-5-tolylazo)-2-naphthol (D&C Red No. 9; CAS Reg. No. 5160-02-1)

2. Function and uses

This colourant has been used in external cosmetics and drugs, including those subject to incidental ingestion, for very many years (since the late 1930s). It is used widely in lipsticks. Use levels are up to 5 %. It is allowed as a colourant under the EEC Cosmetics Directive for all uses except around the eye.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The review does not contain any information on acute toxicity, skin or eye irritation, or sensitization, but concentrates on long term toxicity, especially carcinogenicity, and includes mutagenicity data.

3.7 Subchronic oral toxicity

A subacute (90-day) feeding study in Fischer 344 rats with 0, 0.25, 0.50, 1.0 and 2.0 % in the diet revealed enlargement of the spleen in all dose groups and abnormal red blood cells. In a 20 week study in rats using the same dietary levels splenomegaly and low haemoglobin levels and haematocrit values were observed (in the review these changes are evaluated as 'no significant adverse effects').

3.10 Chronic toxicity

The review tabulates a few details of a 2 year rat feeding study and a 2 year dog feeding study conducted before 1976.

The 2 year rat study (Osbourne Mendel strain) was conducted with dietary levels of 0, 100, 500, 2500 and 10000 ppm. Splenomegaly and abnormal RBCs were seen at the two high dose levels. The NOAEL was 500 ppm, or about 25 mg/kg b.w./day. No neoplasia were observed.

The 2 year dog study comprised groups fed 0, 150, 1000 or 5000 ppm.

Splenomegaly and destruction of RBCs occurred with 1000 and 5000 ppm, increased liver weights with 5000 ppm. The NOAEL was 150 ppm or about 4 mg/kg. Again no neoplasia were observed, but the study was of too short a duration to allow any conclusions to be drawn regarding carcinogenicity in this species.

A 2 year mouse study at dose levels of 0, 50, 250 and 1000 ppm produced changes in cell count, haemoglobin and haematocrit in the high dose group. Other changes were not considered related to treatment.

A second mouse study using feeding levels of 0 and 2000 ppm. The only change attributed to treatment was chronic inflammation of the stomach.

A chronic study in Sprague Dawley rats, and involving *in utero* exposure, used the relatively low feeding levels of 0, 100, 200 and 500 ppm for 30 months. The only change observed was an increased spleen weight accompanied by decreases in red cell parameters, at 500 ppm and observed only at 12 months. The No Effect Level was 200 ppm.

A second chronic (30-months) rat study again in Sprague Dawley rats and with *in utero* exposure used only one high feeding level of 10000 ppm. This level caused changes of the spleen, kidney, liver, pancreas and pituitary. The spleen changes were accompanied by 4 mesenchymal tumours of the spleen in the treated animals two of which were very uncommon

in the rat-strain used; two spleenic tumours were seen in the controls. The incidence was, however, not statistically significant. In the adrenals of the treated rats, the incidence of hyperplasia and of phaeochromocytoma were increased in both sexes but the increase in the latter was not statistically significant.

Mechanisms of induction of spleenic tumours in rats by D&C Red 9

Chronic studies in rats have shown that exposure to high dietary levels of D&C Red 9 results in the induction of spleenic tumours, particularly in Fischer F344 animals, with a high incidence of both fibrosarcomas and angiosarcomas being observed. There was no clear evidence for an increase in any other tumour type in rats, nor of any carcinogenic effect in mice. The spleenic tumours in rats were associated with levels that also resulted in marked toxicity to the spleen (capsular and parenchymal fibrosis), effects being observed in most animals. A number of suggestions regarding potential mechanisms have been made; these have recently been reviewed (Bus and Popp 1987). It was suggested by Goodman et al. (1984) that spleenic toxicity, arising from an initial toxicity to erythrocytes (probably by an amine metabolite), followed by sequestration of the damaged erythrocytes in the spleen, leading to haemosiderin deposition coupled with enhanced delivery of toxic metabolites in the spleen, resulted in fibrosis and subsequently the formation of fibrosarcoma. A slight different mechanism was proposed by Weinburger et al. (1985) namely that acute vascular congestion, resulting from spleenic scavenging of chemically damaged erythrocytes may be an important initial toxic lesion to the spleen. The vascular congestion would lead to spleenic haemorrhage, formation of fibrous tissue mass and, again in conjunction with accumulation of toxic metabolites within the spleen (derived from scavenging erythrocytes) transformation of mesenchymal cells of the spleen, resulting in the expression of spleenic fibrosarcomas and a variety of other lesions. Haemosiderin deposition was not critical for the latter hypothesis, which is pertinent as there was no evidence of increased intra-splenic accumulation of iron containing pigment in the animals treated with D&C Red 9.

These data support the hypothesis that the sarcomas seen at high dose levels were secondary to toxicity, and that a threshold thus exists.

However a crucial question in this regard is whether the compound is acting by a 'non-genotoxic' mechanism. The available published data on mutagenicity of this compound is summarised below.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption was examined by an *in vitro* test with human skin using Franz diffusion cells. The maximum absorption found was 0.06 %, and the calculated total absorption of cosmetic use was ca. 0.07 µg/kg/day.

CTFA calculation of ingested colourant from lipstick (assuming present at 2 %) use (assuming ingestion of 50 % of the amount applied) resulted in a maximum daily intake of 0.4 mg, or 0.008 mg/kg/day. However much higher values would be obtained using other assumptions (0.01 g per application, up top 6 applications per day).

9. Carcinogenicity

Carcinogenicity bioassays were carried out by Battelle (1977-79) as part of the NTP programme. Fischer 344 rats were given dietary levels of 1000 and 3000 ppm, and B6C3F1 mice were given levels of 1000 and 2000 ppm. Animals were exposed to treated diet for 103 weeks and the study terminated 1 week later. D&C Red 9 was carcinogenic in male rats causing an increased incidence of sarcoma of the spleen at the top dose, and a dose related increase in neoplastic nodules of the liver; the significance of the latter is however questionable. There was no evidence of carcinogenicity in female rats. Nor was there any evidence of carcinogenicity in the B6C3F1 mice of either sex. The significance of the spleenic tumours seen in the F344 rats was questioned, as it was argued that the mechanism by which these arose was secondary to marked toxic effects on the spleen at the high dose (fibrosis) (see later for detailed discussion).

8. Mutagenicity

There are a number of reports of this colourant being investigated for its ability to produce gene mutation in bacteria using *Salmonella typhimurium.* Negative results were obtained by Brown et al. (1979) using TA98, 100, 1535, 1537 and 1538, both in the presence and absence of rat S9. Duplicate plating was used, and the results were not confirmed in an independent experiment. Muzzall and Cooke (1979) reported a negative result using the spot test method and also with a plate incorporation assay with strains TA98, TA100, TA1535, TA1537 but using a lipstick containing D&C Red 9 rather than the compound itself. This study was too limited to allow any conclusions to be drawn regarding the mutagenic potential of D&C Red 9. In a recent report on compounds tested as part of the NTP programme (Zeiger et al. 1988) the colourant is reported to be weakly positive against TA97 in the absence of S9. However examination of the data indicates that this result was at most equivocal. Negative results were obtained with the other strains. Thus D&C Red 9 has essentially given negative results in Salmonella assays.

CI 15585 has been investigated for its ability to induce chromosome damage in CHO cells *in vitro.* Concentrations in the range 15-300 µg/ml were used in the presence and 30-150 µg/ml in the absence of S9. Cells were harvested after 6-hour and 24-hour treatments respectively and 100 metaphases analysed. Cytotoxicity precluded cytogenetic analysis at the highest concentrations investigated. An increase in chromosome aberrations was seen in the presence of S9 at 120 and 150 µg/ml. Although this investigation was limited by the use of a single harvest time and the failure to confirm the results in an independent experiment, it did indicate that CI 15585 was clastogenic in the presence of S9.

In addition the ability of the compound to produce unscheduled DNA synthesis (UDS) in rat hepatocytes has been investigated both *in vitro* and also in an *in vivo/in vitro* liver UDS assay using oral doses of 500 mg/kg, with perfusion of the liver and harvesting after 2 and 5 hours. Negative results were obtained in both cases.

There is one brief report of a positive result in a cell transformation assay using Balb/c 3T3 system, but insufficient details were given to assess this study (Tennant et al. 1986).

To summarise the mutagenicity data, CI 15585 has given negative results in assays to investigate its ability to produce gene mutation in Salmonella. Negative results were also obtained when the compound was investigated for its ability to produce UDS assay in cultured primary hepatocytes, and also in an *in vivo* liver UDS assay using an oral dose level of 500 mg/kg. However the compound does appear to have mutagenic potential with positive results being obtained in a metaphase assay for chromosome damage in CHO cells.

11. Conclusions

CI 15585 has been shown to have mutagenic potential in CHO cells and be carcinogenic in rats at high dose levels fibrosarcomas and angiosarcomas of the spleen being produced. No clear evidence of tumour induction at other sites was seen in the rat, nor of any carcinogenic effects in mice. Dietary levels that produced tumours of the spleen in rats were also associated with marked toxic effects (fibrosis), and the tumours may be secondary to toxicity. The No Effect Level in chronic studies in rats and dogs was about 25 and 4 mg/kg/day respectively.

The compound is carcinogenic in rats and has been shown to have mutagenic potential in CHO cells; a genotoxic mechanism could not be ruled out for the induction of malignancies. Furthermore the No Effect Level in the dog is very low, namely 4 mg/kg. The compound should not be used as a colourant for cosmetics.

Classification: D

P 8: *HEXAMIDINE AND ITS SALTS*

1. General

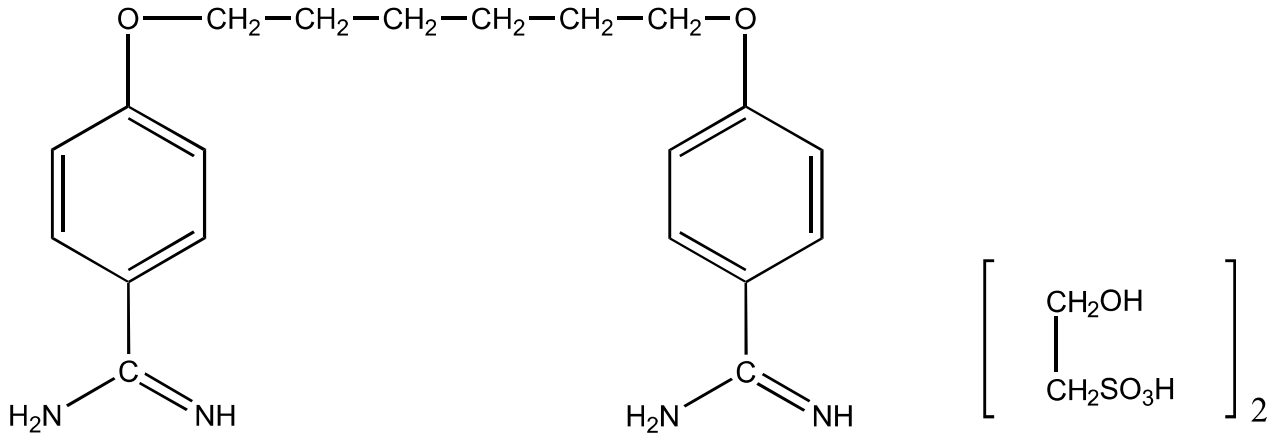
1.1 Primary name

Hexamidine

1.2 Chemical names

1,6-di(4-amidino phenoxy)-n-hexane and its salts including di-isethionate and di(p-hydroxybenzoate)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{20}H_{26}N_4O_2$
Mol weight: 354

1.9 Solubility

Soluble in water. Insoluble in organic solvents.

2. Function and uses

Hexamidine is used in cosmetics as a preservative at a maximum dose level of 0.1 %, and for other uses at concentrations up to 0.3 % in non-rinsed skin products.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

The acute toxicity of hexamidine is considerable. Oral LD_{50} values (in mg/kg b.w.) are 710-2500 in mice, 750 in rats, 500 in rabbits.

Intraperitoneal values of 17-51 and 57 were reported for mice and rats, respectively. Intravenous values are 17 for mice and 8 for rabbits. A dermal value for rats was >4000.

3.4 Repeated dose oral toxicity

A recent short-term (4 wk) oral study was conducted by gavage administration of 50, 100 and 200 mg/kg b.w./day to groups of 5 rats/sex. All test animals showed post-treatment symptoms (salivation, wet fur, brown oral staining). The top-dose rats also showed abnormal position and locomotion, and increased counts of white blood cells and lymphocytes. In the two higher dose groups there were increases in the values of GPT, GOT and calcium in blood plasma. All treated rats showed caecal enlargement. The lungs, heart, liver, kidneys and caecum did not reveal treatment-related microscopical changes. Other organs (including spleen and adrenals) were not examined. The clinical signs and the caecum enlargement were not considered to be of toxicological significance. The no-toxic effect level was established at 50 mg/kg, but the study showed several deficiencies.

3.5 Repeated dose dermal toxicity

A subacute (28 day) dermal toxicity study in rabbits showed that solutions of up to 2 % were only slightly irritant. Daily application of 4 ml/kg b.w. of a 0.05, 0.1 and 2 % solution revealed no systemic toxicity.

3.7 Subchronic oral toxicity

In a 90-day oral study in male rats, daily doses of 400 and 800 mg/kg by gavage induced mortality, growth depression, signs of anaemia, increased liver weight and decreased liver- and kidney function. The lower dose of 200 mg/kg was not a clear no-effect level.

3.8 Subchronic dermal toxicity

A 90-day dermal study in rabbits with the very low dose level of 16 mg/kg b.w. revealed no systemic toxicity.

4. Irritation & corrosivity

A concentration of 0.1 % was slightly irritating to the skin and eye of the rabbit.

5. Sensitization

Hexamidine did not produce any evidence of sensitization in guinea pigs nor of photosensitization using a rabbit model. However there is some evidence for sensitization reactions occuring in man following its use as a topical bacteriocide.

7. Toxicokinetics (incl. Percutaneous Absorption)

Studies using radiolabelled material to investigate skin absorption in the rat indicated very poor absorption. When the compound was applied as a 0.1 % formulation in cold cream under an occlusive dressing for 96 hours a mean of ca. 0.6 % was absorbed (maximum value 1.4 %). Very little absorption is thus likely to occur in use.

8. Mutagenicity

Negative results were obtained in the Ames test *Salmonella thyphimurium* strains TA1535, 1537, 98 and 100 and concentrations up to 500 µg/plate were used. Negative results were also obtained in a metaphase analysis assay to investigate the clastogenicity of the compound in CHO cells. A small increase in aberrations was seen at the intermediate dose but not at the top dose, and the increase was within the laboratories historic control range.

11. Conclusions

Hexamide has moderate acute toxicity by the oral route, but is highly toxic by injection. It is poorly absorbed through the skin and has low toxicity by this route. A 0.1 % solution was slightly irritating to the skin and eyes of rabbits, and there is no evidence of any sensitization potential. The no-effect-level in a 28-day repeated dose oral study was 50 mg/kg. In a 90-day repeated dose study marked toxicity occurred in various organs (especially liver, kidney, haematopoietic system) at 400 mg/kg with marginal effects at 200 mg/kg. Negative results were obtained when the compound was tested for mutagenic potential using the *Salmonella* assay, and also in an *in vitro* assay for chromosome damage in mammalian cells. In view of the very low levels of compound likely to be absorbed through the skin in use studies to specifically investigate effects on the reproductive system are not considered necessary.

Classification: A

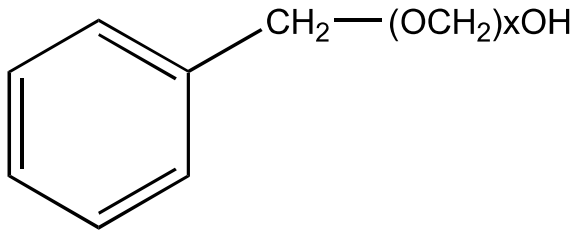
P 21: *BENZYLFORMAL*

1. General

1.1 Primary name

Benzylformal
(mixture of benzyloxymethanol and benzyloxymethoxymethanol; Preventol D_2)

1.5 Structural formula



x = ~ 1.5

1.9 Solubility

Soluble in organic solvents; solubility in water 25 g/l.

2. Function and uses

Used up to 0.2 % in all types of cosmetics.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The oral LD_{50} in rats was 1700 mg/kg; the i.v. LD_{50} in rats was 153 mg/kg. The animals showed sedation, loss of consciousness, paralysis.

3.2 Acute dermal toxicity

The dermal LD_{50} in rats was >1000 mg/kg. In rabbits, dermal LD_{50}-values of 1429 and 2000 mg/kg for males and females respectively were obtained.

3.4 Repeated dose oral toxicity

A 29-day repeated dose toxicity study has been performed in the rat. Compound was given by gavage at doses of 30, 100 and 300 mg/kg as a solution in polyethylene glycol 400 to groups of 10 male and 10 female rats. The only sign of toxicity noted was a slight reduction in body weight gain at 300 mg/kg in the male animals. Haematological examination revealed increased

leucocyte count in the males at 300 mg/kg but no other effects. At autopsy increased adrenal weight was seen in the females at the top dose level only; minor changes were reported in other organs, but there were no dose related trends and these were not significant. Histopathology revealed inflammatory changes in the mucosa of the glandular stomach but no other adverse effects. The NOAEL in this study was 100 mg/kg.

3.8 Subchronic dermal toxicity

A subchronic dermal study has been carried out in the rabbit. Doses of 1, 4 and 16 mg/kg body weight were given to groups of 10 male and 10 female animals 5 days a week for 90 days. Signs of toxicity noted at 16 mg/kg were reduced weight in the females and reduced blood cholesterol in both sexes. Local effects on the skin were seen at 4 and 16 mg/kg, these being dose-related. At autopsy pituitary weight was decreased in the males at the intermediate and high dose level but no pathological changes were seen. There was no histological evidence of damage in any other organ. The NOAEL in this study was thus around 1 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

A skin irritation test in rabbits with 500 mg undiluted substance applied to the intact skin of the ear for 8 hours induced redness and oedema; when applied for only 2 hours, slight redness was observed. A 0.2 % aqueous solution applied for 24 hours did not induce any changes.

4.2 Irritation (mucous membranes)

In an eye irritation test in rabbits 50 mg undiluted substance caused erythema and oedema and an opaque cornea. A 0.2 % aqueous dilution only produced erythema.

5. Sensitization

A sensitization test by the Landsteiner-Draize method with 0.1 % of the test substance in saline both for the induction and for the challenge treatment did not reveal signs of sensitization.

8. Mutagenicity

An Ames test gave positive results when tested at up to 500 µg/plate with *S. typhimurium* TA100; this result was attributed to the presence of 29.7 % formaldehyde in the product. Negative results were obtained in a micronucleus test when mice were given doses of 2 x 250 and 2 x 500 mg/kg with an interval of 24 hours.

11. Conclusions

The substance liberates formaldehyde (at a maximum of 0.004 % under test conditions). Although studies on dermal absorption are not available, appreciable uptake through the skin is suggested by a comparison of the oral and the dermel LD_{50} values and the subchronic study in rabbits using dermal exposure. The NOAEL in a 29-day oral study in the rat was 100 mg/kg.

A much lower value was however obtained in a 90-day dermal study in rabbits, namely 1 mg/kg. Negative results were obtained in an Ames test and a micronucleus test but no *in vitro* data are available on the ability of the compound to produce chromosome damage. Also no data are available on teratogenicity. The maximum exposure to humans in use is about 1 mg/kg, allowing no safety margin. Even if the effects on the pituitary are ignored (and no convincing arguments have been made to support this contention), the NOAEL is 4 mg/kg. The resulting safety margin is also unacceptable. It was thus concluded that this preservative should not be used in cosmetics.

Classification: D

P 71: *BENZALKONIUMCHLORIDE*

1. General

1.1 Primary name

Benzalkoniumchloride

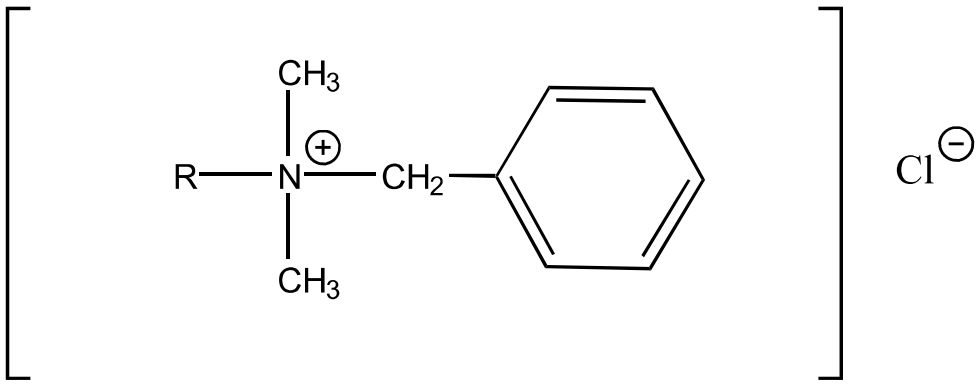
1.2 Chemical names

Alkyl (C_8 - C_{18}) dimethylbenzylammoniumchloride, -bromide and -saccharinate (benzalkoniumchloride)

1.3 Trade names and abbreviations

Colipa no.: P 71

1.5 Structural formula



1.6 Empirical formula

Emp. formula: Dodecyldimethylbenzylammoniumchloride: $C_{21}H_{38}NCl$
Mol weight: 348

1.9 Solubility

Soluble in water and alcohols, poorly soluble in hydrocarbons, oils and fats.

2. Function and uses

Used as a preservative at levels of 0.25 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50} - values for rats and mice obtained for commercial products with different alkyl groups usually vary between 0.5 and 1.0 g/kg b.w. Intravenous LD_{50} - values in mice of 12.8 26 mg/kg have been reported.

Intranasal administration of 0.06 ml of a 0.125 % solution was lethal for rats.

3.7 Subchronic oral toxicity

Sub-chronic (13-wk) oral studies in rats revealed toxicity and mortality at dose levels of 25 mg/kg b.w. and above. With 25 mg/kg b.w., administered to dogs daily for 52 weeks, mortality and gastrointestinal damage was observed.

Further, sub-chronic (90-day) oral studies in rats and dogs, conducted in 1968, became available recently (Colipa subm. IV, ref. 20 and 21). In both studies, dose levels of 5, 12.5 and 25 mg/kg b.w./day were administered; in the rat study by stomach tube, in the dog study by capsule. No changes attributed to treatment were observed. Because the studies showed several deficiencies, the results do not justify to establish a NOAEL.

In a 2 year rat study, 0.5 % in the diet (250 mg/kg b.w.) caused high mortality and pathological changes in the gastrointestinal tract. Microscopical changes of the intestinal tract were seen also in a 2-year study with a second commercial product at dose levels of 25 and 12.5 mg/kg, and in a 2-year study with a third product at a dose level of 30 mg/kg b.w. Dogs given 50 mg/kg b.w./day by gavage (at a concentration of 5 %) showed changes in the intestinal tract after one year.

3.8 Subchronic dermal toxicity

A dermal 90-day study was conducted in rats with a formulation containing 1 % stearyl dimethylbenzylammomiumchloride and 0.2 % benzalkoniumchloride 50 %. Once daily, 5 days/week for 13 weeks the rats received topically 2.4 ml/kg (2.4 mg benzalkonium-chloride/kg). It is stated that no significant local or systemic effects occurred. However, the report is confusing and incomplete.

Dermal life-time studies in mice and rabbits, treated topically with 0.02 ml of 8.5 or 17.0 % solutions twice weekly showed local skin damage in both species, but no skin tumours.

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin irritation tests in rabbits with 0.1 % solutions, and in humans with 1.0 % solutions were negative. With extended contact period in the rabbit, or repeated application in humans these concentrations produced distinct irritation. In rabbits, repeated application of 0.3% induced only mild erythema.

4.2 Irritation (mucous membranes)

Eye irritation in rabbits may occur upon a single application of 0.01 % solution and above and upon repeated application of 0.004 %. Concentrations of 0.01 % and above caused eye irritation in guinea pigs when applied repeatedly on the same day. Single treatment of human eyes with 0.1 %, or daily treatment with 0.03-0.04 % caused irritation.

Soft contact lenses disinfected daily with 0.0025 % benzalkoniumchloride + 0.01 % EDTA induced severe irritation when brought into contact with the rabbit eye for 6 hr/day.

5. Sensitization

A sensitization test in 100 male and 100 female volunteers with 0.1 %, applied daily for 5 days, followed by a challenge treatment with 1 % after 3 weeks, was negative. In the literature only a few cases of sensitization in humans have been reported. Short-term oral administration to several animal species in the diet or the drinking water containing concentrations of 0.02 % or more induced toxic effects.

6. Teratogenicity

In an oral teratogenicity study, groups of 15 pregnant rabbits were treated by gavage with 0, 10, 30 or 100 mg/kg/day (in aqueous solutions of 0.5, 1.5 and 5.0 % respectively) from day 7 through day 19 of gestation. All rabbits of the high dose group died. The intermediate dose caused maternal and embryotoxicity. Signs of maternal toxicity occurred also in the low-dose group. There were no indications of teratogenic properties.

A dermal teratogenicity study was conducted in rats treated topically with 0.5 ml aqueous solutions of 1.6, 3.3 and 6.6 %, (estimated to be about 30, 60 and 120 mg/kg) once daily from day 6 to day 15 of pregnancy. No embryopathic effects were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin penetration tests *in vitro* in pieces of human skin were conducted in aqueous solutions of 0.005 M to 0.1 M benzalkoniumchloride (i. e. 0.17 to 3.4 %). No penetration into the dermis was detected when the solution was unbuffered or acid. Measurable penetration occurred when the epidermal barrier was damaged or with intact skin in solutions of pH 11.

No penetration was found *in vitro* with skin from hairless rats exposed to 2.5 % ^{14}C-dimethylbenzylammoniumchloride for 4.5 hours. In a similar *in vitro* test with human epidermis the mean penetration was 1.47 % of the dose applied.

However results from an *in vivo* study to measure percutaneous absorption in rats indicate much higher absorption than indicated from the *in vitro* data. C^{14} - radiolabelled compound (0.4 ml) was applied to shaved skin of groups of 6 male and 6 female rats under occlusive dressing for 72 hours and the amount of material excreted in the urine and faeces during that time measured; the amount remaining in the carcass of the animals was also determined at that time. In the female animals values of 0.7 ± 0.4 %, 6.1 ± 3.4 % and 7.0 ± 2.2 % were obtained for urine and faecal elimination and remaining in the carcass respectively. The corresponding values in the male animals were 0.8

± 0.3 %, 9.9 ± 2.6 % and 5.3 ± 1.6 % respectively. The bulk of the applied dose remained on the treated skin. These data indicate that 14 % of the applied dose was absorbed in the females and 16 % in the males, giving an overall value of 15 %.

The distribution of the compound was studied after oral, rectal and intramuscular administration of the 10-fold lethal dose to rabbits, dogs and cats. Most of the dose remained at the application site. After oral and rectal administration, small amounts were detected in blood and liver. Upon rectal administration a small amount was found also in the kidneys.

8. Mutagenicity

A mutagenicity test with *S. typhimurium* His G 46-uvr B exposed to 10-100 µg/plate was negative. A micronucleus test in mice treated i.p. with 20 mg/kg b.w., twice, with an interval of 24 hours did not reveal increased numbers of micronuclei. The substance was found to induce repairable DNA damage in the E. coli DNA polymerase A assay, but no mutagenic properties were observed. No forward mutations were induced in Schizosaccharomyces pombe P_1 with or without metabolic activation. A chromosome aberration test with CHO-cells *in vitro* was negative.

11. Conclusions

Benzalkonium chloride possesses considerable irritant properties for the eye and the gastrointestinal tract and was highly toxic under certain conditions of acute exposure. In short and long term toxicity studies effects on the gastrointestinal tract were seen in rats and rabbits with oral doses of 12.5 mg/kg/day, with mortality in rats and dogs at 25 mg/kg/day. In a teratogenicity study in rabbits signs of maternal toxicity were seen, but no evidence of teratogenicity at the lowest dose tested namely 10 mg/kg; both maternal toxicity and embryotoxicity was seen at 30 mg/kg.

It is unlikely that all cosmetic products used would contain this compound. However the maximum allowed concentrations (3 % for rinse off hair products, 0.5 % for other products) appears high in the light of the irritant properties to the mucous membranes and consideration should be given to a reduction to below 0.5 % unless compelling reasons are provided for the use of relatively high concentrations of the compound. It is noted that the available data are still inadequate to firmly establish a NOAEL on repeated exposure.

Classification: B

12. Safety evaluation (Calculation of safety margin)

Benzalkoniumchloride is used in cosmetics both as a preservative (up to 0.1 %) and for other uses. Considering the preservative use, and assuming 30 g application per day, extreme exposure from this route would be ca 0.5 mg/kg/day. Assuming 15 % absorption this is equivalent 0.075 mg/kg; this gives a safety margin of around 125 over a marginal effect level (effects on gastrointestinal tract due to local irritant effects following oral dosing).

Exposure from other routes may be up to 2.5 mg/kg/day, but the bulk of this is from the rinse off hair products; estimation of the absorbed dose from this area is difficult, but very much less than 15 % is likely to be absorbed.

P 84: *SODIUM HYDROXYMETHYLAMINO ACETATE*

1. General

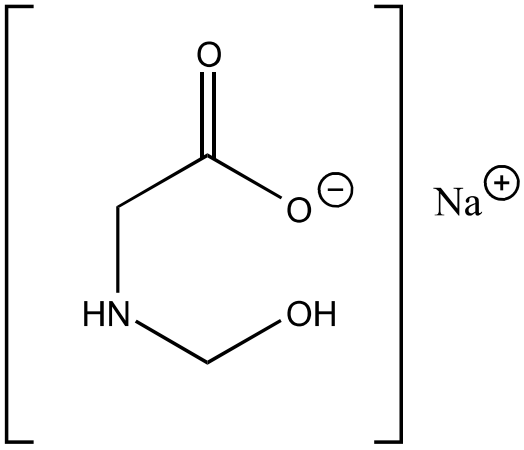
1.1 Primary name

Sodium hydroxymethylamino acetate

1.2 Chemical names

Sodium hydroxymethylamino acetate
(Sodium hydroxymethyl glycinate; Suttocide A)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_3H_6NO_3Na$
Mol weight: 127.1

1.9 Solubility

The compound is strongly alkaline, highly soluble in water, soluble in methanol, propylene glycol and glycerin, but insoluble in most organic solvents.

2. Function and uses

A preservative for use in cosmetics at concentrations of 0.05 % to 0.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}-values in rats were estimated to be 1.067 g/kg b.w., and 1.410 g/kg b.w. in two separate studies.

3.2 Acute dermal toxicity

The dermal LD_{50} in rabbits was > 2 g/kg b.w. The undiluted material applied dermally under occlusion caused a severe reaction to the skin probably as a result of the alkaline properties.

3.4 Repeated dose oral toxicity

A 28 day repeated dose study has been carried out in the rat with the compound administered orally by gavage at dose levels of 40, 160 and 640 mg/kg. There was a decrease in body weight gain in males at 640 mg/kg and in the serum total protein value which was outside the historical range of control values. There were some alterations in haematological parameters in this group which although within the range of historical controls were considered treatment related. Gross findings at necroscopy were reddening of the gastric mucosa in some animals at 640 mg/kg. Histological examination revealed 2 males and 5 females with focal subacute gastritis and 3 females with focal ulcerations at 640 mg/kg. There was also a death in this dose group, probably due to technical error but the possibility that it was compound related could not be ruled out. All other findings were considered coincidental or of no biological significance. The No Effect Level was 160 mg/kg.

3.7 Subchronic oral toxicity

In a sub-chronic oral toxicity study, 4 groups of 10 rats/sex received by gavage 0 (control), 10, 40 or 160 mg/kg b.w./day as a 2 % aqeous solution for 90 days. There were no clinical signs of toxicity or changes in body weight gain, food intake, haematology, clinical chemistry or urine examinations. Gross or microscopic examinations did not reveal any treatment-related effect. The No Effect Level in this study was thus the highest dose used, namely 160 mg/kg (as had been observed in the 28 day study which had been carried out after the 90 day study, primarily to identify toxic effects and target organs).

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin irritation tests in rabbits, showed a 5 % aqueous solution to be moderately irritating, while a 0.5 % solution produced only slight, transient irritation. In a repeated dermal application test, guinea pigs received 0.5 ml aqueous dilutions of 50, 7.5, 0.75 and 0.38 % under occlusion on days 1, 3 and 6 of one week period. No signs of oedema or irritation were observed.

4.2 Irritation (mucous membranes)

Eye irritation tests in rabbits conducted with 100 mg undiluted powder showed moderate irritation when the eye remained unwashed, and mild irritation when the eye was washed after treatment. A 5 % aqueous solution was mildly irritating if not washed out, and not irritating if washing was applied. Relatively mild, transient effects were also seen with a 50 % aqueous solution.

5. Sensitization

Sensitization was examined in guinea pigs, by the Landsteiner test, the maximization test and the Buehler test. In the Landsteiner test, 0.1 ml 0.1 % solution in saline was injected intradermally ten times, once every other day. After a two weeks rest period, the intradermal challenge injection of 0.05 ml 0.1 % solution did not reveal any sensitizing properties. In the maximization test, the induction treatment consisted of 6 intradermal injections of 0.1 ml 5 % solution, followed, 8 days later, by topical application of 0.3 g moistened powder. On day 22, a topical challenge treatment with a 50 % aqueous dilution produced a positive reaction in 7 out of 10 animals. When the challenge was repeated 7 days later, with 5 % and 0.5 %, 4/10 and 2/10 animals respectively reacted positively. These results indicate that the substance has some sensitizing properties. In the Buehler test, 0.5 ml 0.5 % aqueous solution was applied topically 10 times during 3 weeks. After 2 weeks rest, animals were challenged with a 0.5 % solution; there was no evidence of sensitization in any animal.

The ability of a 0.5 % solution of the substance to induce skin sensitization in human volunteers (102) has been investigated. The induction regime consisted of 9 patch applications (24 hour occlusion) over 3 weeks. There was no evidence of skin sensitization in any subject.

6. Teratogenicity

The ability of the compound to produce adverse effects on the developing fetus has been investigated in the rat. In a sighting study deaths were seen at 750 mg/kg, and thus dose levels of 150, 300 and 450 mg/kg were used in the main study. This study used 27 mated females per group given these dose levels on days 6 to 15. There was no significant difference in the percentage gravidity between the groups (92.6 % to 96.3 %). There were 2 maternal deaths at 150 mg/kg both attributable to technical error on dosing. There was post-dose salivation in some animals at all dose levels and there was also decreased activity at 450 mg/kg. There was 1 fetal death at 300 mg/kg which was considered coincidental. There were no significant differences in the total number of implantation sites, corpora lutea, viable and non viable fetuses, fetal sex distribution and body weight, early or late resorptions, number and percent of pre- and post- implantation loss. No soft tissue malformations were observed and there was no significant difference in the skeletal variations. Skeletal malformations were seen in 8 fetuses, 7 from one litter at 150 mg/kg; these were not considered treatment related. The study thus provided no evidence that the substance had any teratogenic potential. The No Effect Level in this study was 300 mg/kg with minor effects seen in the maternal animals at 450 mg/kg.

8. Mutagenicity

A number of mutagenicity studies have been carried out on this substance. An Ames test using up to 0.5 mg/plate in 5 strains of *S. typhimurium*, with and without metabolic activation did not indicate mutagenic properties. A somewhat limited study to investigate unscheduled DNA synthesis in rat hepatocytes using an autoradiographic method and concentrations up to 20 µg/ml gave negative results. Higher concentrations were not used because of cytotoxicity. The positive control gave the expected result. The data obtained were not however confirmed in an independent experiment. No information was available from *in vitro* studies to assess the clastogenicity of the compound. A micronucleus test was conducted in mice treated once orally with 375, 625 or 875 mg a.i./kg b.w. Five mice/group were examined at 30, 48 and 72 hrs after treatment. No increased incidence of micronucleated cells was observed.

11. Conclusions

In summary hydroxymethylamine acetate has low acute toxicity by the oral and the dermal route, and has marked irritant properties due to its alkaline nature. The in use concentration however produced no significant irritant effects. There was evidence that a 5 % solution could induce sensitization using the Magnusson Kligman Maximisation test, but negative results were obtained in the Buehler test at 0.5 % and also in human volunteers at 0.5 %. The No Effect Level in repeated dose studies (28 and 90 days) using the oral route was 160 mg/kg; at 640 mg/kg signs of marked toxicity to the gastrointestinal tract were noted. The mutagenic potential of the substance has been investigated *in vitro* using the *Salmonella* assay and a limited study to investigate the induction of unscheduled UDS in hepatocytes negative results being obtained in both cases. No *in vitro* data are available from metaphase analysis to assess the clastogenicity of the compound, but there is a negative *in vivo* micronucleus assay in the mouse using oral doses of up to 875 mg/kg. Inateratogenicity study in the rat using the oral route the compound gave no evidence of any adverse effects on the developing fetus at dose up to 450 mg/kg; slight effect on the maternal animals were seen at this dose level. The test to investigate chromosome aberrations *in vitro* requested in 1987 has not been provided.

The Committee also noted at that time that the concentration of this compound needed for preservation of cosmetic products is probably considerably less than 0.5 %.

Classification: B.

OPINIONS ADOPTED DURING THE 50TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 2 June 1992

A 97: *1-HYDROXY-2-AMINO-6-METHYL-BENZENE*

1. General

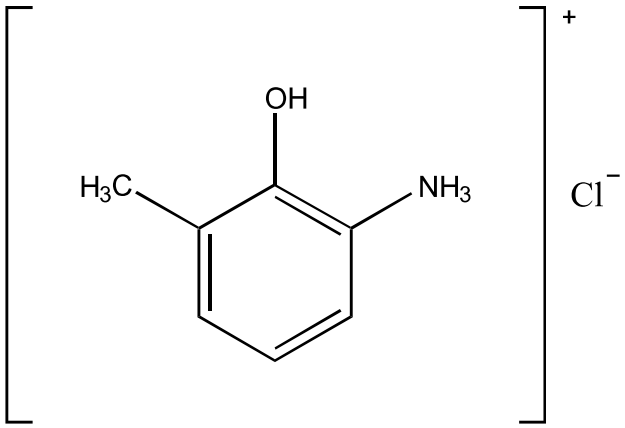
1.1 Primary name

Oxyorange

1.2 Chemical names

1-hydroxy-2-amino-6-methyl-benzene, hydrochloride

1.5 Structural formula



1.8 Physical properties

Appearance: The compound is a yellow-orange powder.

1.9 Solubility

It is soluble in water and some organic solvents but no quantitative solubility data were available.

2. Function and uses

The compound is supplied as an oxidative hair dye at concentrations up to 3 % and used at concentrations of 1.5 % after dilution with hydrogen peroxide. It is supplied as the hydrochloride salt.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats and mice following administration in water. The following LD_{50} values were obtained: female mice 1288 mg/kg, male and female rats

1175 mg/kg. The only sign of toxicity reported in these studies apart from death was a decrease in activity.

3.7 Subchronic oral toxicity

A 90 day study has been carried out in the rat with the compound administered by gavage at dose levels of 15, 30 and 60 mg/kg. No compound related effects were seen on weight gain, clinical chemistry, haematology, urinalysis or an examination of tissues at autopsy at either 15 or 30 mg/kg. There was a reduced body weight gain at 60 mg/kg and a pale-red colouration of the fatty tissue surrounding the testes and histological examination revealed deposits of pigment/haemosiderin in the spleen.

4. Irritation & corrosivity

4.1 Irritation (skin)

No signs of skin irritation were observed in guinea pigs following thrice daily 20 minute application of a 1 % solution for two days to abraded skin. The intensive colouration of the skin by the dye prevented the observation of erythema.

4.1 Irritation (mucous membranes)

Eye irritation has been studied in the guinea pig. A 0.1 ml aliquot of a 1 % aqueous solution was instilled into the right eye. No irritation was seen at this dose.

5. Sensitization

The ability of the compound to induce skin sensitization has been studied in the guinea pig using the maximisation method. The compound was dosed at a concentration of 3 % for both induction and challenge and neither irritation nor sensitization was observed.

6. Teratogenicity

In a teratogenicity study groups of 25 mated female rats received 5, 15 or 40 mg/kg by gavage on days 6 to 15 of gestation. No treatment related effects were reported in either the dams or the fetuses.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption has been investigated in two *in vivo* studies in rats using radiocarbon labelled oxyorange. In the first study the compound was applied under occlusion as a component of a hair dye formulation mixed with hydrogene peroxide for 30 minutes or in a dimethylsulphoxide solution for 24 hours. A total of 1.77 % and 34.85 % respectively were absorbed with the majority renally eliminated. The application site skin contained around 3 % of the dose and the majority was recovered from the application site washings and the dressings. In the second study oxyorange was applied at 2 % in a hair dye formulation with and without hydrogen or as a 6.66 % aqueous solution to the skin for 30 minutes. The dermal absorption was 0.31, 0.36 and 0.75 % in the absence and presence of hydrogen peroxide and

for the aqueous solution respectively with a further 0.13, 1.38 and 0.67 % remaining at the application site. The urine is the major route of elimination after both oral and dermal administration with 90 % of the dose eliminated by this route after oral administration and a similar proportion of the absorbed dose after dermal administration.

8. Mutagenicity

Negative results were obtained in a study to investigate the ability of oxyorange following pre-incubation with hydrogen peroxide to produce gene mutation in Salmonella typhimurium.

Strains TA1535, TA1537, TA1538, TA98 and TA100 were investigated both in the presence and absence of an exogenous metabolic activation system. The compound precipitated at concentrations greater than 0.25 µg/plate or 123 µg/plate. Negative results were also obtained in an investigation of gene mutation in L5178Y mouse lymphoma cells (HGPRT locus) at concentrations up to those causing considerable toxicity (250 µg/ml) both in the presence and absence of an exogenous metabolic activation system. Oxyorange was not mutagenic in the micronucleus test at total doses of 10000, 2000 and 4000 mg/kg given orally in two equal doses 24 hours apart; bone marrow toxicity was only observed at the highest dose. Negative results were obtained in an *in vivo* study to investigate induction of sister chromatic exchange in bone marrow following oral administration of 60, 192 and 600 mg/kg.

9. Carcinogenicity

No data on carcinogenicity studies on oxyorange was available.

11. Conclusions

Oxyorange has moderate acute toxicity by the oral route, however studies suggest that dermal penetration from hair dye formulation is low. There was no evidence of skin or eye irritation with a 1 % solution. There was no evidence of sensitization in a maximisation test in guinea pigs. In a 90 day oral study a no effect level of 30 mg/kg was reported. Mutagenicity data comprised negative results *in vitro* (gene mutation in Salmonella and mouse lymphoma cells) and *in vivo* (micronucleus test and sister chromatid exchange in bone marrow). No adverse effects were reported in an oral teratogenicity study in rats up to 40 mg/kg.

Classification: A

B 29: *4-AMINO-2-NITROPHENOL*

(Colipa no. B29)

The same dossier on this compound is supplied before in 1982. Since no new data on the compound were included in the recently provided dossier, the summary on this compound in 1992 remains as it was made in 1982. Based on the summary of 1982 the following comments can therefore be made 1992:

It is not possible to make an evaluation based on the supplied data on the compound 4-amino-2-nitrophenol. The dossier consists for the greater part of published literature, with no detailed information. The remainder consists of inadequate/incomplete studies or just the summary of the study.

* Data on irritation, sensitization and absorption are not supplied.
* In acute toxicity studies an oral LD_{50}-value of 3300 mg/kg b.w. was found in rats, and an intraperitoneal LD_{50} value of 302 mg/kg b.w. was found in mice.
* For the subacute toxicity only summaries were supplied.
* The semichronic dermal toxicity study in rabbits was incomplete: No detailed information on organ weights and histopathology is presented.
* A reproduction study in rats was incomplete, only tables with results presented.
* Teratogenicity study was incomplete, no details on histopathology and body weights.
* Chronic toxicity/carcinogenicity: oral studies in mouse and rat and dermal studies in mouse and rabbit are available. An increased tumor incidence was seen in the urinary bladder of orally dosed rats. Of these increases that of the transitional cell carcinomas in males dosed with 2500 ppm was significant. A number of tumours in the ovary and uterus and some skin pappilomas near the penis were found in treated DBAF mice.
* Mutagenicity data are incomplete/inadequate. Only a dominant lethal test in rats and an Ames test with only 2 strains (TA 1535 & TA 1538) were supplied.

Conclusion

Because the compound has shown to be carcinogenic in male rats (producing an increase in transitional cell carcinomas of the urinary bladder) and because of similar concerns regarding the very closely structurally related hair dyes B26 and B27, the compound should not be used in cosmetics.

Classification: D

B 49: *ROT X*

1. General

1.1 Primary name

Rot X

1.2 Chemical names

1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene
2-amino-4-chloro-5-(2-hydroxyethyl)-amino-nitro-benzene
3-chloro-4-(2-hydroxyethylamino)-6-nitro-aniline
5-chloro-4-(2-hydroxyethylamino)-2-nitro-aniline
1-amino-2-nitro-4-2-oxyethylamino-5-chloro-benzene

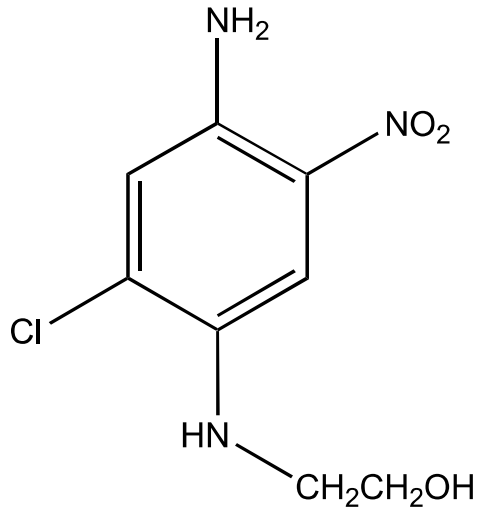
1.3 Trade names and abbreviations

Colipa No. B49

1.4 CAS no.

not given

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}\ H_{10}\ Cl\ N_3\ O_3$
Mol. weight: 232

1.7 Purity, composition and substance codes

sA = commercial product

91 % 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene
5.4 % 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene
3.6% 1-amino-2-nitro-4-amino-5-chloro-benzene

sB = purified recrystallized Rot X according to Colipa (ref. 1) probably sE

sC = main component - 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene (purity ≥ 99 %)

sD = accessory compound - 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene (purity ≥ 99 %)

sE = 90 % 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene
6 % 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene
4 % 1-amino-2-nitro-4-amino-5-chloro-benzene

sF = 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene - dark red powder (According to Colipa sA)

Impurities:

Impurity	Quant	unit
* 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene	5.4 - 6	%
* 1-amino-2-nitro-4-amino-5-chloro-benzene	3.6 - 4	%

Composition of formulation(s):

Formulations (fA) used in 18 month skin-painting study in mice.

	percentage in			
	fA_0	fA_1	fA_2	fA_3
1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene	-	0.5023	0.5023	1.0
1-amino-2-nitro-4-di-(2-hydroxyethyl)-amino-benzene	-	0.013	1.0065	2.0
sodium lauryl ethersulfate	1.05	1.05	1.05	1.05
stearic acid diathanolamide	0.625	0.625	0.625	0.625
copolymer of alkylmethacrylate and methacrylic acid	0.312	0.312	0.312	0.312
isopropanol	15.0	15.0	15.0	15.0
water	83.013	82.995	81.504	80.013

1.8 Physical properties

Subst. code: sA
Appearance: brown-black powder
Melting point: 116°C

1.9 Solubility

Soluble in water.

Freely soluble in: methanol, ethanol, acetone, chloroform, ethylacetate, methylchloride, diethylether.

2. Function and uses

Rot X is included in hair tinting products and colouring setting lotions at a maximum concentration of 1 %.

Rot X is also used in oxidative hair dyeing formulations at a maximum concentration of 2 %; yet in combination with H_2O_2 the maximum concentration at application is 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Unit	Remark
sA	oral	mouse	2850	mg/kg b.w.	10 % in 20 % Arabicum

Groups of 10 female CF1 mice received a single oral dose of 2, 2.5, 3 and 3.5 g sA/kg b.w. as 10 % suspension in 20 % gum Arabic by gavage. Observations time was 14 days. Loss of activity and staggering were seen preceeding exitus. No changes in tissues were seen (macroscopically).

Remark: Test was carried out in 1972.

3.7 Subchronic oral toxicity

Route: oral	Exposure: 90 d	DWE: 10	(unit): mg/kg b.w.
Species: rat	Recov.p.: 28 d	LED: 25	(unit): mg/kg b.w.
Subst.: sA			

Groups of 20-25 male and 20-25 female Wistar rats (b.w. 121-163 g) received daily, 7 days/week, by gavage, for 90 days 0, 10, 25 or 40 mg/ Rot X/kg b.w. as an aqueous solution. After 90 days 20 m and 20 f rats/group were killed. 5 male and 5 female rats of control and 40 mg group were maintained on a control diet for a 4-week recovery period.

Examinations: Daily behaviour and clinical signs. Weekly body weight, food- and water-consumption. At week 0, 6 and 13 in 5 m and 5 f rats/group ophthalmoscopy, hearing test and reflex examinations. At week 0, 6 and 13 in 10 m and 10 f/group and at the end of the recovery period in all remaining animals hematology (Hb, Ht, Er, Leu, Diff, MCV, MCH, MCHC, retics, thromb, prothr. time, inclusion bodies) and clinical chemistry (SAP, ALAT, ASAT, BUN, creatinine, glucose, total bilirubin, total proteins, albumin, serum electrophoresis, uric acid, triglycerides, cholesterol, Na, K, Ca, Fe, inorg. P). At week 0, 6 and 13 in 5 m and 5 f/group and at the end of the recovery period in all remaining animals urinalysis (s.g., pH, proteins, glucose, bilirubin, urobilinogen, blood, nitrate, ketones, sed.). Organs (8) of all animals were weighed in 20 m and 20 f rats/group after 13 weeks and in all remaining animals at the end of the recovery period. Macroscopy was performed in 20 m and 20 f rats/group after 13 weeks and in all remaining animals at the end of the recovery period. Microscopy of ca. 30 tissues was carried out in 10 m and 10 f rats of control and 40 mg group after 13 weeks. In addition trachea, lung, kidneys and uterus of 10 m and 10 f rats in 10 and 25 mg groups after 13 weeks and of all remaining animals at the end of the recovery period were examined microscopically.

Results: Animals on 25 and 40 mg/kg b.w. showed a dose-related slight to moderate increase of locomotor activity within 5-10 min. after administration of the test compound. Skin turgor was reduced in these both groups. Skin and mucous membranes of animals on 40 mg/kg b.w. were slightly discoloured. In all groups red discolouration of the urine was seen; this effect was not seen during the recovery period of 5 m and 5 f of the 40 mg/kg b.w. group. Growth, food- and water-consumption did not show abnormalities. Hematology was normal in all groups. Clinical chemistry revealed a dose-related increase of serum glucose values in males on 25 and 40 mg/kg b.w., but only after 6 weeks on test. Serum bilirubin values showed a significant increase in m and f animals on 40 mg/kg b.w., but again only after 6 weeks on test. Urinalysis did not reveal abnormalities with the exception of the red discolouration in all groups. Organ weights did not show any significant abnormalities. Histopathological examination showed a slight increasing tendency of inflammatory lymphocytic infiltrations in trachea, kidney and also in uterus from control to test animals on 25 and 40 mg/kg b.w. These effects are regarded as possibly related to administration of the test compound. At 10 mg/kg b.w. the frequency of these effects was equal to that in the control group. The no-effect level in this study is 10 mg/kg b.w.

3.10. Chronic toxicity

Route : skin Exposure : 18 mo.
species: mouse Carc.Study: yes
Subst. : fA

Groups of 75 male and 75 female NMRI mice (b.w. 15-33 g) received 3 times weekly for 18 months skin paintings with 0.05 ml of formulations containing 0.5 or 1.0 % 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene together with 0.013, 1.0 and 2.0 % 1-amino-2-nitro-4-di(2-hydroxyethyl)-amino-benzene, respectively (called fA_1, fA_2 and fA_3, respectively). One control group of 75 m and 75 f mice received skin paintings with 0.05 ml of a formulation without active ingredients (fA_0) and another control group of 75 m and 75 f mice received skin paintings with water only.

(for composition of the formulations used in this experiment see 1.7)

Examinations: Daily behaviour and clinical signs. Weekly body weight until week 13, thereafter biweekly. Weekly food consumption. All animals were subjected to weekly clinical examination and were checked for palpable masses. At 12 and 18 months in 10 m and 10 f mice/group hematology (Hb, Ht, Er, Leu, Diff, MCV, MCH, MCHC, retics, thromb.). After 18 months all surviving animals were killed and subjected to macroscopical examination. Microscopical examination of ca. 25 tissues of 50 m and 50 f animals from both control groups and the group receiving fA_3 was performed.

Results: No abnormalities were seen. No increased tumour frequencies were observed.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route : skin Exposure: 2d
Species: guinea-pig
Subst. : sA Concentr: 0.25 %

A group of 10 female guinea-pigs received three skin-paintings per day, with time intervals of 20 min., for 2 consecutive days, on a shaven skin area of 3 x 4 cm with a 0.25 % solution of Rot X in water containing 2 % methylcellulose. 20 min. after each skin-painting the skin was washed. Observations were made up to 3 days after the last application. Draize scoring system for rabbits was used. No skin reactions were seen.

Remark: Guinea-pigs instead of rabbits were used. (The test laboratory claimed a lot of experience with guinea-pigs.)

The concentration tested (0.25 %) was too low compared to the usage concentration (1 %).

No information was given on possible discolouration of the skin which could have been resulted in difficulties at examination of skin reactions.

The study was carried out in 1977.

4.1 Irritation (mucous membranes)

Route: eye
Species: guinea-pig Dose: 0.1 ml
Subst.: sA Concentr: 0.25 %

0.1 ml of a 0.25 % solution of sA in water were applicated in the conjunctival sac of the eyes of 10 female Pirbright guinea-pigs. The eyes were not washed. Observations were made 30 min., 2, 3, 4, 6, 7 and 24 hr after application. After 30 min. in 6 rabbits redness of conjunctiva was seen persisting to 1-2 hr after application in 2 animals. Draize scoring system for rabbits was used.

Remark: No observations were made after 48 and 72 hr. Guinea-pigs instead of rabbits were used. (The test laboratory claimed a lot of experience with guinea-pigs.) The concentration tested (0.25 %) was too low compared to the usage concentration (1 %).

No information was given on possible discolouration of the eye which could have been resulted in difficulties at examination of the ocular effects.

The study was carried out in 1977.

5. Sensitization (1)

Subst.: sA Conc.induc.: 0.5 %
Species: guinea-pig Conc.chall.: > 0.0005 %
Method: Landstein. Draize

A group of 15 f Pirbright guinea-pigs received 2 times daily, 6 days/week, for 3 weeks, intracutaneous injections in the shaven skin with 0.1 ml of an 0.5 % Rot X solution in 50 % ethanol. After a rest period of 4 weeks each animal received intracutaneous challenge injections with 0.1 ml of 1:10, 1:100; 1:500 and 1:1000 dilutions with Ringer's solution of the 0.5 % soln. of Rot X in 50 % ethanol. A control group of 5 f guinea-pigs was used. 24 and 48 hr after the challenge injections observations were made.

Results: During the 3 week induction period Rot X caused slight erythema of the skin. 24 hours after the challenge injections severe erythema was seen both in the test and in the control group diminishing after 48 hr to slight to well-defined erythema. According to the authors the test compound shined through the skin, which made evaluation difficult. No such remark was made with respect to the induction period. The authors concluded that Rot X did not cause sensitization in this test system.

Remark: Control and test group contained too few animals. The test was carried out in 1972.

5. Sensitization (2)

Subst.: sC Conc.induc.: 3 %
Species: guinea-pig Conc.chall.: 3, 2, 1 %
Method: Magnusson Kligman

A group of 10 m and 10 f Dunkan Heartly Pirbright guinea-pigs received during the induction period 2 series of 2 intradermal injections (0.05 ml each) with FCA (1:1 diluted in dist. H_2O) and 3 % Rot X in dist. H_2O, respectively. The next day the animals were pretreated dermally with 10 % Na-laurylsulfate in white vaseline (unoccluded) followed 6 - 8 hrs later by a dermal application with 0.5 ml of 3 % Rot X in white vaseline under occlusion. 48 hrs after the first 2 series of intradermal injections the occlusions were removed and a third series of 2 intradermal injections (0.05 ml each) with 3 % Rot X in FCA, diluted 1:1 with arachis oil, was given. 14 days later the animals were challenged by a closed 24 hr patch test using 0.5 ml of three different concentrations (1, 2 and 3 %) of Rot X in FCA 1:1 diluted in arachis oil per animal. Immediately and 24 hr after the removal of the bandage observations were made. A solvent control and a positive control group (with 1-chloro-2,4-dinitrobenzene) of 5 m an 5 f animals each were included.

Results: No primary irritation or sensitization was seen.

Remark: The test protocol deviated from OECD Guidelines for the Magnusson Kligman test. The dermal applications with Na-lauryl sulphate and the test compound were given on the day

after the first series of intradermal injections instead of on day 7 and 8 and thereafter a second series of intradermal injections with the test compound were given.

No observations were made 48 hr after removal of the bandage of the challenge application.

The solvent control group contained too few animals.

In addition no information was given on possible discolouration of the skin which could have been resulted in difficulties at examination of the skin reactions.

6. Teratogenicity

Route: oral Admin.days: 6-15

Species: rat

Subst.: sF

Groups of 25 pregnant Sprague-Dawley rats received by gavage daily during day 6-15 of pregnancy 0 or 10 mg Rot X/kg b.w. dissolved in distilled water with one or two drops of ammonia. The animals were killed on day 20 of pregnancy.

Examinations:

— Dams:

Daily signs of toxicity and behaviour. Body wt. on day 0, 6, 15 and 20 of gestation. No. of corpora lutea, no. and site of implantations, early intra-uterine deaths, early/late intra-uterine deaths, late intra-uterine deaths.

— Fetuses:

No. live/dead, sex ratio, body wts., gross abnormalities,

1/2 of fetuses - skeletal examination.

1/2 of fetuses - visceral examination.

Results: All dams showed red discoloured urine during dosing period. No maternal toxicity was seen. No irreversible structural abnormalities or embryotoxic effects were seen.

7. Toxicokinetics (incl. Percutaneous Absorption)

An average amount of 52.78 g of a hair dye formulation with 1 % Rot X was applied to washed hair of 5 female human volunteers for 15 min. Blood samples were taken 10, 20, 30, 45 and 60 min. and 2, 3 and 24 hr after the beginning of the treatment period.

Urine was collected 2, 4, 6, 8, 10, 12 and 24 hr after beginning of the treatment period. No Rot X was detected in serum or urine samples (limit of detection 20 and 6 ng/ml), respectively. Based on the values for the detection limit and assuming an average body weight of 62.5 kg for the volunteers, a maximum dermal absorption of 130 mg (0.25 %) has been calculated by the authors.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *In vitro* mammalian)

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	Salm.typh.	TA1535	base-pair subst.	1-1000 µg/pl in DMSO. At 1000 and 10000 µg/pl toxicity.	-	-	r	AR
*sA	Salm.typh.	TA1537	frameshift mut.	1-1000 µg/pl in DMSO. At 1000 and 10000 µg/pl toxicity.	+	+	r	AR
*sA	Salm.typh.	TA 1538	frameshift mut.	1-1000 µg/pl in DMSO. At 1000 and 10000 µg/pl toxicity.	+	+	r	AR
*sA	E.coli	343/133	gene-mut.	liquid test 1, 10, 100 µg/ml in water + 10 % DMSO No toxicity.	-			
*sB	mouse lymf L5178Y		gene-mut.	20,5 - 555 µg/ml in DMSO. At 1666 µg/ml toxicity.	-	-		
*sB	mouse lymf		gene-mut.	500-1250 µg/ml in DMSO.	-			
	L5178Y			> 500 µg/ml toxicity.				
*sC	Chin.Hamst ovary cell		chrom.aber	62.5, 125, 250- µg/ml in DMSO. No tox. Limit of solub 250 µg/ml	-	r	AR	
*sD	Chin.Hamst ovary cell		chrom.aber	200, 400, 800- µg/ml in DMSO. No tox. Limit of solub 800 µg/ml	-	r	AR	

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sE	hum.lymph.		chrom aber	60, 300, 700 µg/ml in DMSO. Toxicity seen at 700 µg/ml	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint; sp = species used for activation (r = rat, m = mouse, h = hamster); res = result of test (+ = pos., - = neg., e = equivocal); ind = inducer (AR = Arocolor, PH = Phenobarbital, MC = Methylcholantrene)

The Ames-test with compound sA was carried out in 3 strains only. In 2 strains both detecting frame-shift mutations, positive results were seen in both the absence and the presence of a metabolic activation system. The test was carried out in 1977.

In the toxicity test with mouse lymphoma cells without metabolic activation survival at 1666 µg/ml was < 1 %. At the next lower dose level of 555 µg/ml no reduction in survival was seen. Therefore a second experiment was carried out with a concentration range of 500-1250 µg/pl. In the second experiment at concentrations ≥ 500 µg/ml survival was ≤ 56 %.

In the first experiment no information was provided on species and inducer used for metabolic activation.

In the chromosome aberration test in CHO cells with sD a non-significant increase in aberrations including gaps was seen at 400 µg/ml in the presence of S-9 mix. Number of aberrations excluding gaps at this concentration and numbers of aberrations excluding and including gaps at all other concentrations without and with S-9 mix were lower than control values.

8.2 Mutagenicity (*In vivo* mammalian, Host mediated).

Sub	Species	Strain	Measured endpoint	Test conditions	Res.
* sA	rats	CFY	micronuclei	oral 2 x 1600 mg/kg b.w. susp in 0.5 % gum tragacanth - interval 24 h	-

In the micronucleus test rats were killed 6 h after last treatment. The animals showed signs of toxicity consisting of lethargy and hypopnoea. Red pigmented urine was observed in all rats treated with Rot X.

Indicator test (Bact., Non mammalian eukaryotic, *In vitro* mammalian):

Sb.	Species	Strain	Meas. endp.	Test condition	res -act	res +act	sp +a	ind +a
*sC	HeLa cells	UDS		0.0064 -500 µg/ml in DMSO. Some toxicity at 500 µg/ml.	-	-	r	AR
*sD	HeLa cells	UDS		0.0064-500 µg/ml in DMSO. Some toxicity at > 100 µg/ml	-	-	r	AR
*sE	Syr.hamst. embryocell		cell trans-formation	35-700 µg/ml-4h 10-100 µg/ml-48 h. Toxicity was observed.	-	-	r	AR

Abbreviations:
sp = species used for activation (r = rat, m = mouse, h = hamster); res = result of test (+ = pos., - = neg., e = equivocal); ind = inducer (AR = Arocolor, PH = Phenobarbital, MC = Methylcholantrene)

In the UDS test in HeLa cells a significant increase in DNA repair at one test level of 4 µg/ml in the absence of a metabolic activation system was seen.

However a dose-relationship was not seen and therefore this single result was considered as biologically insignificant.

Indicator test (*In vivo* mammalian, Host mediated)

Sub.	Species	Strain	Measured endpoint	Test conditions	Res.
*sE	rat	Wistar	UDS in hepatocytes	once 0, 170, 500, 1500 mg/kg b.w. in DMSO. Killing after 24 h.	-

10. Special investigations

UV/VIS, IR and NMR spectra:
UV/VIS spectrum present: yes

Analysis and detection (table)

Medium	Substance	Iso Method detec.	Detec. limit	unit	Minim unit samp.	Recov.
*serum *urine	Rot X Rot X	HPLC HPLC	20 6	ng/ml ng/ml		

11. Conclusions

General
Rot X is a brown-black powder used as a colouring agent in hair tinting products and colouring setting lotions at a maximum concentration of 1 % and in oxidative hair dyeing formulations at a maximum of 2 % (in combination with H_2O_2 maximum concentration at application is 1 %).

Metabolism
In a dermal absorption study human volunteers received an application on the washed hair with 52.78 g of a hair dyeing formulation containing 1 % Rot X during 15 min. No Rot X could be detected in blood serum or urine.

However this study cannot be used for evaluation of dermal absorption of Rot X because fecal excretion was not taken into account and possible deposition in tissues and carcass cannot taken into account. In addition possible breakdown products of Rot X are not taken into account in this study.

Acute toxicity
Rot X was only slightly toxic after a single oral dose to mice (LD_{50} is 2850 mg/kg b.w.). An LD_{50} study in rats is not available.

Irritation and sensitization
A skin- and an eye-irritation study were carried out in guinea-pigs with a 91 % pure product. No irritation was reported. However the Draize scoring system for rabbits was used while no material was supplied to compare reactions seen in guinea-pigs and rabbits. In addition the concentrations tested were too low (0.25 %) compared to the usage concentration (l %). Therefore these tests are not acceptable.

In a Landsteiner-Draize test in guinea-pigs no sensitization was seen, but skin examination was difficult because, according to the authors, the test compound shined through the skin (intradermal injections were given). In addition the control group was too small.

In a Magnusson-Kligman test in guinea-pigs (performed with a product with a purity $\geq$ 99 %) no primary irritation or sensitization was seen. However the test protocol deviated from OECD Guidelines and in addition no information was given on possible discolouration of the skin due to the aqueous solution of the test compound. Both sensitization studies are not acceptable.

Subchronic toxicity
A 90-day oral study in rats revealed a dose-related red discolouration of the urine in all groups (dose levels 10, 25 and 40 mg/kg b.w.) which had been disappeared in the recovery time of the 40 mg group. Increased activity within 5-10 min. after dosing and reduced skin turgor were seen at 25 and 40 mg/kg b.w. At 40 mg/kg b.w. light discoloured skin and mucous membranes were seen. Histopathology showed an increasing trend in the number of inflammatory and immune reactions in trachea, kidneys and probably uterus at 25 and 40 mg/kg b.w. These effects were considered as possibly related to the test compound. The no-effect level in this study is 10 mg/kg b.w.

Chronic toxicity
A 18 month dermal skin-painting study in mice with formulations containing 0.5 or 1 % Rot X together with 0.013 to 2.0 % of 1-amino-2-nitro-4-(2-hydroxyethyl)-amino-benzene did not reveal increased tumor frequencies.

Reproduction data
No embryotoxic or teratogenic effects were seen in a study with rats at the only dose level tested of 10 mg/kg b.w. However the dose level used was too low for evaluation of possible embryotoxic and teratogenic effects of Rot X (10 mg/kg b.w. is the no-effect level in the 13-week oral study). No maternal toxicity was seen at this level in the teratogenicity study.

Mutagenicity
The bacterial assay in *S.typhimurium* was carried out in only 3 strains with a 91 % pure product. In 2 out of 3 strains positive results were seen as well in the absence as the presence of a metabolic activation system. However it is known from scientific literature that aromatic amino/nitro compounds give often false-positive effects in the Salmonella assay (J. Ashby and R.W. Tennant, Mutat. res. 257, (1991), 229-306). In a test in Escherichia coli Rot X (purity 91 %) showed a negative result (only performed without metabolic activation).

In a gene-mutation study in mammalian cells *in vitro* no activity of Rot X (purity 90 %) was seen as well in the absence as the presence of a metabolic activation system. Chromosomal aberration studies in Chinese hamster ovary cells *in vitro* with Rot X (purity (99 %) and the accessory compound 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene (purity ≥ 99 %) showed negative results in the absence and in the presence of a metabolic activation system. In an *in vitro* study with human lymphocytes Rot X (purity 90 %) did not induce chromosomal aberrations. In the *in vivo* micronucleus study in rats no induction of micronuclei was seen after treatment with Rot X (purity 91 %).

Neither in two *in vitro* studies in HeLa cells with Rot X (purity ≥ 99 %) or the accessory compound 1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene nor in an *in vivo* study in rats (hepatocytes) with Rot X (purity 90 %) induction of UDS was seen. In a cell transformation assay *in vitro* Rot X (purity 90 %) gave also negative results.

Conclusion
Rot X is a colouring agent used in hair tinting products, colouring setting lotions and oxidative hair dyeing formulations at a final concentration of 1 %. The acute oral toxicity was studied in mice only and appeared to be slight. The dermal absorption study in human volunteers, the skin- and eye-irritation studies in guinea-pigs and the two sensitization studies submitted are not acceptable due to several deficiencies.

A no-effect level of 10 mg/kg b.w. can be established in a 90-day oral rat study, based on inflammatory and immune reactions seen at histopathological examination of trachea, kidneys and uterus.

An 18-month dermal skin-painting study in mice did not reveal increased tumour frequencies. The study was performed with formulations containing Rot X and a second amino/nitro benzene derivative, viz. 1-amino-2-nitro-4-di-(hydroxyethyl)-amino-benzene.

The teratogenicity study in rats did not reveal an effect at 10 mg/kg b.w. However only one dose-level was tested and this dose-level was too low to evaluate possible teratogenic and/or embryotoxic effects. Therefore this test is not acceptable. With respect to mutagenicity, tests with *S.typhimurium* cannot be used for evaluation of a possible mutagenic effect of Rot X due to the frequent induction of false positive effects by aromatic amino/nitro compounds in this test organism.

Rot X (ca. 90-91 % pure) showed no mutagenic activity in assays detecting gene-mutations in mammalian cells *in vitro*, chromosomal aberrations in mammalian cells *in vitro* and *in vivo*, UDS in rat hepatocytes *in vivo* or cell transformation in Syrian hamster embryo cells *in vitro*. Rot X with a purity ≥ 99 % and one of the impurities (1-(2-hydroxyethyl)-amino-2-nitro-4-(2-hydroxyethyl)-amino-5-chloro-benzene) purity (99 %) did not show activity in assays detecting chromosomal aberrations or UDS in mammalian cells *in vitro*. The other impurity in the commercial product of Rot X was not tested in any mutagenicity assay.

Based on the data mentioned above no definite evaluation of Rot X can be made before adequate studies on dermal absorption, skin- and eye-irritation, sensitization and teratogenicity have been supplied.

B 70: *1-(2'-UREIDOETHYL)-AMINO-4-NITRO-BENZENE*

1. General

1.1 Primary name

1-(2'-ureidoethyl)-amino-4-nitro-benzene

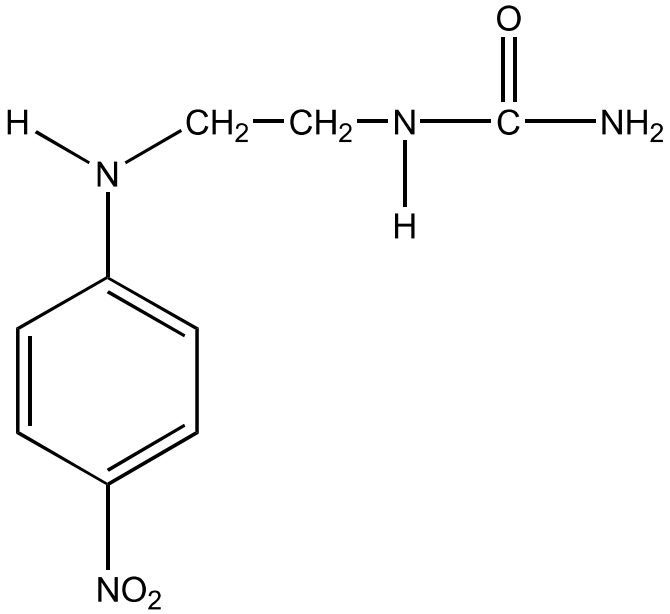
1.2 Chemical names

1-(beta-ureidoethyl)-amino-4-nitro-benzene
4-(beta-ureidoethyl)-amino-nitro-benzene
4-(beta-carbamidoethyl)-amino-nitro-benzene
4-(beta-carbamyaminoethyl)-amino-nitro-benzene
4-(beta-carbamoylaminoethyl)-amino-nitro-benzene
N-ureidoethyl-4-nitro-aniline

1.3 Trade names and abbreviations

sA: 1-(2'ureidoethyl)-amino-4-nitro-benzene (purity > 99 %)
sB: Ureidogelb (LGH 11 0583/2)
sC: Nitrogelb
sD: ureidogelb (batch no. 2912)
sE: 1-(2'-ureidoethyl)-amino-4-nitro-benzene (purity > 97 %)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{12}N_4O_3$
Mol weight: 224

1.8 Physical properties

Appearance: sA: dark yellow powder
Melting point: 178 - 180 ° C

1.9 Solubility

The substance exists as a free base. It is slightly soluble in ethanol, methanol and water; and it is insoluble in chloroform and ether.

2. Function and uses

1-(2'-ureidoethyl)-amino-4-nitro-benzene is included in hair tinting products, colour setting lotions and oxidative hair dye formulations at a maximum concentration of 0.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Unit.	Remark
sA	oral	mouse	7320	mg/kg b.w.	females only
sA	oral	rat	8000	mg/kg b.w.	

The test compound (a 10 % suspension in a 10 % Arabic gum) was given once by stomach tube to 6 male and 6 female Wistar rats at dose levels ranging from 6000 to 8000 mg/kg b.w. and to 10 female CF1 mice at dose levels ranging from 4200 to 11400 mg/kg b.w. The animals were observed for 14 days and organs of all animals were examined.

The calculated LD_{50} values for male and female rats were 8000 and > 8000 mg/kg b.w., respectively. The calculated LD_{50} value for female mice was 7320 mg/kg b.w. During the observation period a limitation of activity, tonoclonic spasms and exitus were seen. No organ changes were detected.

3.7 Subchronic oral toxicity

Route:	oral	Exposure:	13 wk	
Species:	rat	Recov.p.:	4 wk	LED: 5 mg/kg b.w.
Sust.:	sB			

180 young Wistar rats (BOR: WISW (SPF/TNO); 90 males and 90 females) were used in a 90-day oral study. The age at the beginning was 6 weeks and the weight was 120 - 154 g for males and 110 - 148 g for females. 20 males and 20 females received 5 or 20 mg/kg b.w. of ureidogelb (LGH 11 0583/2) daily for 90 days by stomach tube. One group of 25 males and 25 females served as controls and another group of 25 males and 25 females received 60 mg/kg b.w. of the test compound by stomach tube for 90 days. The animals had free access to food and water.

After 90 days all animals were sacrified except for 5 males and 5 females of the control group and the highest dose group, which remained for 4 further weeks under post-treatment observation for an assessment on signs of recovery.

Observations:
Behaviour, general observations and urine and fecal excretion daily. Viability and mortality twice daily. Body weight and food consumption weekly.

Ophthalmoscopic examinations at the start of treatment and after 6 and 13 weeks (5 males and 5 females/group), same for hearing tests and reflex-examinations.

Haematology (20 males and 20 females/group; ery, Hb, Ht, MCV, MCH, reticulocytes, inclusion bodies, throm, leu, prothrombin time), biochemistry (20 males and 20 females/group; alb, SAP, Ca, chol, creat, glu, SGOT, SGPT, inorganic phosph., K, serum electrophoresis, serum Fe, Na, Na/K, total bil, total prot, triglycerides, BUN) and urinalysis (5 males and 5 females/group; SG, prot, pH, glu, bil, urobil, blood, nitrate, ketones, sed) at the beginning of the study and after 6 and 13 weeks.

Gross pathology (all animals), relative and absolute organ weights (all animals; 9 organs) and histopathology (10 males and 10 females of the control and highest dose group; 29 tissues).

Results:
No mortalities were seen. Animals of 5 and 20 mg/kg b.w. dose groups showed yellow discoloured urines and yellow stained perigenital fur from week 9 to the end of the study.

Haematology showed a significant increase in leucocyte value in males of the highest dose group at week 6 and week 13. A slight significant decrease in erythrocyte values and a slight significant increase in reticulocytes in males and females of the middle and highest dose group at 6 and 13 weeks was found. Also a slight significant increase in MCV value at week 13 in males of the highest dose group and in females of the middle and highest dose group.

According to the authors the changes in erythrocyte and MCV values indicated a tendency towards anemia and hyperchromic anemia, respectively.

In biochemistry the changes observed were a significant increase in SAP levels in both sexes of the highest dose group at week 6, a decrease in Na levels in males of the highest dose group in week 6 and in both sexes of the highest and middle dose groups after 13 weeks. Furthermore, an increase in K levels and a decrease in the Na/K ratio in males of the lowest and highest dose group at week 6 and in both sexes of the middle and highest dose groups at week 13 were found.

Urinalysis showed an increased content of urate crystals in animals of the highest dose group at week 6. Urines of the animals of the middle and highest dose groups showed a moderate to severe yellow discolouration (due to the colouring effect of the compound tested).

In gross pathology a slightly swollen, dark coloured spleen was seen in all animals of the middle dose group and a moderately enlarged, black coloured spleen was found in all animals of the highest dose group. The presence of a hydrometra observed in1 control female and 2 or more females in the test groups is considered to be unrelated to the test compound.

A significant, dose related increase in spleen weights was observed in males of the middle and highest dose groups and in females of all dose groups.

In histopathology no differences between control animals and animals of the highest dose group were observed.

The lowest effect dose observed was 5 mg/kg b.w.

During the recovery period the discolouration of urines disappeared. Leucocyte values became normal and the differences in the other haematological parameters disappeared except for the erythrocyte values. These values remained decreased in females of the highest dose group.

In biochemistry SAP, NA and NA/K values did not differ between the control and highest dose animals, the K values of animals of the highest dose group were still increased. In urinalysis no differences were found and at autopsy no alterations in the spleens were observed.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route:	skin	Exposure:	4 hr
Species:	guinea pig		
Subst.:	sA	Concentr.:	0.5 %

The test compound (0.5 ml of a 0.5 % test suspension, kind of suspension unknown) was applied once (epicutaneously), under occlusive conditions) for 4 hours on the clipped dorsal skin (3 x 2 cm) of 5 female Pirbright white guinea pigs (SPF breeding of Messrs. Winkelmann). After 4 hours the test solution was washed off. The skin was observed 1 hour after application and thereafter once daily for a maximum of 14 days (was done for 8 days).

Results:
Due to the colouration of the skin by the test compound, an erythema could not be recognized. No other irritating effects were observed.

Remark:
The guinea pig was used instead of the rabbit.

4.1 Irritation (mucous membranes)

Route:	eye	Exposure:	24 hr
Species:	guinea pig	Dose:	0.1 ml
Subst.:	sA		

0.1 ml of the test compound (1.5 % in H_2O) was instilled into the right eyes of 5 female Pirbright white guinea pigs (SPF breading of Messrs. Winkelmann). The left eyes served as controls. The eye reactions were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after the application. After 24 hours the readings were carried out after installation of 1 drop of 0.1 % fluorescein sodium solution.

Results:
2 animals showed an erythema from 0.5 to 2 hours after application. No other effects were seen.

Remark:
- The guinea pig is used instead of the rabbit, without a proper motivation.
- According to the OECD, the eyes should at least be examined after 1, 24, 48 and 72 hours.

5. Sensitization

Subst.:	sB	Conc. induc.:	3 %	Result: neg.
Species:	guinea pig	Conc. chall.:	1, 2, 3 %	
Method:	MagnussonKligman			

10 male and 10 female Pirbright white guinea pigs (Hoe: dhpk (SPF-LAC)/Boe.) were induced by two intradermal injections on the clipped shoulder with a 3 % dilution of Ureidogelb (LGH 11 0583/2) in aqua dest (0.05 ml). The next day pre-treatment was performed by means of dermal application of 10 % sodiumlaurylsulfate followed by 3 % Ureidogelb in white vaseline (0.5 ml, closed condition). 48 hours after the first two intradermal injections the third intradermal injection followed with a 3 % dilution of Ureidogelb in Freunds's adjuvant diluted 1:1 in Arachis oil (0.05 ml). Two weeks after the last induction the animals were challenged by closed patch using 3 concentrations (1, 2 and 3 %) of Ureidogelb in Freunds's adjuvant and 1:1 Arachis oil (0.5 ml) per animal. 10 animals (5/sex) served as negative controls (treated with distilled water) and 10 animals (5/sex) served as positive controls (treated with 1-chloro-2,4-dinitro-benzene). After 24 and 48 hours the skin reactions were read.

Results:
No signs of irritation or sensitization were observed at either 24 or 48 hour readings.

6. Teratogenicity

Route:	oral	Admin. Days:	6 - 15
Species:	rat		
Subst.:	sD		

25 mated female Sprague Dawley rats received daily by oral route (intragastric intubation) 10 mg/kg b.w. of Ureidogelb (batch no. 2912) for 10 consecutive days (day 6 to 15 gestation). A group of 25 rats received distilled water and served as a control group. The rats had free access to food and water ad libitum. On day 20 of gestation the animals were sacrificed.

Observations:
Appearance, behaviour and general observations once daily. Body weights on day 0, 6, 15 and 20 of gestation. After sacrifice ovaries and uteri were examined for number of corpora lutea, number and position of implantations and placental weights. The individual foetal weights, the sex of each foetus and external visible anomalies of foetuses were determined. All foetuses were examined for external malformations. One half of the foetuses from each litter was eviscerated and fixed in 95 % ethanol for determinations of skeletal abnormalities. The remaining half was fixed in Bouin's fixation for determination of visceral abnormalities.

Results:
No maternal effects were observed. A dilatation of the renal pelvis was observed in 2 foetuses of the treated group. This effect, however, was considered to be unrelated with the administration of the test compound, because, according to the authors, dilatation of the renal pelvis regularly occurred in historical controls. Other changes in the foetuses were seen in the control group also, so these changes are considered to be unrelated to the treatment.

One malformed foetus (kinky tail, reduction deformity of hind limbs, atresia ani) was found in the treated group. This finding was considered to be incidentical because just 1 of the 277 foetuses showed this effect.

Remark:
Only one dose level was tested in this teratogenicity study.

7. Toxicokinetics (incl. Percutaneous Absorption)

*Percutaneous absorption **in vivo**:*
^{14}C-labelled 1-(2'-ureidoethyl)-amino-4-nitro-benzene (sE) was applied for 30 minutes onto the clipped dorsal skin area of 3 x 3 cm of Sprague Dawley rats (Him: OFA(SPF); 3/sex/group) in two different hair dye formulations i* and ii* (mixed with hydrogen peroxide) or as a 0.83 % solution of the test substance in DMSO/water 1:69. The amount of the substance applied was 2.5 mg for the formulations and 2.6 mg for the solution. After 30 minutes the formulation or solution was scraped off using a spatula, followed by rinse-off using about 100 ml of shampoo-solution and water of about 37° C. Rinsing was continued until the rinsing water and the absorbent tissue which was used to dab the skin dry were free of colour. The rinsings were collected. Therafter the skin was covered for 72 hours. After 72 hours the animals were killed. The radioactivity was determined in rinsing water, absorbent tissue, treated skin, collected urine and faeces (collected daily from the metabolism cages), carcass and 12 organs.

Results:
Most of the ^{14}C-labelled 1-(2'-ureidoethyl)-amino-4-nitro-benzene (98 to 106 %) was removed from the skin by rinsing 30 minutes after application. The amounts in the carcass were below detection, and the amounts in organs varied from not detectable to 0.0002 %/g for small organs (thyroid especially) and to 0.00002 %/g for large organs. No accumulation was observed in organs. The absorption was 0.103 % for the applied solution, 0.0132 % for formulation ii (the formulation containing hydrogen peroxide) and 0.0171 % for formulation i.

* formulations i and ii conntained:

	i	**ii**
^{14}C-labelled test compound (sE)	0.25 %	0.25 %
mixture of salts	0.70 %	0.35 %
ammonia, 25 %	0.36 %	2.83 %
isopropanol	3.90 %	1.95 %
WAS	2.00 %	1.00 %
water, deionised	45.19 %	17.40 %
formulation base	47.60 %	23.80 %
p-toluylendiamine, sulfate	-	1.75 %
mixture of resorcinol and m-aminophenol	-	0.68 %
Welloxon (containing 9 % H_2O_2)	-	50.00 %

8. Mutagenicity (Bact.; Non mammalian eukaryotic, *In vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	*Salm typh*	TA1535	base-pair subst	1-6000 µg/plate; solvent DMSO; toxic conc = 6000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1538	frameshift mut	1-6000 µg/plate; solvent DMSO; toxic conc = 6000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	base-pair subst	1-6000 µg/plate; solvent DMSO; toxic conc = 6000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA 98	frameshift mut	1-6000 µg/plate; solvent DMSO; toxic conc = 6000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA97	frameshift mut	1-6000 µg/plate; solvent DMSO toxic conc = 6000 µg/pl	-	-	r	AR
*sB	mouse lymp L5178Y		mutat. HGPRT,NA/K cell membr APTase	12.5-200 µg/ml 20 ml culture medium; solvent DMSO	-	-	r	AR
*sB	Chin hamst ovary cell		chrom aber	5-50 and 25-250 µg/ml solvent DMSO; toxic conc > 250 µg/ml	-	-	r	AR

Abbreviations:

meas. endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test (+ = pos, - = neg., e = equivocal)
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

1-(2'-ureidoethyl)-amino-4-nitro-benzene was tested for mutagenicity in 5 strains of *Salmonella typhimurium* using a dose range of 1 to 6000 µg/plate. Negative and positive

controls were included. The studies were conducted with and without metabolic activation (S_9-mix of Aroclor 1254-induced rats). The toxic concentration was 6000 µg/plate.

Results:
No mutagenic activity was seen neither with nor without metabolic activation.

1-(2'-ureidoethyl)-amino-4-nitro-benzene (LGH 11 0583/2) was tested for mutagenicity in mouse lymphoma L5178Y cells at the HGPRT (6-thioguanine restistance) and NA^+/K^+ cell membrane ATPase (ouabain resistance) locus. The substance was tested both with and without metabolic activation (S_9-mix of Aroclor 1254-induced male Wistar rats) at concentrations that ranged from 12.5 to 200 µg/ml (solvent DMSO) in 20 ml culture medium. Positive and negative controls were included.

Viability was determined after 2 and 7 days for ouabain and 6-thioguanine resistance, respectively. All microtitre plates were then incubated for 2 weeks after which cells containing viable clones were counted. The extended incubation times were needed to enable phenotypic expression of the induced mutations.

Results:
The test substance did not induce a significant increase in mutation to ouabain or 6-thioguanine restistance neither with nor without metabolic activation.

Chinese hamster ovary cells were used to determine whether 1-(2'-ureidoethyl)-amino-4-nitro-benzene (LGH 11 0583/2) induced chromosome aberrations both in the presence and absence of metabolic activation (S_9-mix of Aroclor 1254-induced male Wistar rats). The concentrations tested were 25, 50 and 250 µg/ml with metabolic activation and 5, 25 and 50 µg/ml without metabolic activation in a 20 ml culture medium (solvent was DMSO). Positive and negative controls were included.

Results:
The test substance did not induce chromosome aberrations in Chinese hamster ovary cells neither with nor without metabolic activation.

Indicator tests (Bact., Non mammalian eukaryotic, *In vitro* mammalian):

Sb.	**Species**	**Strain**	**Meas.endp.**	**Test conditions**	**res -act**	**res +act**	**sp +a**	**ind +a**
*sB cells	HeLa S3		DNA repair solvent DMSO	0.125-250 µg/ml;	-	-	r	AR

Abbreviations:
meas. endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test (+ = pos, - = neg., e = equivocal)
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

Indicator test (*In vivo* mammalian, Host mediated):

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sB	rat bone marrow	CD	sister chr exchanges sample at 22 hours	400-4000 mg/kg b.w. solvent dist water	-

1-(2'-ureidoethyl)-amino-4-nitro-benzene (LGH 11 0583/2) was tested in an UDS-test for its ability to induce UDS in HeLa S3 cells in vitro with and without metabolic ability (S_9-mix of Aroclor 1254-induced male Wistar rats). The cells were treated with the test substance (0.125 to 250 µg/ml, solvent DMSO) for 2.5 hours together with tritium labelled thymidine. Positive and negtive controls were included.

Results:
Unscheduled DNA synthesis was not induced, neither with nor without metabolic activation.

1-(2'-ureidoethyl)-amino-4-nitro-benzene (LGH 11 0583/2) was tested for its potential to induce sister chromatid exchanges (SCE's) *in vivo* using the bone marrow of young male CD rats (supplied by Charles River). A bromodeoxyridine pellet was implanted subcutaneously at 0 hours in 5 groups of 5 rats. After 2 hours 3 groups were dosed (orally by gavage) with the test compound at dose levels of 400, 1280, 4000 mg/kg b.w. 2 Groups served as controls and received either the vehicle control substance (distilled water), or the positive control substance (5 mg cyclophosphamide/kg b.w.). Colchicine injections were given after 20 hours from the time of implantation and 2 hours later marrow samples were prepared (1 femur/animal).

Results:
The test substance did not show a SCE inducing potential in rat bone marrow in this *in vivo* assay.

11. Conclusions

A Quality Assurance was included by the sensitization, absorption and mutagenicity tests (except for the Ames test) and by the teratogenicity and semichronic toxicity tests.

Acute toxicity
1-(2'-ureidoethyl)-amino-4-nitro-benzene appeared to be slightly toxic to rats and female mice in acute oral toxicity tests.

Irritation
The eye and skin irritation tests were carried out with guinea pigs and not with rabbits, the species normally used and for which the Draize scorings system is applicable. For this reason the eye and skin irritating potential cannot be properly evaluated. The reason why the guinea pig was used is not motivated (and the motivation was neither found in the "Bundesgesundheitsblatt", 24, Nr.6, 1981, to which the authors referred).

Sensitization
No signs of sensitization were observed in the maximization test of Magnusson and Kligman at either 24 or 48 hour readings after repeated intradermal and topical applications to guinea pigs.

Semichronic toxicity
In a 90 day feeding study rats (m + f) were daily given 0, 5, 20 or 60 mg/kg b.w. of 1-(2'-ureidoethyl)-amino-4-nitro-benzene by stomach tube. Significant changes in blood parameters and biochemistry were seen at the middle and highest dose levels.

A changed colour of the spleen was observed in all animals of the middle and highest dose group, and a significant dose related increase in spleen weight was observed in males of the middle and highest dose group and in females of all dose groups. No differences were observed in histopathology.

5 mg/kg b.w. was the lowest effect level observed.

After the recovery period no differences in spleen colour and weight was observed between the control and the highest dose group.

Teratogenicity
In a teratogenicity study with rats no teratogenic or foetotoxic effects were found after administration of 10 mg 1-(2'-ureidoethyl)-amino-4-nitro-benzene/kg b.w. (the only dose level tested) during day 6 to 15 of gestation.

Mutagenicity
1-(2'-ureidoethyl)-amino-4-nitro-benzene was tested for its mutagenic potential in the *Salmonella typhimurium* and mammalian cells *in vitro* and *in vivo*. The test substance did not show a mutagenic activity in any of these test systems.

Absorption
^{14}C-labelled 1-(2'ureidoethyl)-amino-4-nitro-benzene was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in DMSO/water. Most of the test substance was recovered in the rinsing water (98 to 106 %). The absorption rates observed were 0.103 % for the applied solution, 0.0132 % for the formulation containing hydrogen peroxide, and 0.0171 % for the formulation without hydrogen peroxide.

Conclusion
A proper evaluation of skin and eye irritating properties of the test compound is not possible because of the fact that the guinea pig is used instead of the rabbit and no good motivation is given for this choice. Furthermore no material is supplied to compare the results of the used test species with the conventional test using the rabbit. However, only minor effects were seen and further animal usage for irritancy testing was considered to be not justified.

The test compound showed no signs of sensitization.

The dermal absorption was 0.103 % for the applied solution and 0.0132 % or 0.0171 % for the formulation with or without hydrogen peroxide, respectively.

The compound appeared to be not teratogenic or foetotoxic after administration of 10 mg/kg b.w.

No mutagenic activities were observed when the test substance was tested in various test systems.

Based on the effects found in the 90-day oral study, especially those found in the spleens of females, no no effect level can be established (In male rats no effects were seen at 5 mg/kg b.w.).

For normal use of Ureidogelb the following calculation can be made:

500 mg of Ureidogelb comes in contact with the human skin in permanent hair dye condition and 175 mg in semipermanent hair dye condition (based on a maximum usage volume of 100 ml and 35 ml hair dye containing 0.5 % Ureidogelb, respectively). With a maximum dermal penetration of 0.0132 % this results in a dermal absorption of 0.066 mg per treatment for permanent hair dye and 0.023 mg per treatment for semipermanent hair dye, which is 0.0011 mg/kg b.w. and 0.0004 mg/kg b.w., respectively (assuming a body weight of 60 kg). Because 5 mg/kg b.w. was the *lowest effect level* the safety margin is calculated on 0.5 mg/kg b.w. So a safety margin of 455 can be calculated for the permanent hair dye. For the semipermanent hair dye a safety margin of 1250 can be calculated.

It should be noted that the lowest effect level is based on daily exposure for 90 days, while human exposure to permanent hair dye is unlikely to be more than once a month and human exposure to semipermanent hair dye is unlikely to be more than once a week.

No additional data are needed and the safety margins for both permanent and semipermanent hair dye are considered to be acceptable.

Classification: A

B 74: *FLUORGELB*

1. General

1.1 Primary name

Fluorgelb

1.2 Chemical names

3-nitro-4-[(2,3-dihydroxypropyl-amino]-trifluoromethyl-benzene
1-trifluoromethyl-3-nitro-4-(2,3-dihydroxypropyl)-amino-benzene
1-(2,3-dihydroxypropyl)-amino-2-nitro-4-trifluoromethyl-benzene
3-((2-nitro-4-(trifluoromethyl)phenyl)amino)-1,2-propanediol

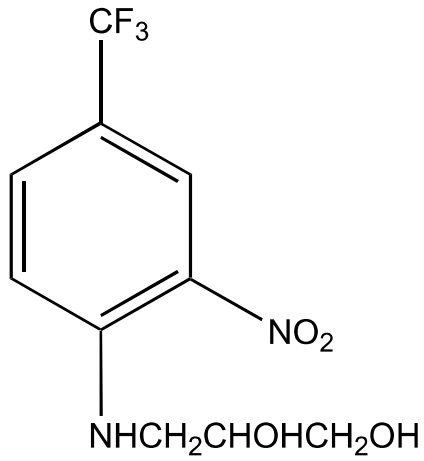
1.3 Trade names and abbreviations

Fluorgelb

1.4 CAS no.

104333-00-8

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_{11}F_3N_2O_4$
Mol weight: 280

1.7 Purity, composition and substance codes

sA: 4-(2,3-dihydroxypropyl)-amino-3-nitro-trifluoromethyl-benzene (purity > 99 %)
sB: Fluorgelb (unspecified)
sC: Fluorgelb (JM 717)

sD: Fluorgelb (Cos 219)
sE: Fluorgelb (G 23/31; purity 99 %)

1.8 Physical properties

Appearance sA: fine yellow powder
Melting point: 119° C

1.9 Solubility

The substance is soluble in dimethylsulfoxide (DMSO) and carboxymethylcellulose (CMC).

2. Function and uses

The substance is included in hair tinting products and colour setting lotions at a maximum concentration of 1 %. In oxidative hair dye formulations the maximum concentration included is 2 %. Since the oxidative hair dyes are mixed 1:1 with hydrogen peroxide before use, the concentration at application is 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Unit
sA	oral	mouse	740	mg/kg b.w.
sA	oral	rat	915	mg/kg b.w.

10 male and 10 female mice (CF1) received (by stomach tube) a single dose of the test compound (5 % suspension) at dose levels of 850, 1150 or 1450 and 500, 900 or 1300 mg/kg b.w., respectively. 10 male and 10 female rats (Wistar) received (by stomach tube) a single dose of the test compound (10 % suspension) at dose levels of 500, 1000 or 1500 mg/kg b.w. The animals were observed for 14 days.

The calculated LD_{50} values for the male and female rats were 1085 and 915 mg/kg b.w. respectively and for male and female mice 740 and 1030 mg/kg b.w. respectively. During the observation period a strong limitation of the animal's activity, abdomal position and yellow colouration of the extremities were observed. No organ changes were detected related to the substance.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Unit
sE	dermal	rat	> 2000	mg/kg b.w.

2000 mg Fluorgelb (G 23/31)/kg b.w. was applied once on the clipped skin of 5 male and 5 female Sprague-Dawley rats. The application site was 5 x 6 cm (10 % body surface) in the

dorsal thoracal region. The test substance was covered by a patch for 24 hours. Thereafter the patch was removed and the residual test substance was wiped off. 1, 10, 30 minutes and 1, 2, 4 and 6 hours after patch removal behaviour, reactions and physical signs of the animals were examined and then at least once a day for 2 weeks. Body weights were recorded before application and 7 and 14 days after application. All animals were sacrificed after 14 days and examined macroscopically.

Results: None of the animals died during the study. So the dermal LD_{50} is greater than 2000 mg/kg b.w.

In 7 animals (4 males and 3 females) signs of general malaise, like chromodakryorrhoea and ruffled fur were noted. No abnormalities were seen within the first 2 days after administration.

3.7 Subchronic oral toxicity (1)

Route:	oral	Exposure: 90 d	DWE:	10 mg/kg b.w.
Species:	rat		LED:	100 mg/kg b.w.
Subst.:	sB			

120 Sprague Dawley rats (15/sex/group) were daily given 0, 10, 100 or 500 mg Fluorgelb (suspended in 1 % aqueous carboxymethylcellulose)/kg b.w. by gavage for a period of 90 consecutive days. The age of the animals at the start of the study was 5 - 6 weeks, the body weight of males was 97 - 134 g and of females 89 - 116 g. Food and water ad libitum. After 90 days the animals were sacrificed.

Observations: General condition and behaviour and clinical signs daily. Body weight weekly (except during week 4, 5 and 6 twice weekly). Food consumption weekly. Water consumption daily by visual inspection and during week 5, 11 and 12 accurate measurements. Haematology (separate group of 10 males and 10 females from the same stock as experimental rats and 5 males and 5 females from the experimental and control groups; Hb, RBC, Ht, MCV, MCH, MCHC, WBC, diff, thrombin time) during week 12 and at the beginning of the study. Blood chemistry (BUN, prot. alb, alb/glob ratio, creat, bil, ALAT, ASAT, gamma-glutaryl transpeptidase, glu, Ca, K, Na, Cl, P) same animals week 12. Ophthalmoscopic observations of high dose and control groups prior to administration of the test material and all animals at the end of the study. Absolute and relative organ weights (all animals; liver, kidney, adrenal, gonads). Gross pathology (all animals) and histopathology (32 tissues and organs showing gross lesions or change in size; animals of control and highest dose groups and the liver and kidney of all animals).

Results: No mortalities during the study. No clinical signs were detected at 10 mg/kg b.w.

At 100 mg/kg b.w. a yellow staining of the fur was seen by day 6, which persisted during the study. Yellow stained urine was seen after day 78 and a diurese in 11 animals of this dose group was seen between day 72 and 78. An increased salivation in all males by day 37 upto half an hour after dosing and now and then in females after day 22. On day 75 all animals were hunched and pilo-erected. At 500 mg/kg b.w. a hunched posture and pilo-erection was seen from the start, the second day of dosing all females and 8 males were lethargic ½ to 5 hours after dosing until day 10. All animals of this dose level developed a decreased respiratory rate between a ½ and 3 hours after dosing until day 7, after day 75 all animals showed this symptom

again until 1 hour after dosing. On day 14 all animals showed an increased salivation 1 - 5 hours after dosing for the duration of the study and several animals of both sexes showed yellow stained urine and all animals had a yellow stained fur. Symptoms of all the effects mentioned before were found now and then in some animals throughout the study. The males of 500 mg/kg b.w. dose group showed a reduced body weight gain (30 % less at the end of the study). An increase in food consumption was seen in females of the 100 and 500 mg/kg b.w. groups. An increase in water consumption was seen in animals of the 500 mg/kg b.w. group in each week for which accurate measurement was taken (48 to 74 %).

In haematology a statistical significant increase in WBC (dose related) and lymphocyte count was found in the high dose females.

In biochemistry significant increased protein levels (dose related) were seen in males of the 100 and 500 mg/kg b.w. group. A significant increase in albumin levels (dose related) were found in males of the 100 mg/kg b.w. group and in females of the 100 and 500 mg/kg b.w. group. Furthermore a significant increase in bilirubin levels in 500 mg/kg b.w. animals and a significant increase in creatinine levels (dose related) in 100 and 500 mg/kg b.w. animals were observed.

The ophthalmoscopic examination showed a slight yellowish appearance of the retina in all animals treated with 100 and 500 mg/kg b.w. This effect was not supported in histopathology.

Significant increased absolute liver weights (dose related) were seen in animals of the 100 and 500 mg/kg b.w. group. Significant increased relative liver weights (dose related) were seen at 100 mg/kg b.w. in males and at 500 mg/kg b.w. in males and females. Significant increased relative kidney weights were seen in males of the 500 mg/kg b.w. group.

The only treatment related observation detected in gross pathology was the yellow stained fur of animals of the 100 and 500 mg/kg b.w. group (which could be due to the stained urine).

In histopathology statistically significant effects on the kidney and the liver were seen. A dilitation of proximal tubules and Loops of Henle was seen in both sexes at 500mg/kg b.w. The tubular nephropathy was seen in males only at 500 mg/kg b.w. The hepatocyte enlargement was seen at 100 and 500 mg/kg b.w. in males and only at 500 mg/kg b.w. in females.

3.7 Subchronic oral toxicity (2)

Route: oral Exposure: 90 d
Species: rat
Subst.: sB

30 SPF-derived Wistar rats (Crl: Wi/Br; 15/sex/group) were daily given 30 mg/kg b.w. of Fluorgelb (Cos 219, suspended in 0.5 % Na-carboxymethylcellulose) by gavage over a period of 13 weeks. 30 rats (15/sex/group; same strain) served as controls and received the vehicle only (0.5 % Na-CMC). At the start of the study the body weight of the males was 112 - 165 g and of the females 122 - 144 g. After 13 weeks all animals were sacrificed.

Observations: Mortality, viability and clinical signs daily. Food consumption and body weights weekly. Ophthalmoscopic and reflex examinations and hearing tests at the start and end of the study of all animals. Haematology (RBC, WBC, throm, Hb, Ht, MCV, MCH, MCHC, diff,

reticulocytes, prothrombin time, inclusion bodies) and biochemistry (glu, TG, chol, total prot, alb, bil, creat, BUN, uric acid, Ca, Cl, P, Fe, K, Na, ASAT, ALAT, SAP, creatinine kinase, glutamat dehydrogenase, SGOT/SGPT and NA/K ratio) of 10 animals/sex/group at the start of the study and at week 6 and week 13. Urinalysis (colour, prot, bil, pH, glu, BUN, blood, nitrite, ketones, sed, spec. weight) at the start of the study and at week 6 and 13 of 5 animals/sex/group. Organ weights (13 organs; all animals), gross pathology (all animals) and histopathology (10/sex/group; > 40 tissues).

Results: No mortalities. All animals showed a healthy habbit and no compound related changes were seen in body weights, food consumption, haematology, biochemistry and urinalysis except for a yellowish discoloured urine during the entire study and a staining of the abdominal fur accordingly during the last 4 weeks of the study.

An increased mean liver weight and reduced mean adrenal weight were observed in treated females. However, because no functional or morphological impairmants were seen these effects could, according to the authors, be considered as biologically insignificant. A compound related yellowish discolouration of the gastric mucous membrane was seen in gross pathology. No compound related effects were observed in histopathology.

Remark: Only one dose level tested. Used strain is the Wistar, while the Sprague Dawley was used in the other 90-day oral study.

4. Irritation & corrosivity

4.1 Irritation (skin) (1)

Route:	skin	Exposure:	5 d
Species:	guinea pig		
Subst.:	sA	Concentr.:	3 %

The test compound was applied daily (epicutaneously, under non-occlusive conditions) during 5 days as a 3 % aqueous solution thickened with 0.5 % tylose on the clipped skin (flank areas of 3 x 4 cm) of 15 female Pirbright white Guinea pigs (SFP breading of Messrs. Winkelmann). Reactions were assessed 5 hours after each application and on the third day after the last treatment.

Results: Due to the colouration of the skin by the test compound, an erythema could not be recognized. No other irritating effects were observed.

Remark: The guinea pig was used instead of the rabbit.

4.1 Irritation (skin) (2)

Route:	skin	Exposure:	4 hr	Pr.Irr. Index: 0
Species:	rabbit	Dose:	0.5 g	
Subst.:	sE			

0.5 g of Fluorgelb (G 23/31) was applied on the intact clipped skin of each 3 female New Zealand White rabbits. The test site was ca. 6 cm^2 median on the dorsal thoracal region. The test substance was covered by a patch for 4 hours. After patch removal the residual test

substance was wiped off. All animals were examined for erythema and oedema as well as for other local and systemic signs approximately 1, 24, 48 and 72 hours after patch removal.

Results: Fluorgelb had no irritating or corrosive effects at any observation time.

4.1 Irritation (mucous membranes) (1)

Route:	eye	Exposure:	24 hr
Species:	guinea pig	Dose:	0.1 ml
Subst.:	sA		

0.1 ml of the test compound (3 % in water) was instilled into the right eyes of 5 female Pirbright white guinea pigs (SPF breading Messrs. Winkelmann). The left eyes served as controls. The eye reactions were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after the application. After 24 hours the readings were carried out after installation of 1 drop of 0.1 % fluorescein sodium solution.

Results: 4 animals showed an erythema after 0.5 hours, and 3 animals showed a slight fluid secretion. Both effects disappeared after 1 hour. No alterations of cornea, iris or fundus of the eyes were seen.

Remark: The guinea pig is used instead of the rabbit, without a proper motivation. According to the OECD, the eyes should at least be examined after 1, 24, 48 and 72 hours.

4.1 Irritation (mucous membranes) (2)

Route:	eye			Pr.Irr. Index: 2
Species:	rabbit	Dose:	ca. 50 mg	
Subst.:	sE			

0.1 ml (41 to 52 mg) Fluorgelb (G 23/31) was applied into the conjunctival sac of the right eye of each of the 3 female New Zealand White rabbits. The left eyes served as controls. Both eyes were examined within 24 hours before application and 1, 24, 48 and 72 hours after application. The entire eye, especially cornea, iris and conjunctivae were examined, using a otoscope-lamp. At all observation times the animals were examined also for other than local changes.

Results: Minimal redness of the conjunctivae in 2 of the rabbits at 1 and 24 hours after application and a minimal oedema of the conjunctivae in 1 rabbit 24 hours after application was observed. No irritating effects could be noted.

Remark: The administered dose was half the usually administered dose!

5. Sensitization

Subst.:	sB	Conc. induc.:	5 / 50 %	Result: neg
Species:	guinea pig	Conc. chall.:	50 %	
Method:	Magnusson Kligman			

20 female albino Dunkin-Hartley guinea pigs were induced by intradermal injection on both clipped shoulders with a 5 % dilution of Fluorgelb in arachis oil followed after a week by a topical application of 50 % Fluorgelb in petroleum jelly under occlusion for two days. Two weeks after the last induction the animals were challenged on the right shoulders with a 50 %

solution of Fluorgelb in petroleum jelly (24-hour closed patch) and with the vehicle alone on the left shoulders. 10 animals served as controls. After removal of the patch (24 hours) and after a further 24 and 48 hours the skin reactions were read.

Results: No signs of sensitization were observed.

Remark: Faint yellow stains were noted at the test material sites of all test and control animals, but according to the authors, the staining did not affect assessment of skin responses at these sites.

6. Teratogenicity

Route:	oral	Admin.Days: 6 - 15	DWE: 10 mg/kg b.w.
Species:	rat		LED: 25 mg/kg b.w.
Subst.:	sA		

Groups of 24 mated female Sprague Dawley rats (Him; OFA SPF; 10 - 12 weeks old) were daily given (by gavage) 10, 25 or 45 mg/kg b.w. of Fluorgelb (suspended in a 0.5 % solution of Na-carboxymethylcellulose) during day 6 to 15 of gestation. 24 females served as controls and were treated with 0.5 % aqueous solution of Na-carboxymethylcellulose only. On day 20 of gestation the female rats were killed and necropsied.

Observations: Clinical signs and behaviour once daily. Body weight, body weight gains and food consumption on day 0, 6, 11, and 20 of gestation. Ovaries and uteri were examined for number of corpora lutea, number and position of implantations and placental weights. The individual foetal weights, the sex of each foetus and external visible anomalie of foetuses were determined. The foetuses were fixed alternatively in Bouin's fluid (soft tissue examination) and in ethanol (skeletal examination). Before ethanoal fixation the foetuses were eviscerated and the viscera were examined for anomalies too.

Results: Maternal body weights and body weight gains were slightly reduced at 25 and 45 mg/kg b.w.

A minor decrease of foetal and placental weight could indicate a slight foetotoxic effect of Fluorgelb, especially at the highest dose level. The dose related decrease in sex ratio (% of male foetuses) is thought to be of no importance because no difference in total number of foetuses was seen. The lowest dose group (10 mg/kg b.w.) differed statistically from the control group in the number of minor anomalies, however, no dose response was observed.

Furthermore the frequency of foetuses with 5 + 5 ossified metatarsi (normally 4 + 4) was found in the lowest dose group.

No irreversible abnormalities were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

*Skin absorption **in vitro**:*
^{14}C-labelled Fluorgelb was integrated into a hair-dye gel at 0.5 % and this was diluted with hydrogen peroxide (1:1) to simulate normal use conditions (as stated in the paragraph 2. "Function and Uses" the concentration at application is 1 %). 1 g of the mixture was applied

for 30 minutes to intact pig skin pieces of 9 - 10 cm^2, with a thickness of 1 mm, fixed in a permeation cell. After 30 minutes the skin pieces were washed with neutral shampoo and water and thereafter the absorption was determined by measuring the radioactivity. The measurement was carried out without addition of hairs, with addition of 0.35 g bleached, 0.5 cm long buffalo hair and also with addition of 0.35 g untreated, 0.5 cm long brown human hair.

Result: The absorption of Fluorgelb was decreased from 0.169 % to 0.098 % in the presence of buffalo hair, whereas the presence of human hair showed a slight increase in absorption from 0.031 % to 0.068 %.

Remark: Only a summary was supplied.

*Skin absorption **in vivo**:*

The absorption of radioactivity has been determined following a single topical application of ^{14}C-ring-labelled Fluorgelb on the shaven back of pigmented male and female Long-Evans rats. The test compound was applied to 3 males and 3 females in a dimethylsulfoxide solution (5 mg, 10 % solution) for a contact period of 24 hours. The test compound was also applied to 3 other males and females as part as a hair dye formulation (mixed with 9 % hydrogen peroxide at a ratio of 1:1) for a contact period of 0.5 hour under occlusion.

Result: The extent of percutaneous absorption of ^{14}C-ring labelled Fluorgelb in the dimethylsulfoxide solution ranged from 21 to 26 % for males and from 26 to 33 % for females, whereas the percutaneous absorption from the hair dye formulation was ≤ 1 % for both sexes.

Remark: In normal conditions of use (0.5 hour after application) 50 - 60 % of the radioactivity was recovered in the dressings and 40 - 50 % of the administered radioactivity was recovered in the washings. Less than 1 % of the radioactivity remained in the skin.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *In vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a
*sC	*Salm typh*	TA1535	base-pair subst	8 - 5000 µg/plate; solvent DMSO; toxic conc. >5000 µg/plate	-	-	r
*sC	*Salm typh*	TA1537	frameshift mut	8 - 5000 µg/plate; solvent DMSO; toxic conc. >5000 µg/plate	-	-	r
*sC	*Salm typh*	TA1538	frameshift mut	8 - 5000 µg/plate solvent DMSO; toxic conc. >5000 µg/plate	-	-	r

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a
*sC	*Salm typh*	TA100	base-pair subst	0 - 5000 µg/plate; solvent DMSO; toxic conc. >5000 µg/plate	-	-	r
*sC	*Salm typh*	TA98	frameshift mut	8 - 5000 µg/plate; solvent DMSO; toxic conc. >5000 µg/plate	-	-	r
*sC	*Sacch cer*	D7	gene mut	31.25 - 1000 µg/ml; solvent DMSO; toxic conc. >1000 µg/ml	-	-	r

Abbreviations:

meas. endp. = measured endpoint

sp = species used for activation (r = rat, m = mouse, h = hamster)

res = result of test (+ = pos., - = neg., e = equivocal)

Fluorgelb (JM 717) was tested for mutagenicity in 5 strains of *Salmonella typhimurium* and in one strain of *Saccharomyces cerevisiae* using a dose range of 8 to 5000 µg/plate and 31.25 to 1000 µg/ml, respectively. Negative and positive controls were included. The studies were conducted with and without metabolic activation (S_9-mix of the rat liver).

Result: No mutagenic activities were seen neither with nor without metabolic activation.

8.2 Mutagenicity (*In vivo* mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sD	mouse bone marrow		micronucleated polychromatic erythrocytes	250 mg/kg b.w. solvent DMSO; samples at 24, 48, 72 hours	-

Fluorgelb (Cos 219) was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of 5 male and 5 female mice (NMRI). 30 mg/kg b.w. cyclophosphamide was given to the positive controls and the solvent alone served as negative control. 1000 PCE's/animal were analysed.

Result: Fluorgelb did not induce micronuclei in the test system used.

Indicator tests (Bact., Non mammalian eukaryotic, *In vitro* mammalian)

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a
*sC	hepatocyte rat		DNA repair (UDS)	1 - 100 µg/ml solvent DMSO	-		
*sC	Chin hamst ovary cell		sister chr exchanges	0.001 - 1 mM solvent DMSO	-	-	r

Abbreviations:
meas. endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test (+ = pos., - = neg., e = equivocal)

Fluorgelb (JM 717) was tested in an UDS-test for its ability to induce DNA repair processes in hepatocytes of male rats (Wistar CF HB; 8 - 12 weeks old) *in vitro*. The aliquotes of hepatocytes (3.5 - 4 x 10^6 hepatocytes/aliquot) were treated with Fluorgelb for 3 hours together with tritium labelled thymidine. Positive and negative controls were included.

Results: Unscheduled DNA synthesis was not induced.

Fluorgelb (JM 717) was tested for sister chromatid exchange in Chinese hamster ovary cells (kl cells obtained from Messrs. Flow). At each concentration 100 stained metaphases were evaluated. Positive and negative controls were included.

Results: Fluorgelb did not induce SCE's in the test system with nor without metabolic activation.

10. Special investigations

General pharmacology of Fluorgelb:
The effects of Fluorgelb were investigated on various systems in the KMF NMRI mice, KFM Han Wistar rats, Dunkin-Hartley Albino Guinea pigs or New Zealand White KFM rabbits. The solvents used were carboxymethylcellulose sodium salt (4 %) in distilled water for peroral application, physiological saline for intravenous and subcutaneous applications and Krebs-Henseleit solution, Tyrode solution and Jalon's solution for isolated organs. The dose levels administered in most of the test systems were 0, 60 and 200 mg/kg b.w.

Central nervous system:
200 mg Fluorgelb/kg b.w. caused transient effects on the central nervous system, such as sedation, apathy and decreased activity in rats and mice; an increased sleeping time and a delayed onset of convulsions and a moderate decrease of locomotor activity in mice; a slightly decreased body temperature in rats and rabbits. 60 mg Fluorgelb/kg b.w. did not cause these effects.

Autonomic nervous system:
Inhibition of concentration by standard antagonists of isolated ileum and vas deferens of the guinea pig and of isolated uterus of the rat was seen at organ bath concentration of 100 µg/ml.

A relaxation of the isolated trachea of the guinea pig was observed after organ bath concentrations of 1 - 100 µg/ml.

Gastrointestinal system:
200 mg Fluorgelb/kg b.w. slightly decreased the gastric secretion in rats.

Cardiovascular system:
The thrombin time was moderately decreased when 200 mg/kg b.w. of Fluorgelb was administered to rats.

Renal function:
At 200 mg Fluorgelb/kg b.w. an increase in urinary volume, and a slight reduce in specific gravity and osmolality was seen 0 - 6 hours after treatment. The excretion of potassium and phosphorus was markedly increased from 0 - 6 hours after treatment and the excretion of urea was moderately increased from 6-24 hours after treatment. At 60 mg/kg b.w. the specific gravity was slightly reduced, the osmolality was moderately reduced and the excretion of phosphorus was moderately increased, all from 0 - 6 hours after treatment.

11. Conclusions

A Quality Assurance was included by the sensitization test, the irritation tests in rabbits and the absorption test *in vivo*. It was also included by 2 mutagenicity tests (UDS and micronucleus), the acute dermal toxicity test in rats, and by the teratogenicity and the two 90-day feeding studies.

General
4-(2,3-dihydroxypropyl)-amino-3-nitro-trifluoromethyl-benzene is used in hair tinting products and colour setting lotions at a maximum concentration of 1 %. The concentration at application as an oxidative hair dye is 1 %.

Acute toxicity
The substance can be classified as moderately toxic, based on the results of the acute oral toxicity tests. In a dermal toxicity test the substance was slightly toxic.

Irritation
At first only eye and skin irritation tests carried out with guinea pigs were supplied and not with rabbits, the species normally used and for which the Draize scorings system is applicable. Because the guinea pig was used instead of the rabbit the eye and skin irritating properties could not be evaluated properly. The reason why the guinea pig was used is not motivated (and the motivation was neither found in the „Bundesgesundheitsblatt, 24, nr. 6, 1981“, to which the authors referred).

Eye and skin irritation test in rabbits have been supplied, and no irritating properties could be noted. Although the eye irritation test was performed with a lower dose level than normally requested, irritation properties are not suspected.

Sensitization
No signs of sensitization were observed in the maximization test of Magnusson and Kligman at either 24 or 48 hour readings. Faint yellow stains were reported to be at the test sites of all

test and control animals, but the authors stated that this did not affect assessment of the skin responses.

Subchronic toxicity
In a 90-day feeding study, Sprague Dawley rats (m + f) were daily given 0, 10, 100 or 500 mg/kg b.w. of Fluorgelb by gavage.

At 100 and 500 mg/kg b.w. yellow stained urine and fur were seen. Increased salivation, hunched posture and pilo-erection were seen at 100 and 500 mg/kg b.w. throughout the study. Males of 500 mg/kg b.w. showed a depressed body weight gain. Changes in haematology (increased WBC and lymphocyte count) and biochemistry (increased protein, albumin, bilirubin and creatinine levels) were seen at 100 and 500 mg/kg b.w. Ophthalmoscopic examinations showed a slight yellow colouration of the retina, but this effect was not supported in histopathology. Increased absolute and relative liver weights were seen at 100 and 500 mg/kg b.w. and increased relative kidney weights were seen at 500 mg/kg b.w. Effects on liver and kidney were also seen in histopathology at 100 and 500 mg/kg b.w. (dilitation of proximal tubules and loops of Henle, tubular nephropathy and hepatocyte enlargement).

At 10 mg/kg b.w. no effects were seen.

In another 90-day feeding study Wistar rats (f + m) received daily 0 or 30 mg/kg b.w. An increased liver weight and a reduced adrenal weight were observed in treated females. A yellow colouration of the gastric mucous membrane was seen in gross pathology. No compound related effects were seen in histopathology.

Teratogenicity
In a teratogenicity study with rats no indications were found for a teratogenic effect. A slight maternal toxic and foetotoxic effect could not be excluded at 25 and 45 mg/kg b.w., respectively. 10 mg/kg b.w. can be considered as the no effect level.

Mutagenicity
Fluorgelb was tested for its mutagenic potential in bacterial cells and mammalian cells *in vitro* and **in vivo**. The substance did not show a mutagenic activity in any of these test systems.

Absorption
The absorption of ^{14}C-labelled Fluorgelb was determined *in vivo* and *in vitro:*

In vitro the substance was integrated in a hair dye gel at 0.5 % and this was diluted with hydrogen peroxide (1:1) to simulate normal use conditions. 1 g of the mixture was applied to intact pig skin pieces in a permeation cell. The absorption was measured in the absence and presence of hair (bleached buffalo or untreated human hair). The percutaneous absorption appeared to be decreased from 0.169 % to 0.098 % in the presence of buffalo hair, whereas in the presence of human hair a slight increase in absorption was seen from 0.031 % to 0.068 %.

In vivo the test substance was applied to the shaven back of rats as a dimethylsulfoxide solution or as part of a hair dye formulation (mixed with 9 % hydrogen peroxide). For the solution in DMSO the measured radioactivity ranged from 21 % to 26 % for males and from 26 % to 33 % for females, for the hair dye formulation less than 1 % was measured for both sexes.

Final conclusions
Based on the eye and skin irritation tests in the rabbit it can be concluded that Fluorgelb has no eye or skin irritating properties.

The test compound showed no signs of sensitization.

The *in vivo* dermal absorption of the solution in DMSO ranged from 21 to 26 % for males and from 26 to 33 % for females, the dermal absorption for the hair dye formulation was less than 1 % for both sexes.

The compound appeared to have a slight maternal and foetotoxic effect at 25 and 45 mg/kg b.w., respectively. No indications were found for a teratogenic effect.

No mutagenic activities were seen in the various test systems used.

Based on the 90-day feeding study and the teratogenicity study 10 mg/kg b.w. can be considered as the level without effect.

For normal use of Fluorgelb the following calculation can be made:

1 gram of Fluorgelb comes in contact with the human skin in normal use condition (based on a maximum usage volume of 100 ml hair dye formulation containing 1 % Fluorgelb). With a maximal dermal penetration of 1 % this results in a dermal absorption of 10 mg per treatment, which is 0.17 mg/kg b.w. (assuming a body weight of 60 kg). So a margin of safety of 59 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in the 90-day oral study / teratogenicity study.

It should be noted that the no effect level found in rats is based on daily exposure for 90 days, while human exposure to oxidative hair dye will be at the most once monthly. The actual usage of the lotion containing Fluorgelb is unknown but is expected to be about once a week.

B 76: *CHLORGELB*

1. General

1.1 Primary name

Chlorgelb

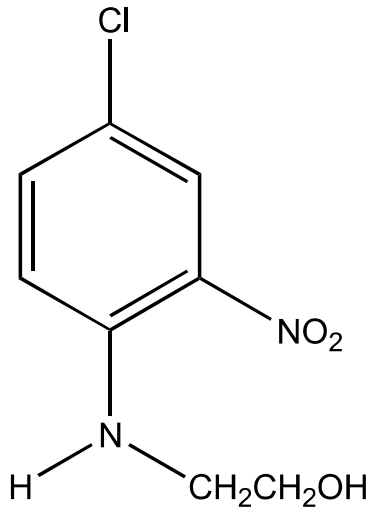
1.2 Chemical names

1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene
4-(2'-hydroxyethyl)-amino-3-nitro-chlorobenzene
4-chloro-1-(2'-hydroxyethyl)-amino-2-nitro-benzene
N-(2'-hydroxyethyl)-4-chloro-2-nitroaniline

1.3 Trade names and abbreviations

sA: 1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene (purity > 99.9 %)
sB: Chlorgelb (GHS 091184)
sC: 1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene (purity > 97 %)
sD: 1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene (LGH 221283/1; purity 99 %)
sE: Chlorgelb (Brä I/314; purity > 99.9 %)
sF: Chlorgelb (not specified)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_9N_2O_3C_1$
Mol weight: 217

1.8 Physical properties

Appearance: sA, orange yellow cristalline powder
Melting point: 99 - 100° C

1.9 Solubility

The substance exists as a free base. It is slightly soluble in water and fully soluble in ethanol and dimethylsulphoxide.

2. Function and uses

1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene is included in semi-permanent dyes and colour setting lotions at a maximum concentration of 0.5 %. In oxidation dyes the maximum concentration is 1 %. As the oxidation dyes are mixed with hydrogen peroxide before application the concentration in use is only 0.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Unit
sA	oral	mouse	1172	mg/kg b.w.
sA	oral	rat	1250	mg/kg b.w.

The test compound (a 10 % suspension in Arabic gum) was given once by stomach tube to 18 male and 18 female Wistar rats at dose levels ranging from 0.9 to 2.5 g/kg b.w. and to 30 male and 40 female CF1 mice ranging from 1.0 to 2.0 and from 1.0 to 2.5 mg/kg b.w., respectively. The animals were observed for 14 days and organs of all animals were examined.

The calculated LD_{50} values for male and female rats were 1.564 and 1.172 g/kg b.w., respectively. For male and female mice the values were 1.250 and 1.650 g/kg b.w., respectively.

During the observation period a limitation of activity and yellow coloured extremities were observed. No organ changes were detected.

3.7 Subchronic oral toxicity

Route: oral Exposure: 90 d
Species: rat Recov. p.: 4 wk LED: 10 (unit): mg/kg b.w.
Subst.: sF

180 Wistar rats (90 males and 90 females; BOR:WISW [SPF/TNO]) were used in a 13 -week oral study. The age at the beginning of the study was 6 weeks and the body weights were 107 - 148 g for males and 100 - 137 g for females. 20 males and 20 females received daily 10 or 30 mg/kg b.w. of Chlorgelb (suspended in 0.5 % carboxymethylcellulose) by stomach tube. 25

males and 25 females received daily only the solvent (controls) or 90 mg/kg b.w. of Chlorgelb (suspended in 0.5 % carboxymethylcellulose) by stomach tube. The animals had free access to water and food.

After 90 days all animals were sacrificed except for 5 males and 5 females of the control and highest dose group (90 mg/kg b.w.), which remained for 4 weeks under post-treatment observation for an assessment on signs of recovery.

Observations:
Mortality, clinical signs and behaviour daily. Body weights, food and water consumption weekly. Ophthalmoscopic examination, hearing test and reflex examination (10 males and 10 females of the control and highest dose groups) at the start of the study and after week 6 and week 13 and on the remaining animals at the end of the recovery.

Haematology (10/sex/group; ery, Ht, Hb, MCV, MCH, MCHC, reticulocytes, throm, leu, diff, prothrombin time), clinical chemistry (10/sex/group; alb, SAP, Ca, Cl, chol, creat, glu, ASAT, ALAT, K, inorg. P, serum electrophoresis, Fe, Na, total bil, total prot, TG, BUN, uric acid) and urinalysis (5/sex/group; sg, prot, pH, glu, bil, urobil, blood, nitrite, ketones, sed).

Absolute and relative organ weights (all animals; 8 organs), gross pathology (all animals) and histopathology (30 tissues of 10 males and 10 females; kidneys and thyroids were examined on all animals of the study, including recovery animals).

Results:
No animals died during the study. A dose related yellow staining of urines, fur, paws and tails was seen in all treated animals throughout the study. The animals of the highest dose group showed a slightly reduced activity, diuresis and pilo-erection from week 8 until termination.

The body weight gains of males of the highest dose level were slightly but significantly reduced at week 13. After 13 weeks slight but significant changes were seen in erythrocytes (decrease in males at 90 mg/kg b.w.), in MCV (decrease in males at 90 mg/kg b.w.). In clinical chemistry slight but significant changes after 13 weeks were seen in total protein (increased in females at 90 mg/kg b.w.), in SGOT (increased in males at 90 and in females at 10 mg/kg b.w.), in Na (increased in females at 90 mg/kg b.w.), in Ca (increased in males at 90 mg/kg b.w. and in females at all dose levels: 2.51, 2.65, 2.69 and 2.76 mmol/l in the control, 10, 30 and 90 mg/kg b.w. group, respectively). Other changes in haematology and clinical chemistry were seen incidentally after 6 weeks only and/or at a lower dose level only.

No changes were seen in absolute or relative organ weights or in gross pathology. In histopathology a slight tubular oedema was found in the kidneys of 3 males and 2 females of the 90 mg/kg b.w. group and this effect can be related to the administered test compound. Probably also related to the test compound was the observed increased number of animals with an activation of the thyroid epithelia (a change from flat to cubic epithelium) in the 90 mg/kg b.w. group. Thyroid slices of the 178 animals of this 90-day study were also examined by another institute which considered the observed epithelial changes to be artefacts caused by autolysis.

10 mg/kg b.w. is a marginal effect level, based on the Ca changes in female rats.

During the recovery period the stained urines disappeared as well as the reduced activity, diuresis and piloerection. The fur, paws and tails remained stained. The body weight gains of the males of the 90 mg/kg b.w. group increased during this period. In haematology no differences were found between the control and recovery animals, but in clinical chemistry still slight but significant changes remained, such as increased glucose in both sexes, decreased Na in both sexes and decreased Ca and P in females and a decreased Cl in males. Furthermore electrophoresis-globulin A1 + A2 was decreased in females and electrophoresis-globulin G and ASAT was increased in females.

Differences between the control and recovery animals in the thyroid epithelium and kidneys were not observed after the recovery period.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route:	skin	Exposure:	5 d
Species:	guinea pig		
Sust.:	sA	Concentr.:	5 %

The test compound (5 % in H_2O) thickened with 0.5 % Tylose) was applied daily during 5 days on the clipped skin (flank area of 3 x 4 cm) of 15 Pirbright white Guinea pigs (SPF-breed of Fa. Winkelmann). Reactions were assessed 5 hours after each application and the third day after the last application.

Results:
Due to severe colouration of the skin by the test compound, an erythema could not be recognized. No other irritating effects were observed.

Remark:
The guinea pig was used instead of the rabbit.

4.1 Irritation (mucous membranes)

Route:	eye	Exposure:	24 hr
Species:	guinea pig	Dose:	0.1 ml
Subst.:	sA		

0.1 ml of the test compound (1.5 % in H_2O) was instilled into the right eyes of 5 female Pirbright white guinea pigs (SPF-breeding of Fa. Winkelmann). The left eyes served as controls. The eye reactions were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after application. After 24 hours (and daily afterwards until no symptoms were seen anymore), after installation of one drop of 0.1 % fluorescin sodium solution, the readings were carried out.

Results:
In all animals an erythema was observed and in 3 of 5 animals a fluid excretion was seen. After 4 hours these effects disappeared.

Remark:
— The guinea pig is used instead of the rabbit, without a proper explanation.
— According to OECD the eyes should be examined until 72 hours, even if the readings are negative.

5. Sensitization

Subst.:	sB	Conc. induc.:	5 %	50 %	Result:	neg
Species:	guinea pig	Conc. chall.:	50 %			
Method:	MagnussonKligman					

20 female albino Dunkin-Hartley guinea pigs were induced by intradermal injection on each side of the midline of the clipped (40 mm x 60 mm) shoulder region with a 5 % dilution of Chlorgelb in arachis oil followed by a topical application one week later of 50 % Chlorgelb in petroleum jelly under occlusion for 48 hours. Two weeks after the last induction the animals were challenged on the shorn right flanks with 50 % solution of Chlorgelb in petroleum jelly (24-hour dosed patch) and with the vehicle alone on the shorn left flanks. 10 animals served as controls. After removal of the closed patch (24 hours) and after a further 24 and 48 hours the skin reactions were read.

Results:
No signs of sensitization were observed.

Remark:
Yellow stains were noted at the test material sites of all test and control animals. The staining did not affect assessment of skin responses at these sites, according to the authors.

6. Teratogenicity

Route:	oral	Admin. Days:	5 - 15	DWE:	10	(unit):	mg/kg b.w.
Species:	rat			LED:	30	(unit):	mg/kg b.w.
Subst.:	sF						

Groups of 24 mated female rats (BOR Wisw-SPF TNO; age 16 weeks, body weight 174 - 226 g) were given daily (by stomach tube) 10, 30 or 90 mg/kg b.w. of Chlorgelb (suspended in carboxymethylcellulose 0.5 %) during day 5 to 15 of gestation. 24 females served as controls and were treated with the solvent only. The animals had free access to water and food. On day 20 of gestation the dams were sacrificed.

Observations:
Clinical signs and behaviour daily. During treatment special reflex examinations were carried out. Body weights and food consumption at the start of the study and at days 5, 10, 15 and 20.

A complete autopsy of the dams and a macroscopic evaluation of organs was carried out. Number, sex and weights of the fetuses, number and weights of placentae, distribution of fetuses in uterus, number of resorptions, number of corpora lutea and implantations, and weight of the uteri were determined. Instantly after dissection ex uteri, all fetuses were examined grossly for external visible deviations from normal condition. About 1/3 of all

fetuses were fixed in Bouin's fluid (soft tissue examination) and about 2/3 of all fetuses were fixed in alcohol and stained in Alizarin (skeletal examination).

Results:

Orange staining of urines was seen in the maternal rats of the 30 and 90 mg/kg b.w. dose groups.

Fetuses of the 30 and 90 mg/kg b.w. showed a significant and dose related increase in mean body weights.

The placenta weights were significantly increased in the females of the 90 mg/kg b.w. dose group.

1 fetus (1 of 224 examined fetuses of this dose level) with malformations (maxilla and bones shortened, occipital bones not ossified, agenetic sternum) was found in the 90 mg/kg b.w. group.

7. Toxicokinetics (incl. Percutaneous Absorption)

*Skin absorbtion **in vivo**:*

^{14}C-ring-labelled Chlorgelb (purity > 97 %) was applied for 30 minutes onto the shaven back (3 x 3 cm) of Sprague Dawley rats (Him:OFA[SPF]; 3/sex/group) in two different hair dye formulations i* and ii* (mixed with hydrogen peroxide) or as a 1.67 % solution of the test substance in DMSO/water 3:1. The amount of the test substance applied was 4.99 mg for formulation i, 2.52 mg for formulation ii and 5.27 mg for the solution.

After 30 minutes the formulation or solution was scraped off using a spatula, followed by a rinse-off using about 100 ml of a shampoo-solution and water of about 37° C. Rinsing was continued until the rinsing water and the absorbent tissue which was used to dab the skin dry were free of colour. The rinsings were collected. Thereafter the skin was covered for 72 hours. After 72 hours the animals were killed. The radioactivity was determined in rinsing water and absorbent tissue, treated skin, collected urine and faeces (collected daily from the metabolism cages), carcass, blood, and 12 organs.

Results:

97.5 to 99.6 % of the applied amount was recovered by rinsing 30 minutes after application. The amounts in the carcass were below or close to the detection limit of 0.003 to 0.006 %. In the organs the radioactivity determined was below or close to the detection limit of 0.00002 %/g for large organs and about 0.0003 %/g for small organs (especially thyroid). A slight indication of accumulation in fat was obtained only after application of the solution. In 1 female of the solution group the radioactivity in the urine, faeces and absorption appeared to be about 7 times higher than in the other 5 females in this group. No possible reason was detected for this observation.

The cutaneous absorption was 0.114 % for formulation i, 0.128 % for formulation ii (with hydrogen peroxide) and 1.02 % or 0.520 % for the solution with or without the deviating female, respectively.

* formulation i and ii contained:

	i	**ii**
^{14}C-labelled chlorgelb	0.50 %	0.25 %
p-toluylendiamine, sulfate	-	1.75 %
mixture of resorcinol and m-aminophenol	-	0.68 %
mixture of salts	0.70 %	0.35 %
ammonia, 25 %	0.36 %	2.83 %
isopropanol	3.90 %	1.95 %
WAS	2.00 %	1.00 %
water, deionised	44.94 %	17.40 %
formulation base	47.60 %	23.80 %
Welloxon (containing 9 % H_2O_2)	-	50.00 %

8.1 Mutagenicity (Bact., Non mammalian eukaryotic. *In vitro* mammalian).

Sb.	**Species**	**Strain**	**Meas.endp.**	**Test conditions**	**res -act**	**res +act**	**sp +a**	**ind +a**
*sD	*Salm typh*	TA1535	base-pair subst	2.4 - 1500 and 500 - 3000 µg/ plate; solvent DMSO	-	-	r	
*sD	*Salm typh*	TA1537	frameshift mut	2.4 - 1500 and 500 - 3000 µg/plate; solvent DMSO	-	-	r	
*sD	*Salm typh*	TA1538	frameshift mut	2.4 - 1500 and 500 - 3000 µg/ plate; solvent DMSO	-	-	r	
*sD	*Salm typh*	TA98	frameshift mut	2.4 - 1500 and 500 - 3000 µg/ plate; solvent DMSO	-	-	r	
*sD	*Salm typh*	TA100	base-pair subst	2.4 - 1500 and 500 - 3000 µg/ plate; solvent DMSO	+	-	r	
*sB	mouse lymp L5178Y		mutat. HGPRT	1.88 - 500 µg/ml solvent DMSO	-	-	r	AR

Abbreviations:
meas. endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

8.2 Mutagenicity (*In vivo* mammalian, Host mediated).

Sub	Species	Strain	Meas. endp.	Test conditions
*sE	mouse	NMRI	micronucleated polychromatic erythrocytes	90, 300, 900 mg/kg b.w. solvent DMSO; samples at 24, 48, 72 hours

Salmonella assay
Chlorgelb (LGH 22 12 83/1; 1 mg of test substance in 1 ml DMSO) was tested for mutagenicity in two experiments in 5 strains of *Salmonella typhimurium.* The toxic concentration was determined in the TA100 and appeared to be 1250 µg/plate (80 % reduction). Based on this result a concentration range of 2.4 to 1500 µg/plate was selected for one experiment and a range of 500 to 3000 µg/plate was selected for the other experiment. The tests were performed both with and without metabolic activation (rat liver homogenate, no details) and positive and negative controls were included.

Results:
TA100 showed a (weakly) positive result without metabolic activation.

Mouse lymphoma fluctuation assay
Chlorgelb (GHS 091184) was tested for mutagenicity in a mouse lymphoma fluctuation assay at the HGPRT locus (6-thioguanine resistance), both in the absence and presence of metabolic activation (S9-mix of Aroclor 1254-induced Wistar rats. Based on a preliminary toxicity test the dose levels used were 18.8, 37.5, 75, 110 and 300 µg/ml in the absence and 31.1, 62.5, 125, 250 and 500 µg/ml in the presence of metabolic activation. The test was carried out in duplicate. Solvent (DMSO) and positive controls were included. 7 days after treatment, all cultures were plated for 6-thioguanine resistance in microtitre plates.

Results:
Precipitation occurred in cultures treated with 1000 µg/ml or more in the absence and with 2000 µg/ml in the presence of S_9-mix.

In the presence of S9-mix, a statistically significant increase in mutation rate was seen in one serie tested at 37.5 µg/ml. No increase was observed in the replicate or at other concentrations, therefore this increase is considered to be of no importance. It is concluded that Chlorgelb has no mutagenic activity at the HGPRT locus of L5178Y mouse lymphoma cells neither with nor without metabolic activation.

Remark:
No background frequencies were given.

Micronucleus assay
Chlorgelb (Brä I/314) was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of NMRI mice. The mice received 90, 300 or 900 mg/kg b.w. by stomach tube (solvent was DMSO) based on a preliminary experiment. Negative (solvent alone) and positive (cyclophosphamide 5 %) controls were included. Samples were taken 24 hours after administration for all dose levels, and at 48 and 72 hours after

administration of 0 (negative control) and 900 mg/kg b.w. In each group 1000 cells from 5 males and 5 females were scored.

Results:
Chlorgelb did not induce micronuclei in the test system used.

Indicator tests (*In vivo* mammalian. Host mediated):

Sub	Species	Strain	Meas. endp.	Test conditions
*sB	rat	Wistar	DNA repair (UDS)	70 - 600 mg/kg b.w. solvent DMSO

Unscheduled DNA synthesis
Chlorgelb (GHS 091184) was tested for its ability to induce DNA repair processes in hepatocytes of Wistar rats (Crl:[WI]BR, SPF) in vivo. 3 groups of rats (6 males and 6 females/group) received a single administration by gavage of 70, 200 or 600 mg/kg b.w. (volume 2 ml/kg b.w., solvent DMSO). Solvent and positive controls were included. After 24 hours liver preparations were made in addition of 185 kBq 3H-thymidine per million cells. 100 cells/animal were examined for unscheduled DNA synthesis.

Results:
The test compound did not show an UDS-inducing effect.

11. Conclusions

A Quality Assurance was included by the sensitization test and the absorption test. It was also included by 3 mutagenicity tests (UDS-test, mouse lymphoma assay and micronucleus test) and by the teratogenicity and 90-day oral study as well as by the additional report on thyroid examination.

General
1-chloro-3-nitro-4-(beta-hydroxyethyl)-amino-benzene is used in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 0.5 %. In oxidation dyes the maximum concentration is 1 %, but because the oxidation dyes are mixed with H_2O_2 before application, the concentration in use is 0.5 %.

Acute toxicity
The substance can be classified as moderately toxic, based on the results of the acute oral toxicity tests.

Irritation
The eye and skin irritation tests were carried out with guinea pigs and not with rabbits, the species normally used and for which the Draize scorings system is applicable. For this reason the eye and skin irritating potential cannot be properly evaluated. The reason why the guinea pig was used is not motivated (and the motivation was neither found in the „Bundesgesundheitsblatt, 24, Nr. 6, 1981“, to which the authors referred).

Sensitization

No signs of sensitization were observed in the maximization test of Magnusson and Kligman at either 24 or 48 hour readings. Yellow stains were noted at the test material sites of all test and control animals, but the authors stated that this did not affect assessment of the skin responses.

Semichronic toxicity

In a 90-day feeding study, Wistar rats (m + f) were daily given 0, 10, 30 or 90 mg/kg b.w. of Chlorgelb by stomach tube. No animals died during the study. All treated animals showed yellow stained urines, furs, paws and tails throughout the study. Animals at 90 mg/kg b.w. showed a slightly reduced activity, diuresis and pilo-erection from week 8 until termination. A significant decreased body weight gain was seen in males at 90 mg/kg b.w. Slight but significant changes in blood parameters and clinical chemistry were seen especially at the 90 mg/kg b.w. level (decreased erythrocytes and MCV in males; increased total protein and Na in females; increased SGOT and Ca in males). The increased Ca-level was seen in all treated females.

No changes were seen in organ weights or gross pathology. In histopathology a slight tubular oedema was found in 5 treated animals of the highest dose group. Furthermore the increased number of animals with activated thyroid epithelium at the highest dose level is considered to be treatment related, especially because no differences in thyroid epithelium were found between control and recovery animals after the recovery period. Also the other (probably) treatment related differences were not observed after the recovery period except for the stained furs, paws and tails.

10 mg/kg b.w. is considered to be a marginal effect level.

Teratogenicity

In a teratogenicity study with rats no indications were found for a teratogenic effect, but fetuses of the 30 and 90 mg/kg b.w. group showed a significant and dose related increase in mean body weights. The placenta weights of females of the 90 mg/kg b.w. group were significantly increased. At 10 mg/kg b.w. no maternal or fetal effects were observed.

Mutagenicity

Chlorgelb was tested for its mutagenic potential in bacterial cells as well as in mammalian cells *in vitro* and *in vivo*. All the tests were negative except for the Salmonella assay in which a (weak) positive result was obtained in the strain TA 100 without metabolic activation. However, it is known that aromatic amino/nitro compounds often give false-positive effects in the Salmonella assay (Ashby and Tennant, 1991, Mutat. res. 257: 229-306). Therefore it is concluded that Chlorgelb does not have a mutagenic potential.

Absorption

The absorption of ^{14}C-labelled Chlorgelb was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in DMSO/water. Most of the substance was recovered by rinsing (97.5 to 99.6 %). A slight indication of fat accumulation was observed only after application of the solution. For an unknown reason the radioactivity in urine and faeces and absorption appeared to be 7 times higher in 1 female treated with the solution. The cutaneous absorption was 0.114 % for the formulation without and 0.128 % for the formulation with hydrogen peroxide and 0.520 % for the solution (or 1.02 % with deviating female included).

Conclusion
A proper evaluation of skin and eye irritating properties of the test compound is not possible because of the fact that the guinea pig is used instead of the rabbit and no good motivation is given for this choice. Furthermore no material is supplied to compare the results obtained in the used species with the conventional test using the rabbit. However, only transient effects were seen and further animal usage for irritancy testing was considered to be not justified.

The test compound showed no signs of sensitization.

The dermal absorption was 0.114 % for the formulation without and 0.128 % for the formulation with hydrogen peroxide and 0.520 % for the solution (without the deviating female).

No indications were found for a teratogenic effect and no foetotoxic effect was found after administration of 10 mg/kg b.w.

It was concluded that the compound had no mutagenic properties when tested in various test systems.

In the 90-day feeding study effects were found especially at the 90 mg/kg b.w. level, but 10 mg/kg b.w. can still be considered as a marginal effect level because of the slight but significant increase in Ca-levels in female rats. No differences were found after the recovery period, except for the stained paws, furs and tails.

Based on the effects found in the 90-day oral study a no effect level cannot be established, because of the marginal effect found in female rats (Ca-levels) at 10 mg/kg b.w.

For normal use of Chlorgelb the following calculation can be made:

500 mg of Chlorgelb comes in contact with the human skin in permanent hair dye condition and 175 mg in semipermanent hair dye condition (based on a maximum usage volume of 100 mg and 35 ml hair dye containing 0.5 % Chlorgelb, respectively). With a maximal dermal penetration of 0.128 % this results in a dermal absorption of 0.64 mg per treatment with permanent hair dye and 0.22 mg for treatment with semipermanent hair dye, which is 0.011 mg/kg b.w. and 0.004 mg/kg b.w., respectively (assuming a body weight of 60 kg). So a safety margin of 909 can be calculated between the figure for human exposure to permanent hair dye and the marginal effect level found in female rats in the 90-day study. For the semipermanent hair dye a safety margin of 2500 can be calculated. It should be noted that the marginal effect level is based on daily exposure for 90 days, while human exposure to permanent hair dye is unlikely to be more than once a month and human exposure to semipermanent hair dye is unlikely to be more than once a week.

No additional data are needed and the safety margins for both permanent hair dye and semipermanent hair dye are considered to be acceptable.

Classification: A

B 78: *1-(2,3-DIHYDROXYPROPYL)-AMINO-2-NITRO-4-(METHYL-(2-HYDROXYETHYL))-AMINOBENZENE*

1. General

1.1 Primary name

1-(2,3-dihydroxypropyl)-amino-2-nitro-4-(methyl-(2-hydroxyethyl))-aminobenzene

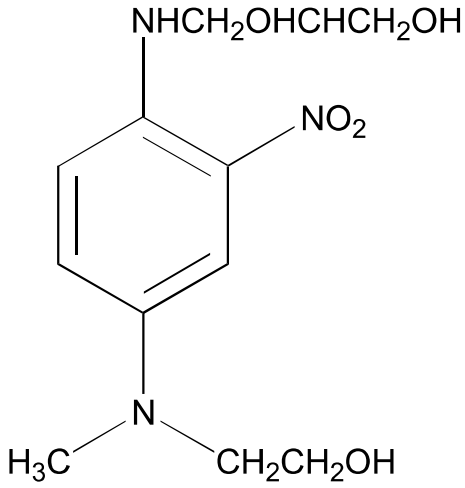
1.2 Chemical names

1-(2,3-dihydroxypropyl)-amino-2-nitro-4-(methyl-(2-hydroxyethyl))-aminobenzene
N-(2,3-dihydroxypropyl)-4-((2-hydroxy-ethyl)-methyl-amino)-2-nitro-aniline

1.3 Trade names and abbreviations

Propylblau

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}\ H_{19}\ N_3\ O_5$
Mol weight: 285

1.7 Purity, composition and substance codes

It exists as a hydrochloride; the commercial product contains 97 % of the compound, the other substances are not identified.

All studies have been carried out with the commercial product alone and with a cosmetic formulation containing the commercial product.

2. Function and uses

Oxidative hair dye; max. use 2.0 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: female mice, oral 3000 (2700-4500) mg/kg
male and female rats, oral 2500-3000 mg/kg

3.7 Subchronic oral toxicity

The compound has been administered orally by gavage to the Sprague-Dawley rats (10 males and 10 females/group) at dose levels of 0-5-20-60 mg/kg/day (in aqua distillate) for 90 days. Due to mortalities one male (0-5-60 mg/kg/day) or two female (5 mg/kg/day) rats have been additionally treated. The following observations or laboratory investigations have been made: clinical condition and behaviour; body weights; food consumption; ophtalmoscopy; haematology, clinical chemistry, urine analysis. At the end of the dosing period the following procedures have been made: necropsy, organ weights evaluation and histopathology. The results showed a slight statistically significant increase ($P < 0.05$) in relative liver and kidney weights in the females tested at the highest dose. Fur and skin of the tail of rats treated with 20 and 60 mg/kg showed partially lilac colouring, especially around the genital region. Due to a dark lilac coloured urine the urine analysis data of rat treated with 60mg/kg in week 13 could not be estimated. The observed mortality was not related to the treatment. For all other investigations no significant differences have been observed between treated and control groups. The dose of 20 mg/kg represents the dose with the NOEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound has been applied epicutaneously as a 3 % suspension in propylene glycol to the clipped flank areas on 15 female guinea pigs once a day for 5 days. The compound resulted not irritant after reading at the end of treatment and 72 h later.

4.1 Irritation (mucous membranes)

The compound has been instilled into one eye of 5 female Pirbright guinea pigs at dose of 1.5 % (0.1 ml suspension in propylenglycol) without rinsing. The irritation of mucous membrane has been evaluated at 0.5 - 1 - 2 - 3 - 4 - 6 - 7 hours after applications, and 24 hours after the instillation of one drop 0.1 % of fluorescin sodium solution. Positive reactions have been revealed in all animals (erythema and fluid excretion). The compound resulted slightly irritating (eye irritation index = 6.0).

5. Sensitization

It has been studied in 20 female Dunkin-Hartley guinea pigs by three intradermal injection, on shoulder region: 1) Freund´s complete with distilled water (1:1); 2) 5 % solution of test compound in arachis oil; 3) a 1:1 solution of the test compound and Freund´s complete adjuvant in distilled water. 10 animals served as negative control. One week later 50 % w/w solution of the test compound in petroleum jelly was topically applied, under occlusion, on the same skin area for 48 hours. Two weeks later the guinea pigs were challenged by a topical application of 0.1 - 0.2 ml of the test compound as 50 % (w/w) dilution in petroleum jelly, under occlusion, to the right flank. No signs of skin reactions have been observed at 24 and 48 h after challenge in any of the test or control animals. The compound resulted not sensitizer in guinea pigs.

6. Teratogenicity

The compound was orally administered by gavage to groups of 23 - 24 SPF albino rats on day 5 to 15 of gestation period at doses of 0, 10, 30, and 90 mg/kg b.w. (10 ml/kg b.w. in aqua deionized). The following observations were made: clinical, weight development and food consumption of mated females. All mated females were sacrificed on day 20 of gestation for evaluation of uterine data: foetuses (alive/dead), birth position (anterior / posterior), early and late resorptions, placentae, implantation sites. The number of corpora lutea was also counted. The following specific investigation of fetuses were performed: gross external examination, visceral imperfections, skeletal defects. During treatment a violet discolored urine of all females has been observed. No differences between treated and control groups have been observed for all parameters and exames performed. The study gave „no-effect-level“ at 90 mg/kg b.w. for maternal toxicity and fetal development adverse effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

The compound (^{14}C-labelled) was applied on the clipped dorsal skin of the Sprague Dawley rats (3 males and 3 females for each experimental group) as a 6.66 % water solution (group C), or included in two different hair dye formulations without (2.0 % in form.I., group A) or with 9 % hydrogen peroxide (2 % in form., II. group B). The compound has been also applied as a 2.0 % aqueous solution on two different treatment groups for the analysis of urine, faeces, organs, carcass without GIT (group D) or blood levels (group E). The radioactivity has been estimated (groups A, B and C) in rinsing water, treated skin, urine, faeces, organs and carcass. The percutaneous absorption has been evaluated after 72 h (group A, B, C, D) or 24 h (group E) from the amount of ^{14}C eliminated already from the body plus the amount of ^{14}C still being present in the carcass. The results showed a mean percutaneous absorption of: 0.021 % (form.I., group A), 0.025 % (form.II., group B), 0.088 % (water solution, group C). The excretion in the urine and faeces was 75 - 79 % and 22 - 25 % respectively. In the first 24 h, 74 - 90 % of the eliminated ^{14}C-labelled compound (total mean excretion) has been excreted. After 72 h the blood or organs levels of ^{14}C labelled compound were near or under the detection limit (0.0004 % of dose/g for thyroids - 0.00003 % dose/g for large organs). In the application site these values of ^{14}C-labelled compound have been revealed: 0.38 % (form.I., group A), 0.69 % (form.II.

group B) and 1.56 % (water solution, group C). In the oral study 74 % and 24 % of the compound has been eliminated in the urine and faeces respectively: 95 % of the compound was eliminated in the first 24 hours.

8. Mutagenicity

The compound tested for gene mutation in five strains of *Salmonella typhimurium*, in the absence and in the presence of S9 mix, showed positive results in TA 98 and TA 1538 strains (± S9 mix). No mutagenic effects have been observed in TA 98 NR (nitroredutase-deficient) *Salmonella* strain. The compound did not induce gene mutation in mouse lymphome L 5178 Y cells line (HPRT), micronuclea on mice (1250 mg/kg stomach intubation, analysis at 24 h) produced negative results.

11. Conclusions

The ingredient has low acute toxicity by the oral route for mice and rats. There is no evidence of skin irritation with a 3 % solution and skin sensitization in guinea pigs. The ingredient resulted a slightly eye irritating agent on guinea pigs. In a 90 day oral study (gavage) on rats a no effect level of 20 mg/kg was observed.

Mutagenicity data included positive results on TA 98 and TA 1538 strains of *Salmonella*, in the presence and absence of a metabolic system, but not in TA 98 NR, a nitroreductase-deficient strain of *Salmonella*. Negative results were obtained in mouse lymphoma L 5178 Y cell line (HPRT-gene-mutation), in the Micronucleus test on mice treated with 1250 mg/kg by stomach intubation (24-48-72 hours), in the SCE test on CHO cell line, in the UDS test on *in vitro* rat hepatocytes, and *in vivo* rat liver. No teratogenic effect was observed in rats treated up to 90 mg/kg b.w.

The ingredient applied on clipped dorsal skin of rats as a water solution, or as a hair dye formulation with and without an oxidising agent (H_2O_2) showed a cutaneous absorption of less than 1 %, after 72 hours.

The exposure level during a hair dye application with this ingredient is of 1 g: with a cutaneous absorption percentage of 1 % or less, the human exposure to this ingredient is less than 10 mg/person, equivalent to 0.16 mg/kg b.w.

Considering a no effect level found in rats is based on daily exposure for 90 days subcronic toxicity study on rats, the safety margin for this compound is more than 125.

It should be noted that the no effect level found in rats is based on daily exposure for 90 days, while human exposure to this oxidative hair dye is unlikely to be more than once a month.

On the basis of the overall available information the use of this ingredient does not present a risk for the consumers.

Classification: A

B 82: *RK-BLAU*

1. General

1.1 Primary name

RK-Blau

1.2 Chemical names

N-(2,3-dihydroxypropyl)-4-[ethyl-(2-hydroxyethyl)-amino]-2-nitroanilin hydrochloride
1-[(2,3-dihydroxypropyl)-amino]-2-nitro-4-[ethyl-(2-hydroxyethyl)amino]-benzene hydrochloride

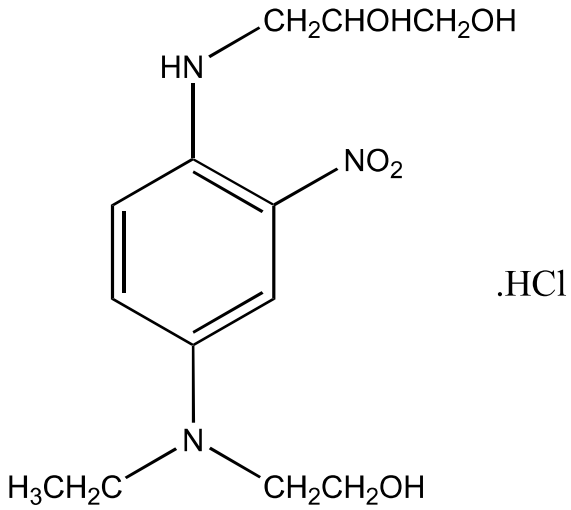
1.3 Trade names and abbreviations

Cos 316

1.4 CAS no.

114087-42-2

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{13}H_{21}N_3O_5xHCl$
Mol weight: 335.6

1.7 Purity, composition and substance codes

sA = 98.7 % N-(2,3-dihydroxypropyl)-4-[ethyl-(2-hydroxyethyl)-amino]-2-nitroaniline hydrochloride
1.2 % 4-[2,3-dihydroxypropyl)-amino]-N-ethyl-3-nitroaniline hydrochloride
0.1 % not identified

Impurities:

Impurity	CASnr.	Quant	unit
* 4-[(2,3-dihydroxypropyl)-amino]-N-ethyl-3-nitroaniline hydrochloride	114087-40-0	1.2	%

1.8 Physical properties

Appear. sA: yellow to yellow-green powder
UV-VIS spectrum present: yes

1.9 Solubility

Solubility in
water: > 100000 mg/l
DMSO: > 10 %
Propylene glycol: 0.8 %

2. Function and uses

RK-Blau is used in oxidative hair dye formulations at a maximum of 2 %. When mixed with hydrogen peroxide the final concentration in the dyeing formulation will be 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LD_{50}	Unit	Remark
sA	oral	rat, male	4470	mg/kg b.w.	as 25 % soln in water
sA	oral	rat, female	4580	mg/kg b.w.	as 25 % soln in water

Disturbances in consciousness, with apathy, abnormal posture and position, disturbances in coordination, slightly reduced reflexes and slight piloerection. Blue-violet discolouration of skin and mucous membranes and blue-violet urine. Symptoms mentioned above occurred 10 min. after administration and lasted for 24 hrs. in survivors. Macroscopy of animals, died, showed blue-violet discolouration of all tissues.

3.7 Subchronic oral toxicity

Route: oral Exposure: 90 d DWE: 15 mg/kg b.w.
Species: rat Recov. p.: 28 d LED: 30 mg/kg b.w.
Subst.: sA

Groups of 20 - 25 male and 20 - 25 female Wistar rats (b.w. 141 - 194 g) received daily, 7 days/week, by gavage, for 90 days 0, 15, 30 or 90 mg RK-Blau/kg b.w. as an aqueous

solution. After 90 days 20 m and 20 f rats/group were killed. 5 male and 5 female rats of control and 90 mg group were maintained on a control diet for a 4-week recovery period.

Examinations
Daily behaviour and clinical signs. Weekly body weight, food- and water-consumption. In 10 m and 10 f/group ophthalmoscopy at week 0 and 13 and hearing test and reflex examination at week 6 and 13. At week 0, 6 and 13 in 10 m and 10 f/group and at the end of the recovery period in all remaining animals hematology (Hb, Ht, Er, Leu, Diff, MCV, MCH, MCHC, retics, thromb, prothr. time, inclusion bodies) and clinical chemistry (SAP, ALAT, ASAT, BUN, creatinine, glucose, total bilirubin, total proteins, albumin, serum electrophoresis, uric acid, triglycerides, cholesterol, Na, K, Ca, Fe, inorg. P, Cl). At 0, 6 and 13 weeks in 5 m and 5 f/group and at the end of the recovery period in all remaining animals urinalysis (s.g., pH, proteins, glucose, bilirubin, urobilinogen, blood, nitrate, ketones, sed.). Organs (9) of 20 m and 20 f rats/group were weighed after 13 week and of all remaining animals at the end of the recovery period. Macroscopy was performed in 20 m and 20 f rats/group after 13 weeks and in all remaining animals at the end of the recovery period. Microscopy of ca. 30 tissues was carried out in 10 m and 10 f rats of control and 90 mg group after 13 weeks. In addition liver, kidneys and ovaries of 10 m and 10 f rats in 15 and 30 mg groups after 13 weeks and of all animals at the end of the recovery period were examined microscopically.

Results
In the 90 and 30 mg groups violet staining of urine, fur, paws and tails of all animals was seen. A secondary staining of cages and bedding was observed in these groups. In the 15 mg group a slight bluish discolouration of the urine was seen. Urine of recovery animals in the 90 mg group was not coloured during the recovery period, but fur paws and tails remained stained. During week 7 - 13 males in the 90 mg group showed a significantly increased growth. Food- and water-consumption were normal in all groups. Hematology did not reveal significant treatment related changes. Clinical chemistry showed in males on 30 and 90 mg/kg b.w., after 6 weeks only, a slight but significant decrease in albumin and a slight but significant increase in globulin alpha-1 and alpha-2, both with a dose-relationship. Urinalysis showed dose-related blue-violet discolouration of the urine. In the 90 mg group urine could not be analyzed due to this discolouration. Organ weights were normal. Histopathology of the liver showed increased translucence and reactive cellular response of Kupffer's cells, most intensively and most frequently in rats on 90 mg/kg. b.w. and also to a certain degree in animals on 30 mg/kg b.w. In 2 out of 5 male animals in the 90 mg recovery group the liver changes were still present after a 4-week recovery period.

Remark: Results of determinations of uric acid values in serum were not given.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route:	skin	Exposure:	4 h
Species:	rabbit	Dose:	0.5 g
Subst.:	sA	Concentr.:	undiluted

6 rabbits received during 4 hrs an application on the shaven intact and on the shaven scarified skin with 0.5 g RK-Blau under occluded condition. Observations were made immediately after

exposure and 24, 48 and 72 hr after exposure. No dermal reactions were observed according to the authors.

Remark: No information was given on possible discolouration of the skin which could have been resulted in difficulties at examination of the skin reactions.

4.1 Irritation (mucous membranes)

Route: eye
Species: rabbit Dose: 0.1 g
Subst.: sA Concentr.: undiluted

0.1 g RK-Blau was applicated in the conjunctival sac of the eyes of 6 rabbits. The eyes were not washed. After 1, 2 and 8 hrs and after 1, 2, 3, 4, 5, 6 and 7 days observations were made. During the first 8 hrs after application slight conjunctival erythema was seen in all rabbits. After 8 hrs no reactions were seen according to the authors.

Remark: No information was given on possible discolouration of the eye which could have been resulted in difficulties at examination of the ocular reactions.

5. Sensitization

Subst.: sA Conc. induc.: 3 % 3 %
Species: guinea-pig Conc. chall.: 3 %
Method: Magnusson Kligman

A group of 20 Dunkin Hartley guinea-pigs was induced with 3 series of 2 injections (0.05 ml each) with 3 % RK-Blau in dist. H_2O, 3 % RK-Blau in FCA (1:1 in arachis oil) and FCA (1:1 in arachis oil), respectively. On day 7 the animals received on the shaven injected area a dermal application with 0.5 ml of a 3 % RK-Blau soln. in dist. H_2O during 48 hr under occluded condition. 14 days later the animals received a dermal challenge application with 0.5 ml of a 3 % soln. of RK-Blau in dist. H_2O during 24 hr under occluded condition. 24 hr and 48 hr after removal of the challenge application observations were made. A control group of 20 animals was used. No primary irritation or sensitization was observed in the treated group.

Remark: The skin of the guinea-pigs in this maximization test was not treated with sodium lauryl sulphate during the induction period as is prescribed for non-irritation substances. According to the authors RK-Blau did not reveal skin irritation in rabbits (see 4.1).

In addition no information was given on possible discolouration of the skin which could have been resulted in difficulties at examination of the skin reactions.

Microscopical examination of the skin in the treated area could have given an unequivocal result of this test.

6. Teratogenicity

Route: oral Admin. Days: 5 - 15 DWE: 90 mg/kg b.w.
Species: rat
Subst.: sA

Groups of 24 pregnant Wistar rats received daily during day 5 - 15 of gestation 0, 15, 30 or 90 mg RK-Blau/kg b.w. as an aqueous solution by gavage. The animals were killed on day 20.

Examinations:

— Dams: Daily signs of toxicity and behaviour. Body wt. on day 0, 5, 10, 15 and 20. Food consumption for 5-day intervals during 20 days. Macroscopy of organs, wts. of placenta and uteri, no. and sites of implantation, no. of corpora lutea, no. of early and late resorptions.

— Fetuses: No. live/dead, sex ratio, body weights, gross abnormalities. 2/3 of fetuses - skeletal abnormalities (Alizarin red) 1/3 of fetuses - visceral abnormalities (Wilson).

Results: In all treated groups urine of dams was slight to dark violet discoloured with a dose-relationship. No maternal toxicity was seen. No irreversible structural abnormalities or embryotoxic effects were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

3 groups of 3 m and 3 f anesthetized Sprague-Dawley rats received during 30 min. an application on the shaven dorsal skin (3 x 3 cm) with 10 mg ^{14}C-labelled (in benzene ring) RK-Blau as a hair dyeing formulation, as a hair dyeing formulation with H_2O_2 or as an aqueous solution. After the application period the skin was scraped off with a spatula, rinsed with a 3 % solution of shampoo and thereafter with water (37° C). Rinsing was continued until water and cellulose absorbent, used to dab the skin dry, were free of colour. Subsequently the animals were placed in a metabolism cage and urine and feces were collected for 72 hr. Thereafter the animals were killed. Radioactivity was determined in scrapings, rinsings and absorbents, urine, feces, organs (15), treated skin + surrounding skin and carcass. Total recovery was 96.6 - 97.4 %. Majority of the applied ^{14}C was found in scrapings, rinsings and absorbents i.e. 96.0 - 96.9 % of the applied dose. Mean ^{14}C content at the site of application was 0.36, 0.61 and 0.77 % of the applied dose after application of a hair dyeing formulation, a hair dyeing formulation with H_2O_2 and an aqueous solution, respectively.

In urine 0.012, 0.027 and 0.09 % of the applied dose and in feces 0.006, 0.011 and 0.039 % was excreted within 72 hr after application of the hair dyeing formulation, the hair dyeing formulation with H_2O_2 and the aqueous solution, respectively. Excretion of RK-Blau was fast; 82, 81 and 89 % of the radioactivity, recovered in urine and feces, was excreted within 24 hr. ^{14}C in carcass and organs was beneath the detection limit. Mean cutaneous absorption (calculated from ^{14}C eliminated via urine and feces and ^{14}C in carcass) was 0.019, 0.039 and 0.130 % of the applied dose from a hair dyeing formulation, a hair dyeing formulation with H_2O_2 and an aqueous solution, respectively.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *In vitro* mammalian).

Sb.	Species	Strain	Meas.endp	Test conditions -act	res +act	res +a	sp +a	ind
*sA	*Salm.typh.*	TA97	frameshift mut.	1-6000 µg/pl in water	-	-		

Sb.	Species	Strain	Meas.endp	Test conditions -act	res +act	res +a	sp +a	ind
*sA	*Salm.typh.*	TA98	frameshift mut.	1-6000 µg/pl in water	-	-		
*sA	*Salm.typh.*	TA100	base-pair subst.	1-6000 µg/pl in water	-	-		
*sA	*Salm.typh.*	TA100 NR	base-pair subst.	10-6000 µg/pl in DMSO	-	-		
*sA	mouse lym. L5178Y		gene-mutations	50-1580 µg/ml in DMSO. At 5000 µg/ml no survival	-	-	r	AR
*sA	Chin.hamst ovary cell		chrom.aber	165, 500, 1650 µg/ml in in DMSO. 5000 µg/ml not toxic.	-	-	r	AR

Abbreviations:

Meas.endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

8.2 Mutagenicity (*In vivo* mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	NMRI	micronuclei	orally 20, 70 and 200 mg/kg b.w. in water	-

8.3 Mutagenicity tests (text).

In the Ames-test only three Salmonella strains were tested. No preceeding toxicity test was performed. The origin of the S_9-mix showed less than 2x increase in number of revertants/plate. No Quality Assurance Declaration was supplied. The test does not meet the current standards and therefore is not acceptable.

The Ames-test with the nitroreductase deficient strain TA 100 was performed with S_9-mix only. No preceeding toxicity test was carried out. The origin of the S_9-mix was not given. No Quality Assurance Declaration was supplied. The test does not meet the current standards and therefore is not acceptable.

In the assays with *Salmonella typhimurium* strains no information was provided on species and inducer used for metabolic activation.

Indicator tests (Bact., Non mammalian eukaryotic. *In vitro* mammalian):

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	Chin.hamst. ovary cell		SCE's	3.35-335.6 µg/ml	-	-	r	AR

Abbreviations:

Meas.endp. = measured endpoint
sp = species used for activation (r = rat, m = mouse, h = hamster)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

In the SCE-test in Chinese hamster ovary-Kl cells no preceeding toxicity test was performed. Furthermore no information on cell cycle time is available, the origin of the S_9-mix is not given and the positive control assays showed doubtful results. No Quality Assurance Declaration was supplied. This test does not meet the current standards and therefore is not acceptable.

11. Conclusions

General

RK-Blau is a yellow to yellow green powder used as a colouring agent in oxidative hair dye formulations at a maximum of 2 %. Mixed with H_2O_2 the final concentration of RK-Blau in the dyeing formulation is 1 %. With respect to physical properties only data on solubility in water, DMSO and propylene glycol are supplied.

Metabolism

A dermal absorption study in rats showed a mean cutaneous absorption of 0.019, 0.039 and 1.30 % of the applied dose (10 mg) from a hair dyeing formulation, a hair dyeing formulation with H_2O_2 and an aqueous solution, respectively.

Acute toxicity

RK-Blau was slightly toxic at acute oral administration to rats (LD_{50} about 4.5 g/kg b.w.). The animals showed blue-violet discolouration of skin, mucous membranes and urine. Macroscopical examination revealed a blue-violet discolouration of tissues.

Irritation and sensitization

No skin- or eye-irritation in studies with rabbits or sensitization in a study in guinea-pigs was seen. Some concern existed about the interpretation of the results due to possible colouration of the skin by the dyestuff. Since no signs of reaction had been observed when the compound itself had been used (rather than a dilution) the results of these studies were considered as reassuring and no further animal studies could be justifiable.

Subchronic toxicity
A 90-day oral study in rats (dose levels 15, 30 and 90 mg/kg b.w.) showed dose-related violet staining of the urine, disappearing during the recovery period at the 90 mg level. Violet staining of fur, paws and tails was seen at 90 and 30 mg/kg b.w. not disappearing during the recovery period at the 90 mg level. Histopathology revealed effects on the liver in 30 and 90 mg/kg b.w. groups. The no-effect level in this study is 15 mg/kg b.w.

Chronic toxicity
No data available.

Teratogenicity
An oral teratogenicity study in rats did not reveal teratogenic or embryotoxic effects up to the highest dose of 90 mg/kg b.w. In dams a dose-related violet discolouration of the urine was seen. No maternal toxicity was seen in this study.

Mutagenicity
The supplied bacterial assays in *S. typhimurium* and the SCE test in mammalian cells *in vitro* showed severe deficiences and are not acceptable. Adequate *in vitro* studies in mammalian cells detecting gene-mutations and chromosomal aberrations and an adequate *in vivo* micronucleus assay in mice showed negative results.

Conclusions
RK-Blau is a colouring agent used in oxidative hair dyeing formulations with a final concentration of 1 %. Cutaneous absorption (determined in a rat study) from an oxidative hair dyeing formulation with H_2O_2 is 0.039 %. RK-Blau possesses only slight acute oral toxicity.

The skin- and eye-irritation studies in rabbits and the sensitization study in guinea-pigs did not reveal an effect.

In a sub-chronic oral study in rats the no-effect level is 15 mg/kg b.w. based on effects on the liver.

RK-Blau did not reveal any teratogenic or embryotoxic activity in a study with rats to the highest dose level of 90 mg/kg b.w. No maternal toxicity was seen. RK-Blau induced neither gene-mutations or chromosomal aberrations in mammalian cells *in vitro* nor micronuclei in mice *in vivo.*

In spite of the deficiencies of the bacterial assays mentioned above a repeat of the mutagenicity assay in *Salmonella typhimurium* would only provide limited information because scientific literature (J. Ashby and R.W. Tennant, Mut. Res. 257, (1991), 229-306) showed that aromatic amino/nitro compounds give often false-positive effects in the Salmonella assay.

For normal use of RK-Blau the following calculation can be made:

1000 mg of RK-Blau comes in contact with the skin in permanent hair dye condition and 350 mg in semipermanent hair dye condition (based on a maximum usage volume of 100 ml and 35 ml hair dye formulation containing 1.0 % RK-Blau). At a maximum dermal penetration of 0.039 % this results in a dermal absorption of 0.4 mg for permanent treatment and 0.14 mg for semipermanent treatment, which is 0.007 and 0.002 mg/kg b.w., respectively (assuming a body weight of 60 kg). With respect to the no-effect level of 15 mg/kg b.w. in rats, this means

that there is a safety margin of 2100 and 7500, respectively for the use of the permanent hair dye formulation and the semipermantent hair dye formulation. It has to be noted that the no-effect level in rats is based on daily exposure for 90 days while human exposure to permanent hair dye formulation is unlikely to be more than once a month and human exposure to semipermanent hair dye formulation is unlikely to be more than once a week.

No additional data are needed and the safety margins for both permanent and semipermanent use were acceptable.

Classification: A

S3: *ETHOXYLATED ETHYL-4-AMINOBENZOATE*

1. General

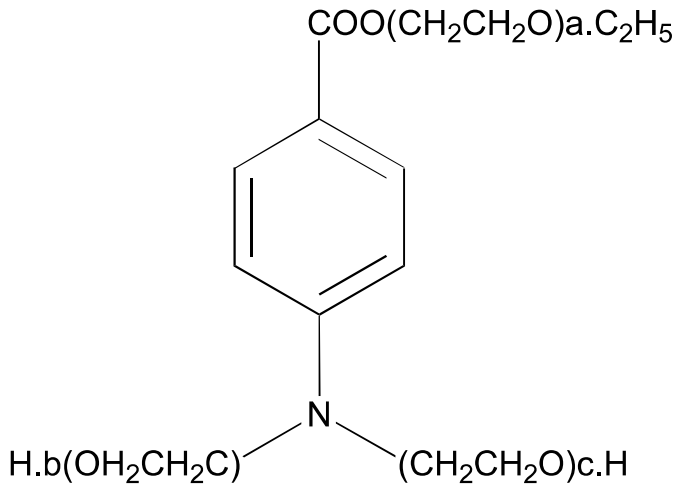
1.1 Primary name

Ethoxylated ethyl-4-aminobenzoate

1.2 Chemical names

Ethoxylated ethyl-4-aminobenzoate

1.5 Structural formula



(a+b+c) = 25

1.6 Empirical formula

Emp. formula: $C_{59}H_{111}NO_{27}$
Mol weight: 1266.6

1.7 Purity, composition and substance codes

The compound is manufactured by reacting the ethyl ester of para-aminobenzoic acid with ethylene oxide. Free ethylene oxide is then blown away by a stream of nitrogen. The content of ethylene oxide in the end product is less than 1 ppm. Purity greater than 99 %.

1.8 Physical properties

Appearance: A clear slightly viscous yellow liquid at room temperature.

1.9 Solubility

Soluble in water; poorly so in ethanol or anhydrous isopropanol.

2. Function and uses

Proposed use level: up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute toxicity is low: in the mouse (i.p.) and the rat (oral) the LD_{50} is greater than 1.9 g/kg b.w. Exposure of rats to air saturated with a.i. for up to 8 hours produced no abnormality.

3.7 Subchronic oral toxicity

Rat: a 3 month test using a.i. in the diet was carried out according to GLP in groups of 10 male and 10 female Wistar rats. The dose levels were 0, 1000, 4000 and 16000 ppm, approximately 70, 290, 1130 mg/kg b.w./day (males) and 80, 360 and 1350 mg/kg b.w./day (females).

The main abnormal findings were as follows. The total bilirubin in dosed males fell progressively with dose. There was no obvious reason for this. Histological examination of the liver showed cellular infiltration and fatty changes in all groups, including controls; and tubular mineralisation of the kidneys was found in all female animals, both test and control. It was concluded that no drug related abnormality had been produced.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit: A patch with about 0.5 ml of test solution was applied to the shaved skin of the back. With undiluted a.i., exposure was for 1, 5 and 15 minutes and 20 hours; using 10 % and 50 % aqueous solutions, exposure was for 20 hours. The undiluted material caused slight erythema which faded over 8 days. The diluted solutions caused no irritation. Undiluted a.i. or 10 % or 20 % aqueous solutions were applied to the inner skin of the ear in groups of 2 animals for 20 hours. The results were similar to those of the preceding experiment.

A patch soaked in 50 % aqueous solution of a.i. was applied for 8 hours a day for 5 days, always to the same area. No abnormality was produced.

Groups of 6 male albino rabbits were used; sites on either flank were prepared, and those on the left side scarified. A 20 % aqueous solution was applied on a patch for 24 hours without occlusion. Reading was at 24 and 48 hours. There was very slight erythema in 4/6 animals.

Two animals had 6 applications in a week of a 50 % aqueous solution to an area of 36 cm^2 of depilated dorsal skin. Each application was for 8 hours. There was no evidence of irritation.

Man: Twenty subjects, some suffering from skin disease, were tested. Undiluted a.i. and aqueous solution of 1 %, 5%, 10% and 50% were applied on patches for 20 hours over an area of 1 cm^2. No irritation was produced.

4.1 Irritation (mucous membranes)

Rabbit: the undiluted a.i. was applied to the conjunctiva in a dose of 50 mm^3. There was a slight redness and opacity at 1 hour and 24 hours, but appearances were normal at 8 days. The use of 10 % and 50 % aqueous solution was followed by no abnormality.

Further tests were carried out on the chorio-allantoic membrane of the chick at 10 days incubation. Concentrations of 1 % and 10 % in olive oil were applied. Rinsing was carried out after 20 seconds. The substance is stated by the authors to be „practically non irritant“ at these concentrations, but details of the scoring system are not given.

5. Sensitization

Guinea pig. Ten animals were used for the test, and 3 were subjected to challenge only, without induction. The a.i. was dissolved in acetone, and applied to the flank; the same area was used throughout the induction. The first application was of a 50 % solution, and subsequent ones were of 80 %. Nine applications were made over 2 weeks. After a 12 day rest, a challenge application with a 50 % solution was made to the opposite flank. Reading was at 12 hours. There was no evidence of sensitization, or of primary irritation.

Man. A maximisation method was used in 27 male and female subjects; 3 subjects failed to complete the test. The test site was pretreated with aqueous 5 % sodium lauryl sulphate for 24 hours with occlusion, for 48 hours at a time. Five such applications were made. After a two week rest, 5 % lauryl sulphate was applied to a fresh site, with occlusion, for 30 minutes. The challenge applications were the same as those used for induction, and were applied for 48 hours, with occlusion, to the newly prepared site and to a fresh previously untreated site. Control application was of soft paraffin. There were „very few“ cases of mild irritation due to the sodium lauryl sulphate. There was no evidence of sensitization or of primary irritation.

Photosensitization

Guinea pig. Twenty albino animals were tested, in groups of 4 (2 male and 2 female). A daily application of 0.5 ml of a 20 % aqueous solution of a.i. was made to the shaven skin of one flank daily for 5 days. Each application was followed by 15 minutes of UV irradiation, from lamps with a maximum output at 260 nm. After a 10 day rest, an application to the shaven skin of the opposite flank was followed by the same irradiation. Reading was at 24 and 48 hours. The test was negative.

6. Teratogenicity

Test for teratogenic activity and embryotoxicity.

Fertile hen eggs were used; a suspension of a.i. in olive oil was injected on day 1 or day 5 of incubation. The doses of a.i. used (µl/egg) were: 0.25, 0.625, 2.5 and 6.25; the control was olive oil.

There was a dose related increase in mortality. The chicks hatched from eggs injected on day 1 of incubation showed no abnormalities; those injected on day 5 showed a significant increase in both absolute and relative weights of the heart, but the absolute increase was small and probably not of biological significance.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man: Two sets of tests are reported in which the technique of photoacoustic spectrometry was used. A 2.5 % concentration of a.i. was applied and the technique was used to follow the disappearance of a.i. from the stratum corneum. It was concluded that all the a.i. had disappeared from the stratum corneum in 56 hours. No quantitative data were obtained.

An investigation using the stripping technique was carried out in 10 subjects. A gel containing 10.8 % of a.i. was applied to both forearms, for 15 minutes on one and 30 minutes on the other. The areas were stripped 12 times. It was found that about 0.07 mg/cm^2 of a.i. was absorbed into the stratum corneum.

Rougier *et al* found that the amount of benzoic acid absorbed in 96 hours could be determined by the stripping method by the use of the formula $y = 1.38 x - 0.52$.

If this is applicable to the a.i., the amount absorbed would be about 100 nmoles/cm^2; extrapolated to 1.6 m^2, this would imply an absorption of about 33 mg/kg b.w.

8. Mutagenicity

An Ames test was carried out using strains TA 98, 100, 1535 and 1537. There was no evidence of mutagenicity.

Mouse. A micronucleus test was carried out according to GLP standards. The doses used were 2500, 5000 and 10000 mg/kg b.w., given orally. There was no evidence of clastogenic activity.

10. Special investigations

Phototoxicity.

Man: Ten subjects were used. Each had 3 applications made to the skin at discrete sites: 10 % aqueous solution of a.i.; 10 % solution of the di-isobutyl ester of diethylaminophtalate; and a control solution. The treated areas were exposed to UV radiation in a stepwise manner to determine the m.e.d. The two compounds were equiactive as sunscreens, and there was no evidence of phototoxicity. The report gives little detail.

11. Conclusions

The compound appears to have low acute and subchronic toxicity. It shows no evidence of being irritant to the skin or the mucosa, and tests for sensitization are negative. The test for photosensitization was carried out at 260 nm, which is inappropiate. The data on phototoxicity are too scanty to be evaluated in a satisfactory manner. Tests for photomutagenicity were not carried out. The method used for testing for teratogenic activity is not a validated one. The tests for percutaneous absorption were not conclusive. A chromosomal aberration test *in vitro* should be carried out.

Classification: C

S66: *4-TERT.-BUTYL-4′-METHOXYDIBENZOYLMETHANE*

1. General

1.1 Primary name

4-tert.-butyl-4′-methoxydibenzoylmethane

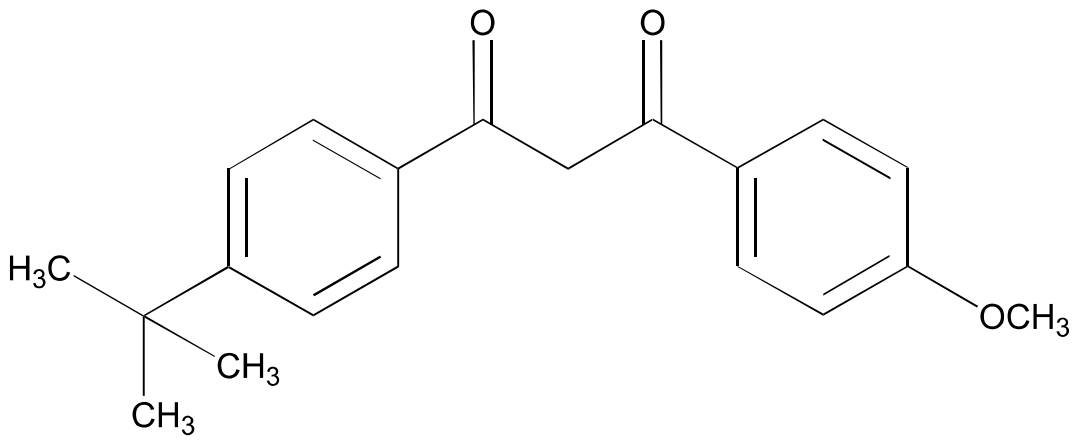
1.2 Chemical names

4-tert.-butyl-4′-methoxydibenzoylmethane

1.3 Trade names and abbreviations

“Parsol 1789”

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{20}H_{22}O_3$

Mol weight: 310.4

1.8 Physical properties

Appearance: Crystalline substance

Absorption spectrum 310 - 340 nm; maximum 355 nm.

1.9 Solubility

Soluble in carbitol 15 %, chloroform 15 %, aceton 5 %, ethanol 2 %; insoluble in water.

2. Function and uses

Use level: up to 5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. The LD_{50} was greater than 16 g/kg b.w. in males and females. There was a relative absence of sperm from the epidydimes of treated animals.

Mouse (oral and intraperitonal). Up to 8 mg/kg b.w. did not cause mortality, although some abnormal clinical signs were observed.

3.2 Acute dermal toxicity

Rat. Up to 1000 mg/kg b.w. with occlusion for 24 hours did not cause any deaths. No evidence of compound related skin damage was found. The LD_{50} was estimated at greater than 1 g/kg b.w.

3.5 Repeated dose dermal toxicity

Rat. Four week study.

Four groups, each of 5 male and 5 female animals, were studied. Dose levels and conditions were: control (abraded skin); 120 mg/kg b.w./day (abraded skin); 200 mg/kg b.w./day (abraded skin); 230 mg/kg b.w./day (intact skin). Exposure was for 5 hours a day, with occlusion, followed by rinsing. There was some skin irritation in all groups. Some changes in haematological findings occurred, but they do not appear to be significant. No significant changes were found in biochemical values. Necropsy was grossly normal, and histological examinations revealed no abnormality, either in internal organs or the skin.

3.7 Subchronic oral toxicity

Rat. In a 13 week study, 4 groups of 12 male and 12 female rats were given the a.i. in the diet in amounts equivalent to 0, 200, 450 and 1000 mg/kg b.w./day.

There were no treatment related deaths. Food consumption was reduced in the intermediate and top dose groups. There was a fall in the red cell count in females at the intermediate and top dose groups. There plasma protein levels were somewhat higher in all dosed animals, but this did not seem to be dose related. At necropsy, the relative liver weights of females were increased in both the intermediate and top dose animals.

Supplementary groups of 6 rats were treated with the top dose and then allowed a 4 week recovery period. At sacrifice, the liver weights of these rats were similar to those of control rats.

Depending on the view taken of increase in liver weights, the NEL may be 200 or 450 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit: Five groups, each comprising 10 male and 10 female animals, were studied. In each group, 5 animals had the skin abraded, and 5 had not. There were 3 test groups, a solvent

control group and a procedural control group. Applications were made to the test groups of 30, 60 and 360 mg/kg b.w./day, with occlusion, for 6 hours each day for 21 consecutive days. The concentrations of a.i. used were 1.5 %, 5 % and 18 %, respectively, in carbitol. Some irritation was found in the vehicle control animals. There was a dose related erythema in treated animals, being slight at 30 mg/kg b.w./day. Abrasion did not affect the findings. No changes due to treatment were found in body weight, food or water consumption, or in haematological examination, except at the sites of application.

Rabbit. Two groups of 6 rabbits were used, one test and one solvent control. The a.i. was dissolved in ethanol/ 2-phenylethanol (50/50) in a concentration of 10 %; 0.5 ml was applied over 4 cm^2 to an abraded and a non-abraded site on each animal for 4 hours with occlusion. The primary irritation index with the vehicles was 1.17, and of the solution of a.i. was 1.39.

4.1 Irritation (mucous membranes)

Rabbit: A standard Draize test was carried out, using a.i. dissolved in diethyl phtalate, without rinsing. There was no adverse effect up to the limit of solubility, 20 %.

5. Sensitization

Guinea pig: A Freund´s complete adjuvant (FCA) test was carried out. Two groups of 8 to 10 animals were used. Induction was by 3 intradermal injections, on days 0, 4 and 9, of a 50 % suspension of a.i. in FCA. Animals of the control group received FCA only. On days 21 and 35 a challenge was made by epicutaneous application of 0.025 ml of a.i. at the minimal irritant concentration and at 3 lower concentrations (each 1/3 of the preceding concentration).

There was no evidence of sensitization.

Guinea pig: A Magnusson-Kligman maximisation test was carried out on 2 groups of 20 and 25 animals, one test and one control. Induction was by intracutaneous injection of 0.1 ml of 5 % a.i. in FCA, 5 % a.i. in saline, and FCA alone. This was followed 7 days later by an epicutaneous application of a 20 % suspension of a.i. with occlusion for 2 days. The challenge was carried out on day 21; 20 % and 6 % solutions of a.i. were applied for 24 hours. There was no evidence of sensitization.

Guinea pig: Open epicutaneous test. The 2 experimental groups consisted of 20 animals each and the control group of 10. Solutions of 20 % and 6 % were applied daily to one flank for 21 days. Challenge was made on days 21 and 35 by application of the minimal irritant dose, the maximal non-irritant dose, and on third and one ninth of the maximal non-irritant dose, to the opposite flank. The report is in summary form only. There was no evidence of sensitization.

Man: Repeated insult patch test. Eleven male and 40 female subjects were recruited; 8 failed to complete the study. About 0.2 ml of a 10 % solution was applied under occlusion for 24 hours on 10 occasions, with rest intervals of 24 or 48 hours. On completion of this course, a 10 days rest period was allowed, and then challenge applications were made to the original site and to a new site. No adverse reaction was observed.

Photo-allergenic effects

Guinea pig: Four groups of 10 animals were used, 2 test groups, a negative control and a positive control. In the test animals, induction was carried out as follows: (a) Four injections of 0.1 ml of FCA were made in the neck to delineate a square. (b) In animals of the first test group, applications of 0.1 ml of 10 % a.i. in acetone were made over 8 cm^2 in this area, and 30 minutes later UVA irradiation at 10 J/cm^2 applied. In animals of the second test group, the concentration of a.i. was 1 %. (c) Procedure (b) was repeated 5 times over the subsequent 2 weeks.

Challenge: On days 21 and 35, 0.025 ml of a 10 % solution of a.i. was applied to both flanks over an area of 2 cm^2. The left flank of each animal was then irradiated as above. For the negative control, the induction applications did not contain a.i.; for the positive control, 3 % tetrachlorosalicylanilide replaced the a.i. This gave a well marked positive reaction. There was no evidence of photo-allergenicity due to the a.i.

Photosensitization

Man: Twenty-five volunteers were used. The a.i. was incorporated into petrolatum at 2 % to which 2 % DMSO was added as a maximising agent. A minimum erythema dose (med) for each subject was determined by finding the time taken to produce erythema using UVA + UVB, 285-400 nm.

Induction: This was carried out by application of the preparation of a.i. to 2 areas of the skin of the back, with occlusion. One of these was a test area, the other an irritancy control. The patches were removed after 24 hours. After a further 24 hours, the test area was exposed to 3 meds. This entire procedure was carried out 6 times, beginning on days 1, 4, 8, 11, 15 and 18.

Challenge was carried out about 10 days after the completion of induction. The test material was applied to 2 fresh sites and occluded for 24 hours. The sites were exposed to 10 J/cm^2 of UVA, 320-400 nm. A further site which had not been pre-treated was used as a radiation control. There was no evidence of photosensitization.

6. Teratogenicity

Rat: Four groups of 36 animals were used. Doses of 0, 250, 500 and 1000 mg/kg b.w./day were given by gavage from day 7 to 16 of pregnancy. The numbers pregnant in each group were (respectively) 33, 35, 31 and 34. At day 21, the animals were divided into approximate half groups; one half was selected for sacrifice and the other to continue to delivery and rearing. There was no evidence of teratogenesis.

Rabbit: Four groups of animals were used: control, 80, 200 and 500 mg/kg b.w./day. The doses were given by gavage on day 7-19 of pregnancy. The numbers present in each group were (respectively) 17, 19, 17 and 19. There were some maternal deaths, which did not seem to be compound related. In the low dose group, there was a high incidence of resorptions: the reason for this is not clear. There was no evidence of teratogenesis.

7. Toxicokinetics (incl. Percutaneous Absorption)

Naked rat: isolated skin *in vitro*. ^{14}C-labelled a.i. was applied at a level of 180 $\mu g/cm^2$, using a 1.5 % solution of a.i. in acetone or deltyl. Experiments were carried out for 1 and 6 hours.

Amounts of a.i. were estimated in the stratum corneum by stripping, in the remainder of the skin, and in the chamber fluid. In none of the experiments was any activity found in the chamber. With the acetone vehicle, the amounts in the stratum corneum were about 10 % (of the applied dose) at 1 and 6 hours; the amount in the skin was 4 % at 1 hour and 11 % at 6 hours; the amount in the skin was 4 % at 1 hour and 11 % at 6 hours. With the deltyl vehicle, the figures were 4 % and 5 %, and 4 % and 7 %.

In another similar experiment, the concentrations used were 120, 360 and 1200 µg/cm^2 for 1, 6, 16 and 24 hours. The concentration did not seem to make any difference to the amount found in the skin, but the amount found in the skin increased with time. Little or no activity was found in the chamber in any experiment. Representative figures for the amounts in strippings + skin were: 1 hour 7 %; 6 hours 17 %; 16 hours 28 %, 24 hours 44 %.

The above experiment was repeated to see whether the concomitant incorporation of „Parsol MCX“ made any difference. The solvent was carbitol. The results (at 1 and 6 hours) were broadly similar.

Rat *in vivo:* A 1 % solution of labelled a.i. dissolved in carbitol was applied at a dose of 120 mg/cm^2 for 6 hours. The amounts found in the stratum corneum and in the deeper layers were 1.4 % and 2.3 % respectively (Summary report only: origin of report not stated).

Minipig skin *in vitro.* A concentration of 2 % a.i. in 3 different vehicles (o/w lotion, o/w cream, and w/o cream) was used at 120 µg/cm^2 for 6 hours. The results showed that the total amounts found in the skin for each vehicle were (respectively) 2.6 %, 3.7 % and 2.9 %.

Man: Isolated human abdominal cadaver skin was used in a chamber experiment. Labelled a.i. was used as a 2 % formulation in a w/o cream. The amount applied was equivalent to 50 µg a.i./cm^2. The total amounts in the skin were: 1 hr: 4.5 %; 6 hr: 7 %; 16 hr: 16 %.

No activity was found in the chamber at any time. The skin was cut horizontally by microtome, and it was found that the lower corium contained only 0.35 % after exposure for the longest period.

Man *in vivo:* The a.i. was labelled with ^{14}C. The skin of the back of 4 subjects was treated with 200 µl of a 10 % solution in carbitol, giving an exposure of 2 mg/cm^2 of a.i. Exposure was for 8 hrs. Occlusion was employed in 1 subject. The amounts of a.i. found in the strippings and the urine were estimated. For the occluded experiment, these were 0.48 % and 0.08 %, respectively. In the absence of occlusion, the mean values were 0.17 % and 0.013 %. In a second experiment, unlabelled “Parsol MCX” was added to the a.i., and the recoveries were 0.32 % and 0.04 % (occluded) and 0.56 % and 0.03 % (without occlusion). No radioactivity was found in the blood or faeces in any subject.

8. Mutagenicity

An Ames test was carried out with up to 500 µg a.i. dissolved in DMSO. The test was negative with and without activation.

Tests were carried out on V79 Chinese hamster lung cells to see whether the a.i. induced mutation at the HGPRT locus. The solvent (methanol) caused toxicity at a concentration of a.i. greater than 20 µg/ml. Up to this level there was no evidence of mutagenic effect.

A test for photomutagenicity was carried out using *Saccharomyces cerevisiae* D7. This organism permits testing for crossing over, gene conversion and reverse mutation. Testing was carried out with UVA, but with UVB as well, since the a.i. has some activity at the latter wavelengths also. Intensities used generally were up to 500 000 J/cm^2 for UVA and up to 10000 J/cm^2 for UVB; these were taken to represent the maximum likely exposure of consumers in practice. Occasional experiments were carried out at higher intensities. Negative controls were used, and chlorpromazine was used as a positive control. The experiments seem to have been well carried out, and showed that (a) the a.i. alone is not photomutagenic in this system; (b) radiation alone causes mutagenic change; (c) as concentrations of a.i. increase, the degree of radiation induced mutagenesis decreases.

A test for photomutagenic activity was carried out using Chinese hamster ovary cells (CHO-K5) *in vitro.* Concentrations of a.i. from 15 to 90 µg/ml were used. The cultures were exposed to SSR sufficient to give UVA levels of up to 4 000 J/m^2, and UVB up to130 J/m^2. The positive control was 8-methoxypsoralen. The tests are reported to have shown (a) that the a.i. was devoid of photomutagenic activity but that suchan activity was shown by the positive control; and (b) that the positive control showed no mutagenic activity in the absence of irradiation.

Mouse. A micronucleus test was carried out using oral doses of 1000, 2000 and 5000 mg/kg b.w. given 30 and 6 hours before sacrifice. The test was negative.

11. Conclusions

This compound has low acute toxicity. A 4 week dermal toxicity study in the rat, with occlusion, showed some skin irritation, but no other significant findings. A 13 week oral toxicity study was carried out in the rat with doses of 200, 450 and 1000 mg/kg b.w./day. There were changes in food consumption at the top and intermediate doses, and a fall in red cell count in females, at the intermediate and top doses. The absolute liver weight in males was elevated at the top dose, and the absolute and relative liver weight in females at the intermediate and top doses. In animals allowed a 4 week recovery period, these changes reverted to normal. The no effect level may be 200 or 450 mg/kg b.w./day.

The compound did not produce irritation of mucous membranes at concentrations up to 20 %. Slight irritation was caused in rabbit skin by repeated applications (up to 18 % of a.i.) under occlusion; in man, a repeated insult patch test was negative, as was a rechallenge after 10 days. Tests in the guinea pig for sensitization, phototoxicity, and photoallergenicity were negative. Percutaneous absorption seems to be low in relation to the toxicity findings.

An Ames test, a test with *S. cerevisiae* and with V70 Chinese hamster lung cells and ovary cells showed no evidence of mutagenicity or of photomutagenicity. A micronucleus test was negative. Tests for teratogenic activity in the rat and the rabbit were negative. Clinical experience has shown the compound to be a rare allergen and photoallergen.

Classification: A.

OPINIONS ADOPTED DURING THE 51ST PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 7 October 1992

A 28: *3,4-DIAMINOBENZOIC ACID*

1. General

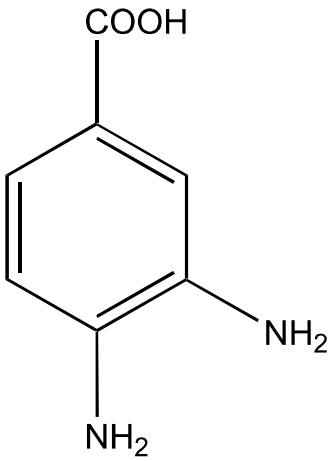
1.1 Primary name

3,4-diaminobenzoic acid

1.2 Chemical names

3,4-diaminobenzoic acid

1.5 Structural formula



1.7 Purity, composition and substance codes

The compound is supplied in oxidation hair dye formulations at concentrations up to 2 % and used at concentrations up to 1 % after dilution with hydrogen peroxide. The compound exists as the free base, the hydrochloride and dihydrochloride salts, the commercial product is usually the free base.

1.8 Physical properties

Appearance: The compound is a grey brown cristalline powder.

1.9 Solubility

It is sparingly soluble in alcohols and cold water and readily soluble in hot water.

2. Function and uses

Substance is used as a permanent hair dye.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats following administration in 0.5 % gum tragacanth containing 0.05 % sodium sulphite adjusted to pH 7. The LD_{50} value obtained was 13.5 g/kg. The signs of toxicity observed were piloerection accompanied by lethargy, ptosis, lack of coordination of voluntary movements and diuresis at the highest doses. Gasping was observed prior to death in some animals and pallor of the liver was found in all the mortalities. There were no histological findings in the survivors.

3.4 Repeated dose oral toxicity

Repeated administration studies on 3,4-diaminobenzoic acid comprise a 90 day and a 28 day study in the rat. A 90 day study has been carried out in the rat with the compound administered orally by gavage at dose levels of 500, 2500 and 5000 (increased to 6000 from week 9) mg/kg to groups of 25 males and 25 females. A satellite group of 30 males and 30 females to investigate reversibility received 7000 mg/kg for 9 weeks and a four week recovery period. No treatment related findings were observed at 500 mg/kg during the study however on histological examination changes were observed in the thyroid and kidney. In the thyroid the epithelia showed an increased tendency towards cubic and cubic/cylindrical cells and slight pigmentation was seen in 7 of 49 thyroids examined. The kidney findings were alteration of tubuli in 3 of 50 animals and focal nephritis in 4 of 50 animals.

There was a dose releated increase in histological findings with a tendency towards cylindrical cells and an increase in the intensity of pigmentation in the thyroid and alteration of the tubuli, focal nephritis and cicatricial retraction in the kidney. The pigment was identified as methaemoglobin. There was an increase in urine deposition, perineal staining, water consumption and urobilinogen at the higher doses. The weights of the liver and kidneys were increased and that of the prostate decreased. The histological effects were not reversed by a four week recovery period. A No Effect Level was not established in the 90 day study. A single dose level of 50 mg/kg was administered by gavage in the 28 day study. No treatment related differences were seen in this study except for discolouration of the urine by the dye.

4. Irritation & corrosivity

4.1 Irritation (skin)

No signs of skin irritation were observed in rabbits following 24 hour exposure under occlusion to a 2.5 % aqueous solution containing 0.05 % sodium sulphite adjusted to pH 7 to intact and abraded skin.

4.2 Irritation (mucous membranes)

Eye irritation was studied in the rabbit. A 0.1 ml aliquot of a 2.5 % aqueous solution containing 0.05 % sodium sulphite adjusted to pH 7 was instilled into one eye which was irrigated with

water 10 seconds after instillation. Mild conjunctival irritation was observed on the first day but no significant irritation occured.

5. Sensitization

The ability of the compound to induce skin sensitization has been studied in the guinea pig using the Magnusson and Kligman maximisation test but only 10 animals were used in the test group. A 0.1 % solution was used for intradermal injection during the induction phase and a 30 % DMSO solution applied dermally under occlusion. The challenge was made in a 20 % DMSO solution. No signs of sensitization were observed in the animals in the test group.

6. Teratogenicity

In a teratogenicity study groups of 22-24 pregnant female rats received 15, 45 and 90 mg/kg by gavage on days 5 to 15 of gestation. No treatment related effects were reported in either the dams or the fetuses.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption has been investigated in an *in vivo* study in rats using radiocarbon labelled 3,4-diaminobenzoic acid. The compound was applied under occlusion as a component of a hair dye formulation both with and without hydrogen peroxide and as an aqueous solution for 30 minutes. A total of 0.261 %, 0.097 % and 1.152 % respectively were absorbed with the majority renally eliminated. The application site skin contained 1.16 %, 0.59 % and 3.89 % respectively and the majority of the dose was recovered from the application site washings and dressings. The highest tissue levels 72 hours after an oral dose were seen in thyroid, lung, kidney and liver.

8. Mutagenicity

Negative results were obtained in studies to investigate the ability of 3,4-diaminobenzoic acid to produce gene mutation in *Salmonella typhimurium* or *Eschericia coli* 343/113. *Salmonella* strains TA1535, TA1537 and TA1538 were investigated in the presence and absence of an exogenous metabolic activation system. The compound was tested at concentrations up to 100 µg/plate. The *E.coli* study was a fluid test carried out at concentrations up to 100 µg/ml apparently only in the absence of an exogenous metabolic activation system. The compound was not mutagenic in a micronucleus test, however no signs of bone marrow toxicity were observed. A total dose of 2000 mg/kg was given orally as two equal doses 24 hours apart to 5 male and 5 female rats. The cells were harvested 6 hours after the second dose and 2000 polychromatic erythrocytes per animal were examined.

9. Carcinogenicity

No data on carcinogenicity studies on 3,4-diaminobenzoic acid were available.

11. Conclusions

3,4-diaminobenzoic acid has low acute toxicity by the oral route and studies suggest that dermal absorption from hair dye formulations is low. There was no evidence of skin or eye irritation following rapid (10 seconds) wash out with a 2.5 % solution. No data were available on animals not subject to rapid irrigation. There was no evidence of sensitization in a maximisation test in guinea pigs. In a 28 day oral study a no effect level of 50 mg/kg was reported. In a 90 day study effects on the thyroid and kidney were seen following histological examination at all three dose levels. These increased with increasing dose and were accompanied by other signs of dose related toxicity at doses above 500 mg/kg. The histological findings were not reversed by a four week recovery period after dosing. Mutagenicity data was limited to negative results *in vitro* in gene mutation assays in Salmonella and Eschericia coli and *in vivo* in a micronucleus test. No adverse effects were reported in an oral teratogenicity study in rats at up to 90 mg/kg.

However further data on mutagenicity are required (an *in vitro* study to measure gene mutation and a study to measure chromosome aberrations by metaphase analysis, both in mammalian cells) to provide adequate reassurances in this regard.

Classification: B

12. Safety evaluation

Exposure per application based on maximum volume (100 ml) and in use concentration (1.0 %) and assuming 60 kg human = 16.66 mg/kg. Amount absorbed assuming 1 % absorption through the skin = 0.166 g/kg. Comparison with a NOAEL of 50 mg/kg in a 28 day study in the rat gives a safety margin of about 300. Furthermore frequency of application is unlikely to exceed once a month, whereas the NOAEL is based on repeated daily exposure. There is an adequate margin of safety.

B 28: *1-HYDROXY-2-AMINO-4,6-DINITROBENZENE*

1. General

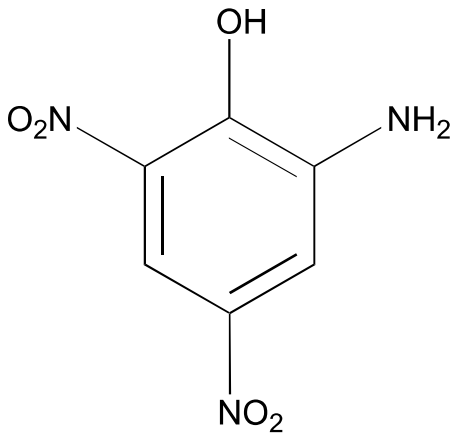
1.1 Primary name

1-hydroxy-2-amino-4,6-dinitrobenzene

1.2 Chemical names

1-hydroxy-2-amino-4,6-dinitrobenzene
Picramic acid

1.5 Structural formula



1.8 Physical properties

Appearance: The compound is a crystalline brown powder.

1.9 Solubility

It is soluble in water, ethanol, benzene and glacial acetic acid but no quantitative solubility data were available.

2. Function and uses

The compound is supplied in both semi-permanent and oxidation hair dye formulation at concentrations up to 1.25 %. The oxidation hair dye formulations are mixed with an equal amount of hydrogen peroxide and the final use concentration is 0.625 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats following oral administration in aqueous gum tragacanth. The LD_{50} value was 110 mg/kg in male and female rats. The reported signs of

toxicity were piloerection, orange staining of the extremities and gasping. Autopsy revealed discolouration of the liver, pallor of the kidney and spleen and orange staining of the inner body wall.

3.7 Subchronic oral toxicity

There are two 90 day studies reported; a dermal application study in the rabbits with a hair-dye formulation containing 0.1% picramic acid and an oral study in the rat. In the oral study the compound was administered by gavage to the rat at dose levels of 20, 40 and 80 mg/kg. There was an increase in water consumption and orange or dark gold colouration of the urine at 20 mg/kg. The livers of 5/15 females and 8/15 males and kidneys of 4/15 females and 12/15 males had a coarse structure on macroscopic examination but no histological findings were reported. Similar changes were observed in the livers of 14/15 females and 12/15 males and the kidneys of 4/15 females and 9/15 males at 40 mg/kg in addition to dark discoloration of the thyroid in 1 female and 2 males and dose related effects on clinical chemistry parameters. A number of treatment related deaths were seen at 80 mg/kg with toxic effects on the thyroid gland, testicles, livers, kidney and small intestine. These effects were not reversible in the recovery group. No biologically significant changes were seen between the test and control group in the rabbit following dermal application of a hair dye formulation containing 0.1% picramic acid. This dermal study in the rabbit was inadequate for assessment of the systemic toxicity of picramic acid after repeated exposure.

4. Irritation & corrosivity

4.1 Irritation (skin)

No signs of skin irritation were observed in rabbits following 24 h application under occlusion of a 2.5 % solution containing 0.05 % sodium sulphite adjusted to pH 7 to intact and abraded skin.

4.2 Irritation (mucous membranes)

Eye irritation has been studied in the rabbit with a 2.5 % solution containing 0.05 % sodium sulphite adjusted to pH 7. The eyes were irrigated with 20 ml of distilled water 10 seconds after instillation of 0.1 ml of the test solution. Mild conjunctival inflammation was observed in all three animals. This mild inflammation lasted to between 4 and 7 days. No significant inflammation occurred.

5. Sensitization

The ability of the compound to induce skin sensitization has been studied in the guinea-pig using the Magnusson and Kligman maximisation method. The compound was dosed at a concentration of 2 % for induction and dilutions of this were dosed during the challenge phase. Four of the 15 animals developed erythema during the challenge phase and the compound was classified as a mild sensitiser.

6. Teratogenicity

There are two reported teratogenicity studies with oral administration of 15 mg/kg to 26 pregnant female rats on days 5 to 15 of gestation and dermal administration of a hair-dye formulation containing 0.1 % picramate to 20 pregnant female rats on days 1, 4, 7, 10, 13, 16 and 19 of gestation. No treatment related effects were reported in either the dams or the fetuses in either of these studies.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption has been investigated in an **in vivo** study in rats using radiocarbon labelled picramic acid. The compound was applied under occlusion as a component of a hair dye formulation for 30 minutes or in a dimethylsulphoxide solution for 24 hours. A total of 0.38 % and 17.78 % respectively were absorbed and around 60 % of the absorbed material was excreted in urine with the remainder in faeces. The application site skin contained 1.67 % and 3.68 % of the dose respectively and the majority was recovered from the application site washings and the dressings (93.66 % and 71.45 % respectively).

8. Mutagenicity

The compound was not mutagenic to *Eschericia coli* strains 343/113 when tested in the absence of an exogenous metabolic activation system at concentration up to 100 µg/ml. There are three studies in *Salmonella typhimurium* of the ability of picramic acid to produce gene mutation. In the first study strains TA1535, TA1537 and TA1538 in the presence and absence of an exogenous metabolic activation system were tested at concentrations up to 1000 µg/ml. The compound was mutagenic to TA1537 and TA1538 in this study in a dose dependent manner. In the second study strain TA98 was used at up to 150 µg/plate again a positive result was obtained. The third study using strains TA1535, TA1538, TA98 and TA100 at concentrations up to 200 µg/plate was also positive. A study of unscheduled DNA synthesis using the method of Williams in primary rat hepatocytes at concentrations of picramic acid up to 10^{-2} M was reported as producing a negative result with toxicity seen at concentrations greater than 10^{-3} M. No evidence for single strand breaks in DNA was found in transformed epidermal cells of C3H mice incubated with up to 3 mM picramic acid for 30 minutes or 2.5 hours. Negative results were obtained in an *in vivo* study to investigate induction of sister chromatid exchange in bone marrow following up administration up to 80 mg/kg, oral administration up to 100 mg/kg and dermal administration in water (100 mg/kg and 5 x 100 mg/kg) and DMSO (250 mg/kg). Picramic acid was not mutagenic in the micronucleus test at a total dose of 200 mg/kg given orally in two equal doses 24 hours apart to 5 males and 5 females. The cells were harvested 6 hours after the final dose and 2000 polychromatic erythrocytes per animal were examined.

9. Carcinogenicity

The only data available on carcinogenicity studies of picramic acid is from a study involving dermal application of a hair dye formulation, containing 0.1% picramate to mice. The occasional observations of ulceration and hyperplasia of the skin may have been related to irritation by the formulation but no other signs of toxicity or induction of tumours were observed.

11. Conclusions

Picramic acid has moderate acute toxicity by the oral route, however, studies suggest that dermal penetration from hair dye formulation is low. There was no evidence of skin irritation with a 2.5 % solution. Mild transient conjunctival irritation was seen with a 2.5 % solution instilled into the eye and rinsed out after 10 seconds; although no data are available from animals not subjected to very rapid washout. The compound was a mild sensitiser in a maximisation test in guinea pigs. In a 90 day oral study a minimal effect level of 20 mg/kg was reported. The compound clearly has mutagenic potential. Positive results were consistently obtained in assays for gene mutation in Salmonella. It is essential to ascertain whether this potential can be expressed *in vivo*. Studies in the whole animal have been limited to the bone marrow. Negative results were obtained from a micronucleus test but only one harvest time was used. Negative results were also reported in an assay for SCE induction in bone marrow. No conclusions can be drawn regarding the carcinogenicity of picramic acid. No adverse effects were reported in an oral teratogenicity study in rats at up to 15 mg/kg.

In vivo data are needed from a well conducted micronucleus test, to a current protocol, and also from an *in vivo* liver UDS assay.

Classification: C

12. Safety evaluation

Permanent hair dye use
Exposure per application based on maximum volume (100 ml) and in use concentration (0.625 %) and assuming 60 kg human
= 10.2 mg/kg

Amount absorbed assuming 1 % percutaneous absorption
= 0. 102 mg/kg

The marginal effect level from the 90 day study in rats was 20 mg/kg. Assuming a NOAEL of 2 mg/kg gives a safety margin of 20.

However the frequency of application is unlikely to exceed once a month, whereas the NOAEL is based on repeated daily exposure.

Thus the safety margin is considered adequate.

Semi-permanent hair dye use
Exposure per application based on maximum volume (35 ml) and in use concentration (1.25 %) and assuming 60 kg human
= 7.28 mg/kg

Amount absorbed assuming 1 % percutaneous absorption = 0.073 mg/kg. The marginal effect level from the 90 day study in rats was 20 mg/kg. Assuming a NOAEL of 2 mg/kg this gives a safety margin of 28.

However the frequency of application is unlikely to exceed once a week, whereas the NOAEL is based on repeated daily exposure.

Thus the safety margin is considered adequate.

However further mutagenicity data are needed from a bone marrow micronucleus test and from an *in vivo* liver UDS assay to provide assurances that the mutagenic activity seen *in vitro* could not be expressed *in vivo*.

PHENOLPHTHALEIN

1. General

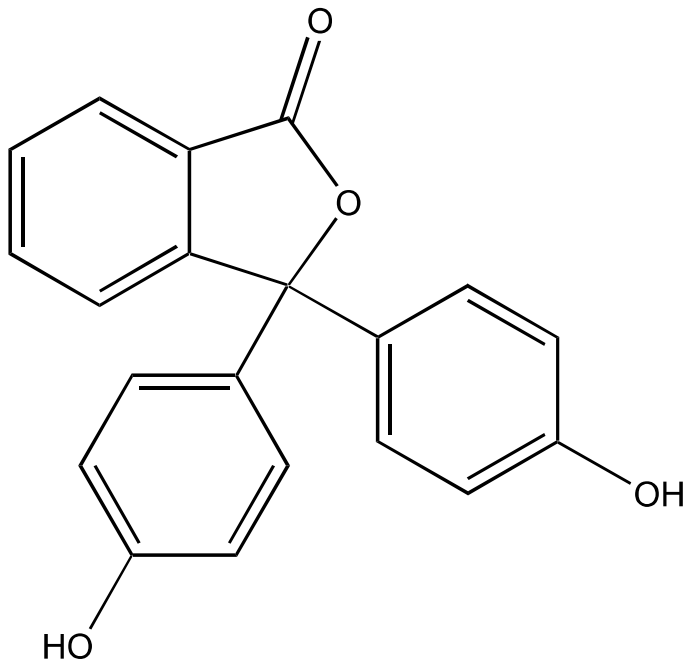
1.1 Primary name

Phenolphthalein

1.2 Chemical names

Phenolphthalein
3,3-bis-(4-hydroxyphenyl)-1-(3H)-isobenzofuranone

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{20}H_{14}O_4$
Mol weight: 318.33

1.8 Physical properties

Appearance:. Phenolphthalein is a yellowish-white powder.

1.9 Solubility

Almost insoluble in water and readily soluble in alcohol and diethyl ether.

2. Function and uses

Industry are requesting approval for use at 0.037 % in 'motivational' toothpastes for children. It has apparently been used for this purpose within the EC since 1988. The estimated worst case ingestion by a child from such use is 0.86 mg.

The compound has been widely used since the early 1900s as a laxative and it is available in non-prescription medicines for this purpose. The recommended daily dose level is in the range 30-200 mg for adults.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The minimum lethal dose in the rat is greater than 1000 mg/kg when given by the oral route, and greater than 500 mg/kg when given by the intra-peritoneal route.

3.4 Repeated dose oral toxicity

No data are available from animal studies.

4. Irritation & corrosivity

4.2 Irritation (mucous membranes)

No data are available from animal studies but a tolerance study involving 50 healthy volunteers (aged 10-18 years) who used a toothpaste containing 0.0185 % phenolphthalein for seven days revealed no differences compared to a matched group using a 'standard' toothpaste. The phenolphthalein containing toothpaste was well tolerated with no induction of compound related mucosal irritation.

8. Mutagenicity

The ability of phenolphthalein to produce gene mutations in Salmonella has been investigated in comprehensive studies by 2 separate groups of investigators. Negative results were obtained in both cases. No data are however available from studies to investigate clastogenicity, or any other end-point.

10. Special investigations

Adverse effects in humans from therapeutic use

Phenolphthalein is a diphenylmethane stimulant laxative usually given in daily doses of 30-200 mg for short periods to adults. Doses of 270 mg or more should be avoided.

Laxative products include 'chocolate' squares and a number of cases of accidental ingestion of large single doses by children have occurred which give rise to particular concern with regard to the potential for severe diarrhoea and fluid depletion. A retrospective review of 204 cases

reported to a Regional Poisons Information Centre in the USA has been published. Mean amounts ingested were about 300 mg. When symptoms occurred these were minor and did not persist for more than 24 hours. The authors concluded that children aged 5 years or under and who acutely ingest 1 g of less of a phenolphthalein-containing laxative product are at minimal, if any, risk of developing dehydration.

There are however occasional reports of marked adverse effects following therapeutic use of phenolphthalein, specifically relating to allergic reactions. Various types of acute skin reaction have been noted following oral ingestion of phenolphthalein, in some cases followed by persistent pigmentation. More rarely serious systemic effects have been reported that may be due to allergic effects. Fatalities have occurred in 2 children following the ingestion of amounts of the order of 1 gram; these may have been due to an anaphylactic type reaction and were associated with pulmonary and cerebral oedema. Hypotension, hypothermia, severe acidosis and pulmonary oedema occurred in an adult after ingestion of 2 grams of the compound in chocolate.

The FDA Advisory panel on OTC laxatives and related compounds published its conclusions on phenolphthalein in 1975. These were that the compound was safe and effective in amounts of 15-20 mg per day for children of 2-5 years of age, and 30-60 mg for children of 6 years and older, when taken orally in laxative products for occasional use only.

11. Conclusions

Phenolphthalein has low acute toxicity. It does not produce any mucosal irritation when used in toothpastes at 0.018 %. No data are available on repeated dose effects to allow a NOAEL to be determined for repeated daily use. No data are available on reproductive effects. Experience in humans from therapeutic use relates essentially to single exposure to high levels (accidents) or occasional use as a laxative. The compound is usually well tolerated at levels of up to 200 mg/day in adults (and ca. 20 mg per day in children up to 5 years) but there are occasional reports of skin reactions due to allergic effects with rare cases of systemic reactions. There are no data available to assess the effect of repeated daily exposure to relatively low levels. The mutagenicity data are inadequate.

It is recommended that data from a 90 day oral toxicity study in the rat, and an *in vitro* assay for clastogenicity in mammalian cells are needed before any meaningful safety assessment of this area can be made.

Classification: C

REPORT ON STRONTIUM PEROXIDE

Submission No 1 for strontium peroxide requests permission for its use at a strength of 6 %, exclusively as a hair product by hairdressing professionals, to be removed by washing.

The data supplied, referring to the method of application, and other data supplied by people within the profession, indicate that a mixture of powders is used, containing strontium peroxide (SrO_2), probably together with other peroxides and masking and thickening agents. The product is diluted and mixed with the required quantity of H_2O_2 (30 vol. %) until a smooth, creamy consistency is reached. This is then immediately applied with a brush onto the hair, from root to tip.

Its pH is highly alkaline (>10) and the liberation of reactive oxygen brings about bleaching of the darker shades of hair after approximately 30 minutes contact. Both the hair and scalp are then thoroughly washed with shampoo and rinsed with water.

The dossier submitted includes an acute toxicity study relating to topical application on rats (limit test), enabling the lethal dose to be established at over 2000 mg per kilo. Given the method of use of the product, this figure may be considered satisfactory.

The primary skin irritation test, carried out over 24 hours on albino rabbits using the occlusive patch test with SrO_2 at 6 % (diluted in water) resulted in a primary skin irritation index of 0.7/8; the product should therefore be considered slightly irritating to the skin of a rabbit.

An identical study, carried out using H_2O_2 in place of water, places SrO_2 in the same category, but the index is slightly higher, at 1/8.

Bearing in mind that the likely length of contact for SrO_2 with the scalp is only 30 minutes, and that the conditions of use do not involve any form of covering, the risk of irritation may be considered very slight.

The sensitisation study, carried out using albino guinea pigs, gave rise to a clear skin sensitization reaction in one of the 20 test animals, and orthogenic reactions in another two. These results indicate that SrO_2 has a slight sensitization potential.

It is important to note, however, that, in contrast to the tests referred to earlier, the latter was carried out using not SrO_2 but a formula (a mauve-coloured powder) of which all we are told is that it contained 11.5 % strontium peroxide.

It is difficult to regard sensitization tests carried out using a finished product, the composition of which is not fully known, as definitive, since the unknown ingredients may affect the response.

Finally, the dossier contains an *in vivo* penetration study carried out on rabbits, using the same formula as in the sensitization study, with contradictory results which appear to us to be of very little use in assessing SrO_2.

Conclusions:

- **Classification A**
- Label: irritant product
- Concentration: 4.5 % of strontium in the preparation ready for use
- References: See SPC/365/92 (Sr.)

OPINIONS ADOPTED DURING THE 52ND PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 12 February 1993

S 27: *PROPENOIC ACID 3-(4-METHOXYPHENYL)-3-METHYLBUTYL ESTER, MIXED ISOMERS*

1. General

1.1 Primary name

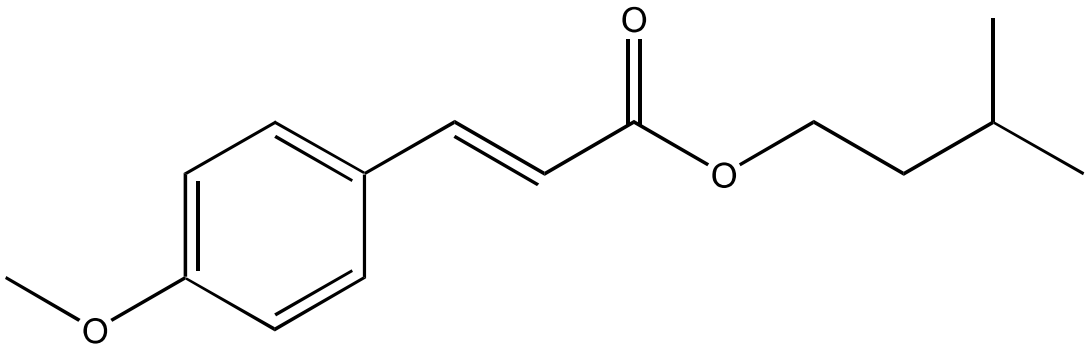
Propenoic acid 3-(4-methoxyphenyl)-3-methylbutyl ester, mixed isomers

1.2 Chemical names

Propenoic acid 3-(4-methoxyphenyl)-3-methylbutyl ester, mixed isomers

Isopentyl-4-methoxycinnamate

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{20}O_3$

Mol weight: 248.4

1.7 Purity, composition and substance codes

Not less than 98 % pure.

1.8 Physical properties

Appearance: Clear yellowish liquid.

SG 1.037 - 1.041

Absorption maximum 308 nm.

1.9 Solubility

Soluble in oils, ethanol, isopropanol. Immiscible with water.

2. Function and uses

Proposed for use as a sunscreen in concentrations up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. Values of 9.6 to 9.9 g/kg b.w. were found. No details are given.

3.2 Acute dermal toxicity

Rat. Acute dermal toxicity testing was carried out according to OECD guidelines. No abnormalities were found up to 20 g/kg b.w.

3.4 Repeated dose oral toxicity

Rat. A 3 week oral toxicity study was carried out as a range finding study. Four groups, each of 5 male and 5 female animals, were used. The doses were 0.3, 0.9 and 2.7 ml/kg b.w./day suspended in 0.8 % hydroxypropylmethylcellulose and given by gavage (in mass units, 312, 935 and 2805 mg/kg b.w./day). There were no deaths. There was decreased weight gain in both sexes at the high dose. All animals were subjected to necropsy. At the top dose, the absolute and relative weights of the spleen and thymus were significantly decreased in both sexes. In males, the weights of the gonads were significantly reduced at the top dose. At 2.7 ml/kg b.w./day in males, and at 2.7 and 0.9 ml/kg b.w./day in females, the weights of the liver were increased significantly, and those of the spleen and thymus reduced. (b) Relative: relative organ weights were difficult to interpret because the tables did not give any statistical analysis. In the text of the report the relative weights of spleen, thymus and gonads in males are stated to be decreased significantly at the top dose, and of the spleen and thymus in females. In the tables of the report, increased relative liver weights are seen at all dose levels, most pronounced in males at 2.7 ml/kg b.w. and in females at 2.7 and 0.9 ml/kg b.w. The NOAEL may be 0.3 ml/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Following a preliminary study, the doses chosen were 0, 20, 200 and 2000 mg/kg b.w./day, administered daily by gavage 7 days a week for 13 weeks. Four groups of animals were used, each containing 15 m and 15 f. All animals were subjected to necropsy after sacrifice, and animals dying during the trial were subjected to necropsy as soon as possible after death. A wide range of tissues was fixed, and all from the control and top dose groups were subjected to histological examination. There were 4 deaths during the experiment: 1 control, 2 at 20 mg/kg b.w./day, and 1 at 200 mg/kg b.w./day. Weight gain was reduced in all animals at the top dose. Haematological changes were found, which were rather variable; in summary, it may be stated that the haemoglobin and MCHC values were increased at the top dose in both male and female animals at the end of the first and third months. There were many changes in the values obtained by clinical chemical analysis. The main ones, which may be significant, were: at 1 month, AP and GOT were increased at the top dose in both sexes, and cholesterol was reduced. The same finding was made at 3 months, and in addition the GPT was raised in female animals at that time. There were no urinary abnormalities.

Organ weights: (a) Absolute weights. At the top dose, both sexes showed increase in the weight of the liver; in females, the weight of the spleen was reduced, and in males the weight of the testis was reduced. (b) Relative weights. At the top dose, the weights of the liver and kidneys were increased. In males, the weight of the heart was increased and that of the spleen and adrenals slightly reduced. In females, the weight of the spleen was reduced.

The histological findings at the top dose showed patchy areas of increase in size of hepatocytes with clear cytoplasm and large nuclei. There was also increased iron-containing pigment in the spleen of both sexes and in the Kupfer cells of the liver in females. These changes were not seen at the lower dose levels. In sum the findings indicate that at the top dose there are effects on the liver, and possibly increased breakdown of red cells. The no effect level is set at 200 mg/kg b.w./day. This appears to have been a well conducted study carried out according to OECD guidelines.

4. Irritation & corrosivity

4.1 Irritation (skin)

Guinea pig. Twelve animals were used. The material applied is not specified: it may have been undiluted a.i. It was rubbed into the clipped skin of the flank for 30 seconds daily for 5 days. The test is stated to have been negative; no details are given.

Man. Thirty subjects were tested by applying undiluted a.i. to the skin of the back or of the inside of the forearm, followed (probably) by occlusion for 24 hours. No irritation is said to have been produced. No details are given.

Man. Tests were carried out on 65 male and 45 female patients hospitalised for various skin diseases. Three concentrations of a.i. in soft paraffin were tested: 1 %, 5 % and 10 %. They were applied to disease free areas of skin of the back by means of a Finn chamber. Contact time was 24 hours; reading was at 24 and 48 hours. In 15 subjects, the test was repeated one or more times. No adverse reaction was found in any test.

4.2 Irritation (mucous membranes)

Chick. Applications of 0.2 ml of dilutions of a.i. in olive oil were made to the chorioallantoic membrane. The text gives data for tests in 1 egg only for each of the concentrations 1 %, 10 %, and control. The results were negative. This test is not yet officially recognised for this purpose.

Rabbit. Eight animals were subjected to a Draize test. A 50 % solution in olive oil was instilled into the conjunctival sac. In 4 animals rinsing was carried out. The result was reported as negative. No details are given.

5. Sensitization

Guinea pig. Twelve animals were used. The concentration used is not stated: it may have been undiluted a.i. It was rubbed into the flank skin for 30 seconds daily, 5 days a week, for 3 weeks. After a 5 day rest, the a.i. was applied to the skin of the opposite flank daily for 3 days. The test is reported as negative. No details are given.

Man. Ten subjects had undiluted a.i. applied twice weekly to the same site for 7 applications. After 12 days a challenge application with undiluted a.i. was made. No abnormality was found. No details are given.

6. Teratogenicity

Embryotoxicity and teratogenic activity

Fertile hen's eggs. Groups of 20 eggs were tested. The dose applied was contained in 0.1 ml of olive oil. The amounts applied were 0, 0.25, 0.625, 2.5 and 6.25 µl a.i. per egg. Injections were given into the white of the egg on day 1 of incubation in one series and on day 5 in another. The LD_{50} of injections on day 1 was 5.8 µl, and on day 5, 1.15 µl (approximately 120 and 25 ppm respectively). Deaths of embryos during the incubation were dose related. Following hatching, the chicks were anaesthetised and bled. The only abnormality found was a statistically significant reduction of blood glucose at 0.25 and 6.25 µl, but its biological significance is doubtful. [This test is not regarded as adequate for an evaluation of teratogenic or embryotoxic effects. In addition, injections are usually made into the yolk sac, or sometimes into the air space, and not into the white of the egg, as here.]

Rat. A study of the teratogenic and embryotoxic properties of the a.i. was carried out according to GLP. The a.i. was dissolved in 3 ml of olive oil and given daily by gavage in doses of 0, 0.25, 0.75 and 2.25 ml/kg b.w./day, from days 6 to 15 (inclusive) after mating. A positive control was used: tretinoin, similarly administered, at a dose of 15 mg/kg b.w./day. At day 20 the animals were killed by ether anaesthesia and subjected to post mortem examination. The foetuses were weighed, and about half of them were subjected to visceral examination and the remainder to skeletal examination.

The chief findings in the dams during the experiment were: a loss of weight in the high dose animals; an increase in water consumption in the high dose animals throughout the experiment, and in the low and intermediate dose animals in the second half of the experiment; a decrease in food intake in the intermediate dose animals in the first half of the experiment, and in the high dose animals throughout the experiment; and a dose related increase in hair loss in all dosed groups and in the positive control animals.

At necropsy, the weight of the adrenal glands was increased in the high dose animals; the weight of the liver was increased in the low dose animals, but this was not thought to be of biological significance.

The effects on foetuses were as follows. There was a dose related increase in intra-uterine mortality. There was a fall in foetal weights in the high dose animals and in the positive control animals. This was a well conducted study, and the a.i. does not show any teratogenic activity; a no effect level of 780 mg/kg b.w./day is found. The positive control animals showed numerous foetal abnormalities.

7. Toxicokinetics (incl. Percutaneous Absorption)

Rat. Five experiments in all are reported; they are designated by the author by the letters A, B, C, D and E.

Experiment A. A 10 % formulation of ^{14}-C a.i. in a w/o emulsion was applied (weight of formulation applied 210 mg) to the clipped skin of 3 m and 3 f rats for 24 hrs, covering an area of 2.5 x 3.5 cm (this area was the same for all the subsequent experiments). A non-occlusive dome was applied over the area. A large number of organs was examined after sacrifice, but the account is confusing. The authors seem to suggest that absorption may be determined by summing the radioactivity in carcass + urine + faeces; this amounts to 11.24 %. Although there seems to have been some radioactivity in the various organs examined, the data given do not permit of any calculation of the amounts.

Experiment B. The same formulation was used in 1 female animal (weight of formulation applied 230 mg). The area was covered with an occlusive polyethylene sheet for 3 days. The total amount of radioactivity over the period in urine + faeces was 15.8 %. The carcass value was 0.7 %, so that the total absorbed over the period is taken to be 16.5 %.

Experiment C. A 10 % o/w formulation was used (weight of formulation applied 220 mg). One female animal was tested. A non-occlusive dome was sutured to the skin under anaesthesia, and the preparation allowed to remain in contact for 7 days. The total of the percentages of radioactivity for urine + faeces over the period was 64.8 %.

Experiment D. This was the same as C except that a 10 % w/o formulation was used (weight of formulation applied 180 mg). The total of radioactivity for urine + faeces over the period was 70.5 %.

Experiment E. One animal was used. A 10 % o/w emulsion was used (weight of formulation applied 200 mg) and the area of application covered with a non-occlusive dressing. After 6 hrs, the area of application was washed and the dressing reapplied, and allowed to remain in place for 7 days. The amount found in the urine + faeces over the period was 3.18 % of the amount applied.

The report is difficult to interpret. It may be concluded that over a period of 6 hrs, about 3 % of a.i. is absorbed from an application area of 8.75 cm^2, using a 10 % formulation; over 7 days about 70 % is absorbed.

Man. After 30 minutes exposure to formulations containing 10 % a.i., the skin was repeatedly stripped at the site of application. It is stated that OECD guidelines were followed. The formulations were w/o emulsions, one of which contained 13.5 % of liquid paraffin; the other contained 10.5 % of liquid paraffin + 3 % “Eusolex 8020” (the sunscreen 4-isopropyldibenzoylmethane). The a.i. was labelled with ^{14}-C. About 3 mg of each formulation was applied without occlusion to two different areas of the forearm, each measuring 2 cm^2. The period of exposure was 30 minutes. The subjects were 2 males and 4 females. Using the first formulation, the amount in the first 2 strips were 42.27 % and 13.28 % respectively. (The area of application is not stated to have been washed before stripping). The authors suggest this may be ignored as being present only in the most superficial layers of the skin. The remaining strips yielded 42.21 % of the applied radioactivity. The amounts found in the strippings with the second formulation were not significantly different. There was a significantly higher amount of radioactivity in the strippings from the females than from the males. There was slight or definite erythema for up to 24 hrs in 4 of the subjects treated with the first formulation. The results are difficult to interpret; if the amounts in all the strippings are taken into account, the

formulae developed by Rougier and his coworkers suggest an absorption of 60 to 70 % over 4 days, or 60 to 70 mg/kg b.w.

8. Mutagenicity

A standard Ames test was carried out, using a.i. dissolved in DMSO, up to 10 mg/plate. No evidence of mutagenic activity was found. With strains TA 1538 and TA 98, the level of revertants was some 3 to 5 times higher after activation, both with the vehicle control and the a.i. This may be related to the fact that the investigator used phenobarbitone + 5,6-benzoflavone as an inducing agent, instead of the customary Aroclor.

A second test using strains TA 98, TA 100, TA 1535 and TA 1537 was carried out. In this case precipitation was noted at levels greater than 5 mg. There was no evidence of mutagenic activity.

Mouse. Micronucleus test. The dose levels were 750, 1500 and 3000 mg/kg b.w., dissolved in olive oil and given as a single intraperitoneal injection. All animals showed toxic effects, most marked at the top dose. There was no evidence of abnormal micronucleus formation.

Human lymphocytes *in vitro*. The test was carried out according to GLP standards. Human lymphocytes were cultured and exposed to concentrations of a.i. in DMSO determined by preliminary toxicity testing, as follows: without activation 0, 10, 30, 100 µg/ml; with activation 0, 30, 100 & 300 µg/ml.

The top doses gave 55 % to 70 % toxicity. Positive controls were cyclophosphamide and mitomycin C. Tests were carried out in duplicate. The cells were exposed to a.i. for 24 hours; they were then washed and cultured for a further 24 hours. At least 100 metaphases from each culture were counted.

There was a slight tendency to an increase in the number of gaps with increasing dose of a. i., but the authors report the test as negative, by comparing the values with those of the historic controls.

10. Special investigations

Phototoxicity & Photoallergy

Guinea pig. Fifty animals were used in a maximisation procedure, according to the method of Guillot et al. GLP guidelines were followed. From preliminary experiments, it was decided to use a 50 % solution of a.i. in ethanol/DEP 1:4 as a nonirritant concentration for the tests. Irradiation was delivered from two lamps. One had a range of 400 to 310 nm, maximum 360 nm, and the other 350 to 285 nm, maximum 310 nm. The 2 control groups (Ia & Ib) consisted each of 3 male and 2 female animals, and were treated identically with the respective test groups except that they were not irradiated. The 2 test groups (IIa & IIb) each contained 10 male and 10 female animals. Animals of group IIa had applications of the solution containing a.i.; those of group IIb had vehicle only. Both of these groups were irradiated.

(a) Phototoxicity. A single application of 0.5 ml of the solution of a.i. (test animals) or of vehicle (control animals) on a piece of gauze 2 cm x 2 cm was made to the depilated skin of the back. After 90 minutes, this was removed, and, in animals of groups IIa and IIb,

immediately followed by irradiation. This consisted of exposure to both lamps for 5 minutes, followed by a 90 minute exposure to the lamp with the longer wavelength. The total irradiation was 12.5 J/cm^2 , and amounted to a minimal erythema dose. The site was inspected after 24 hours. Any reaction was compared with that produced in the area surrounding the patch, which had also been exposed to a m.e.d.

(b)Photoallergy. Four days after the first test, using the same animals, intradermal injections of Freund's complete adjuvant (diluted with saline 50/50) were made at each corner of the site previously tested. The patches and irradiation were repeated. Further applications of patches and irradiation were made on days 7 and 9. A rest period of 14 days ensued. On day 23, a new site on the back was depilated and patches applied as before. The irradiation on this occasion, however, was from the lamp with the longer wavelength only, for 90 minutes. Tests on other guinea pigs had shown that this irradiation did not of itself produce any skin reaction. Readings were made at 6, 24 and 48 hours.

Result: There was no evidence of any phototoxic or photoallergic reaction in any animal. There were no formal positive controls, but in an appendix the findings of a series of experiments using the same protocol are given. In these a wide range of chemicals capable of producing phototoxic and photoallergic reactions was tested (e.g. 8-mop, 5-mop, angelica extract; and promethazine, 3,5,4-tribromosalicylamide, etc.). These gave the expected positive results.

Man. Ten subjects had undiluted a.i. applied by means of an occluded patch for 24 hrs. The area was then exposed to UV irradiation of an intensity slightly below the m.e.d. No abnormality was seen. No details are given.

11. Conclusions

Acute and subchronic toxicity are low. Tests for irritation of mucous membranes and skin were negative. Tests for sensitization were unsatisfactory, but the results of the tests for photoallergenicity permit the deduction that sensitization is unlikely; the substance is a very rare allergen and photoallergen in clinical practice. Tests for teratogenicity were negative. There was no evidence of mutagenic activity, but tests for photomutagenic activity were not carried out. The tests for percutaneous absorption are difficult to interpret.

Classification: B

S 28: *2-ETHYLHEXYL-4-METHOXYCINNAMATE*

1. General

1.1 Primary name

2-ethylhexyl-4-methoxycinnamate

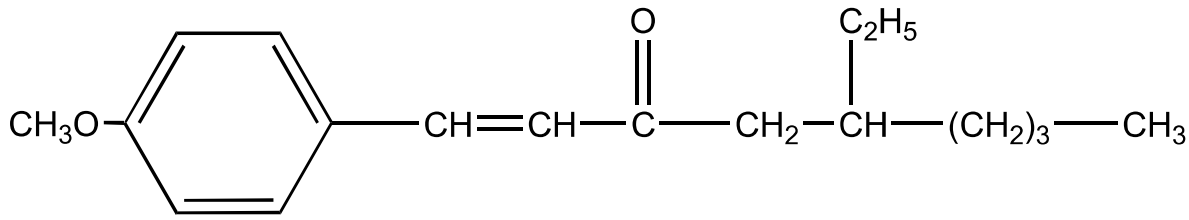
1.2 Chemical names

2-ethylhexyl-4-methoxycinnamate

1.3 Trade names and abbreviations

Parsol MCX

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{18}H_{26}O_2$

Mol weight: 290

1.8 Physical properties

Appearance: Colourless pale yellow slightly oily liquid.

1.9 Solubility

Miscible with alcohols, propylene glycol, etc.

Immiscible with water.

2. Function and uses

Use level up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}: Mouse, greater than 8 g/kg b.w. Rat, greater than 20 ml/kg b.w.

3.4 Repeated dose oral toxicity

Rat. Three week oral study. Groups of 5 male and 5 female animals were given 0, 0.3, 0.9 and 2.7 ml/kg b.w./day by gavage for 3 weeks. All animals of the top dose groups exhibited loss of body weight and a reduced relative and absolute weight of the thymus. Male rats showed a decrease in absolute weight of the left kidney and female rats showed a decrease in the absolute weight of the heart. At the two lower doses, the only significant alteration observed was an increased absolute weight of the pituitary gland in male rats receiving the lowest dose. As the number of animals was small, the investigators considered this not to be biologically significant. The NOAEL was put at 0.9 ml/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Four groups of 12 male and 12 female SPF rats received the compound in the diet at levels of 0, 200, 450 and 1000 mg/kg b.w./day. During the experiment the usual clinical observations were carried out, as well as extensive haematological and biochemical studies. Full gross necropsy was carried out on all survivors. Histological investigations were carried out in half the animals of the control and top dose groups. The organs studied included the heart, lungs, liver, stomach, kidneys, spleen, thyroid and retina. In the remaining animals histological examination of the liver only was carried out. Six control animals and six top dose animals were allowed to recover over 5 weeks, and then examined.

The results of the experiment showed no dose related mortality. The kidney weights of top dose animals were increased, but were normal in the recovery animals; the increase was attributed to a physiological response to an increased excretion load. There was a diminution of glycogen in the liver, and a slight increase in iron in the Kupfer cells in the high dose animals. Two of these also showed minimal centrilobular necrosis of the liver with some infiltration; similar less marked findings were made in 2 of the control animals as well. These findings were attributed to infection. High dose females had increased GLDH which reversed during the recovery period. The NOAEL was put at 450 mg/kg b.w./day.

3.8 Subchronic dermal toxicity

Rat. Thirteen week dermal study. Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55.5, 277 and 555 mg/kg b.w./day applied to shaved skin 5 days a week for 13 weeks (The top dose is used daily by the average consumer). Various laboratory and clinical tests were carried out during the experiment.

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination. The authors put the NEL at 555 mg/kg b.w./day, but in view of the liver findings this may be 227 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Guinea pig. The a.i. was applied undiluted twice daily to 20 animals for 16 days. There were no signs of irritation.

Man. Occlusive applications of undiluted a.i. were made to 60 subjects, of whom 20 had sensitive skin. The applications were made for 24 hours. Observations at removal of the patches, and 24 and 48 hours later, showed no evidence of a reaction.

In 51 male and female subjects, similar patch tests were carried out. The dilution of the a.i. (if any) was not stated. There was no irritation.

A formulation (concentration not stated) tested on the skin of 50 subjects caused no adverse effect.

In 53 subjects, a Draize repeated insult patch test at a concentration of 2 % caused no irritation.

In 54 subjects, a Draize repeated insult patch test of a 7.5 % dilution of a.i. in petrolatum caused no irritation.

A 10 % solution of a.i. in dimethylphthalate was used. A total of 58 subjects was recruited, 12 males and 46 females, aged 18-63. Of these, 6 subjects failed to complete the test for reasons unconnected with the experimental procedure.

Induction applications were made on the skin of the back, for 24 hours with occlusion. The area was inspected at 0, 24 and 48 hours after removal of the patch. No adverse reaction was noted at any stage of the experiment.

4.2 Irritation (mucous membranes)

Rabbit. Groups of 4 animals had 0.1 ml of a test preparation instilled into the conjunctival sac (concentration not stated). No further treatment in one group; in the other, the instillation was followed by washing out. There were no signs of irritation.

A Draize test carried out with undiluted a.i. was found to be practically non-irritant.

5. Sensitization

Guinea pig. Twenty animals received applications of undiluted a.i. twice daily for 16 days. After a 3 day interval without treatment, a daily challenge application was made for 3 days. There was no evidence of sensitization.

Two groups of 4 animals were used. Animals of one group were exposed to 0.05 ml injections of undiluted a.i. daily for 5 days. In the other group, 0.025 ml of a 50 % acetone solution of a.i. was applied to 2 cm^2 areas of shaved skin on either side. There was no evidence of sensitisation.

Man. A Draize repeated insult patch test was carried out at a concentration of 2 % in 53 subjects. There was no sensitization.

In 54 subjects, a formulation of 7.5 % a.i. in petrolatum was applied for 48 hours under occlusion for 11 applications. After a 14 day rest, a challenge application of a single dose was made. There was no adverse reaction.

In an extensive series of patch tests carried out in man, the a.i. was found to be very rarely responsible for allergic contact effects.

Test for capacity to produce photosensitization

Test which "showed that the product did not provoke photosensitization." No details supplied.

6. Teratogenicity

Rabbit. Groups of 20 female animals were mated and given a.i. in doses of 0, 80, 200 and 500 mg/kg b.w./day by gavage during the period of organogenesis. Except for a slight reduction of maternal and foetal weight in the top dose animals, no abnormality was found.

Rat. Following a pilot study, groups of 36 rats were mated and treated with 0, 250, 500 and 1000 mg/kg b.w./day of a.i. (probably by gavage) during days 6-14 of pregnancy. Owing to an error, the preparation of the control foetuses led to their destruction, so this part of the test was repeated under identical conditions. Subgroups of each dose group were allowed to litter normally and rear the offspring. The percentage of resorptions in the high dose group was elevated by comparison with the other groups. The investigator records, however, that this relatively high rate is the usual one with this strain of rat in this laboratory, and he attributes the difference to an unusually low level of resorption in the other groups. No other abnormality was found.

7. Toxicokinetics (incl. Percutaneous Absorption)

Tests for percutaneous absorption

(a) *In vitro* tests.

Rat. Naked rat skin. This was studied in a chamber experiment. Most of the material was found in the stripped skin; there was less in the stratum corneum, and least in the chamber. The approximate amounts found in the chamber were: after 6 hrs, 1.13 %; after 16 hrs, 11.4 %; and at 24 hrs 17.9 %. The figures for the horny layer and the strippings combined were, respectively, 31.4 %, 44.4 % and 45.7 % (percentages of applied doses). Solutions of 3 % and 20 % of a.i. gave similar results.

In another set of experiments, various amounts of "Parsol 1789" (4-*tert*-butyl-4'-methoxydibenzoylmethane) were added to the a.i. in the formulation. There seemed to be no effect on the absorption of the a.i.

Pig. A similar experiment using mini-pig skin was carried out in which "Parsol 1789" was used as well as the a.i. Using 3 sorts of formulation, about 3 % of a.i. was found in the chamber in 6 hrs. Using the concentrations proposed for a particular commercial use (i.e., 7.5 % of "Parsol 1789" and 2 % of a.i.) about 2.2 % was found in the chamber. It is calculated by the authors that the total absorption for a 75 kg consumer would be about 70 mg, or 0.9 mg/kg b.w. (Note however that the maximum proposed use level of a.i. is 10 %).

Man. A test on human abdominal skin in a chamber was carried out. With 7.5 % a.i., about 0.03 % is found in the chamber in 2 hours, 0.26 % in 6 hours, and 2.0 % in 18 hours. Various combinations of a.i. and "Parsol 1789" were investigated.

(b) *In vivo* tests.

Man. Eight healthy volunteers had small amounts of radioactive a.i. applied to the interscapular region. One group of 4 had the material applied under a watch glass; the other 4 had it applied on gauze, whith occlusion in one case. Tests for absorption of a.i. were negative except for about 0.2 % in urine. The concentrations used were not stated.

In a preliminary experiment, a capsule containing 100 mg of a.i. was taken orally. As a lipophilic substance, the a.i. is very likely to be metabolised; it is known in any case to be hydrolysed by plasma esterases, although slowly. The cumulative excretion of 4-methoxycinnamate in the urine over 24 hours was studied by GC/MS of the methyl ester derivative (This method would also detect 4-hydroxycinnamic acid). Over 24 hours, 13.2 % of the amount ingested was recovered, equivalent to 21.5 % of the amount that would be expected if the a.i. were completely absorbed. In the main part of the experiment, an o/w cream containing 10 % a.i. was used. Applications of 2 grams of this material (= 200 mg a.i.) were made to the interscapular area of each of 5 male subjects, aged 29 to 46. The area of skin covered was 25x30 cm. After application, the area was covered with 3 layers of gauze, left in place for 12 hours. Blood was taken at times 0, 0.5, 1, 2, 3, 5, 7, and 24 hours. Urine was collected at 0, 1, 2, 3, 4, 5, 6, 7, 12, 24, 48, 72 and 96 hours.

The control plasma samples showed a level equivalent to about 10 ng/ml before any application had been made. There was no evidence of any rise in plasma levels during the experiment. The urine showed a "physiological" level of 100 to 300 ng/ml. No significant increase in this amount was found in any sample. The authors conclude that very little, if any, of the compound was absorbed under the conditions of the experiment.

8. Mutagenicity

Salmonella mutagenesis assays were performed on the usual strains. There was a positive result with TA 1538 without metabolic activation. This was thought to have been a batch effect. From another laboratory, a very weak positive was found with TA 1538 without activation, at 10 µl/plate; it was not found in 2 replicates, nor in a second Ames test.

A test for mutagenesis and crossing over in *S. cerevisiae* was negative.

A test using Chinese hamster V 79 cells showed a very slight increase in mutant colonies with dose.

A test in human lymphocytes *in vitro* was negative.

A test for cell transformation in Balb/c 3T3 cells was negative.

A test for unscheduled DNA synthesis was negative.

Tests in *Drosophila:*

There was an increase in the frequency of sex-linked recessive lethals.

There was no evidence of mutagenicity in feeding tests (adults and larvae).

Somatic mutation and combination tests using wing structure were negative.

Mouse. Micronucleus test. No effect was found up to 5000 mg/kg b.w.

Test for photomutagenic activity

These were carried out in cells of *S. cerevisiae,* which had previously been shown not to be affected by a.i. *(supra)*. Doses of a.i., dissolved in DMSO, ranged from 0.06 to 625 µg/ml, and radiation up to 500000 J m^{-2} UVA and up to 12000 UVB (50 and 1.2 J cm^{-1}). Chlorpromazine was used as the positive control. Suitable negative controls were also employed. The experiment appears to have been well carried out. The results show that the a.i. is not mutagenic under these conditions; that UVA and (more markedly) UVB are mutagenic; and that the a.i. protects against this effect in a dose dependent manner.

10. Special investigations

Test for capacity to produce phototoxicity

Man. In 10 subjects, patches were applied for 24 hours and the areas then exposed to a suberythematous dose of UV irradiation. There was no evidence of phototoxicity.

Test for inhibition of UV-induced tumors

Hairless mouse. The animals were exposed to repeated doses of UV simulating the solar energy spectrum. After a rest period, 3 applications a week were made to an area of skin of 12-o-tetradecanoyl phorbol-13-acetate (at first at 10 µg/ml, but later at 2 µg/ml, as the higher concentration was found to be irritant). Suitable controls were used. The test group was completely protected by 50 % a.i., and 7.5 % gave an effect equivalent to reducing the insolation four-fold. It had been suggested that the a.i. could itself have been a promoter, but there was no evidence of this.

11. Conclusions

The compound appears to have low acute and subchronic toxicity, orally and dermally; it does not irritate the mucous membranes in conventional animal tests. The data presented suggest that the compound is not an irritant or sensitizer in animals; however, tests for sensitization were carried out at levels below the proposed maximum use level. Clinical investigation shows that this compound is very rarely responsible for allergic contact dermatitis in man. There is no carcinogenicity study, but an extensive range of mutagenicity studies were nearly all negative.

A test for photomutagenicity was negative, although the dose of UVB used was rather low. Animal studies for teratogenic activity were negative. Percutaneous absorption in man appears to be very low.

12. Safety evaluation

Calculation of safety margin. Evidence from experiments with human and pig skin *in vitro* suggests that about 0.26 % and 2.6 % is absorbed over 6 hours, respectively; hairless rat skin *in vitro* suggests about 1.1 % absorption. A reasonable figure overall is about 1.3 % absorption. Human experiments *in vivo* show that about 13 % is absorbed following oral ingestion; using identical analytical techniques, no absorption could be found following application of a 10 % formulation to 750 cm^2 of skin under semi-occlusive conditions for 12 hours.

Taking the maximum absorption as 1.3 %, and assuming the application of 0.5 mg/cm^2 of a 10 % formulation over the entire surface of the body, the amount absorbed in use would be about 12 mg, or about 0.17 mg/kg b.w. Oral No Effect Levels of 250 and 900 mg/kg b.w./day, and a dermal No Effect Level of at least 230 and possibly 550 mg/kg b.w./day are found; teratogenic tests in rabbit and rat show a No Effect Level of more than 500 mg/kg b.w./day. Taking all these findings into account, the safety margin may be conservatively estimated at about X 1450.

Classification: A

S 59: *ALPHA'-(2-OXOBORN-3-YLIDENE)-TOLUENE-4-SULPHONIC ACID*

1. General

1.1 Primary name

alpha'-(2-oxoborn-3-ylidene)-toluene-4-sulphonic acid

1.2 Chemical names

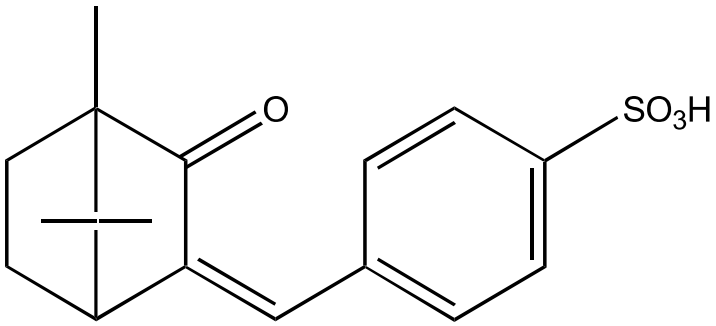
alpha'-(2-oxoborn-3-ylidene)-toluene-4-sulphonic acid

3-(4'-sulpho)benzylidenebornan-2-one

1.3 Trade names and abbreviations

Mexoryl SL

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{17}H_{20}O_4S.3H_2O$

Mol weight: 374.5

1.8 Physical properties

Appearance: Crystalline substance, absorption maximum 294 nm

1.9 Solubility

Highly soluble in water and ethanol.

2. Function and uses

Use level up to 6 %, expressed as acid.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat, oral: 2.2 to 3.2 g/kg b.w.; 1.29 g/kg b.w.

Mouse, oral: 1.83 g/kg b.w.

3.4 Repeated dose oral toxicity

Rat. Oral. Following a pilot study, doses of 0, 150, 300 and 600 mg a.i. (expressed as acid)/kg b.w./day were chosen. These were given to groups of 5 male and 5 female animals 7 days a week by gavage for 28 days.

All animals were subjected to necropsy. Food consumption was reduced at the intermediate and high doses, more marked in males. Salivation following dosing was noted in all high dose animals. High dose males showed a significant fall in serum sodium and a rise in chloride values. There was a fall in blood glucose levels in all female dosed animals, and in males of the high and intermediate dose groups. The authors put the No Effect Level at 150 mg/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Oral. A 3 month study was carried out according to GLP guidelines. Groups of 10 male and 10 female animals were given 0, 100, 250, and 625 mg/kg b.w./day by gavage. The doses are expressed as the triethanolamine salt; as a.i. they are 0, 71.5, 179 and 447 mg/kg b.w./day.

The main clinical finding was ptyalism after dosing in high dose animals. There was a significant fall in body weight gain and food consumption in high dose animals. High dose males showed an increase in urea and cholesterol. Although there was some fall in blood sugar in dosed animals, it always remained within the physiological range. In all dosed male animals there was an increase in the blood levels of alanine aminotransferase, but this was found in females at the high dose only. This increase became even more marked in the recovery period after the termination of dosing. There was also an increase in aspartate aminotransferase in dosed males, but not in females. Organ weights showed a marked increase in the liver weight of high dose females, and microscopy showed hepatocellular hypertrophy. In view of the enzyme findings, the livers of all the experimental animals were examined by an independent histologist. The conclusion was that the appearances in every case were within normal limits. In view of this, and the fact that (a) some enzyme changes were not found in female animals, and (b) that the levels continued to increase even after dosing had stopped, the No Effect Level was put at 250 mg/kg bw/day, equivalent to 179 mg/kg b.w./day of the a.i.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. 6 NZW animals were used. A 6 % neutral aqueous solution of a.i. was applied under occlusion for 24 hours to 2 areas, one of which was abraded. No adverse reaction was seen. A similar test using a 4 % solution was also negative.

Test for capacity to cause irritation on repeated application

Rabbit, guinea pig, rat. A 4 % solution of a.i., neutralised, was applied daily for a month. The result was negative. No details of the tests are given.

4.2 Irritation (mucous membranes)

Rabbit. A neutralised aqueous solution containing 6 % a.i. was used in a Draize test in 6 NZW animals. The application was judged to be very slightly irritant.

5. Sensitization

Guinea pig. Thirty Dunkin-Hartley animals were used in a Magnusson-Kligman procedure. The concentration of the test solution was 6 %. Freund's complete adjuvant, 50 %; a.i. diluted 50 % in water, and a.i. diluted 50 % in FCA/water were injected around the test area. One week later the area was treated with a 6 % solution of a.i. as a patch with occlusion for 48 hours. Two weeks later, challenge applications of 1 % and 0.5 % solutions were made to a fresh site for 24 hours with occlusion. The test was negative.

6. Teratogenicity

Rat. Oral. A standard study was carried out in groups of 20 pregnant female animals. Doses of 0, 50, 150 and 450 mg/kg b.w./day were administered by gavage from days 6 to 15 of gestation. There was no evidence of teratogenic or embryotoxic activity.

7. Toxicokinetics (incl. Percutaneous Absorption)

Tests for percutaneous absorption

(a) Tests *in vitro*. Using ^{14}-C a.i. in a concentration of 6 %, the skin of hairless rats was studied in a Franz chamber over 24 hours, with frequent regular sampling of the saline/bovine albumen fluid in the receiving chamber. Eight replicates were carried out. From an alcoholic vehicle, absorption was 3.0 %, and from a cream formulation 1.6 % of the applied amounts.

A further series of experiments was carried out using human female breast skin, removed at plastic surgery. Here, the respective figures were 0.007 % and 0.18 % respectively.

(b) Tests *in vivo*. The hairless rat was again used. Six animals were tested, using radioactive a.i. (i) A saline solution of the radioactive a.i. was given intravenously, and its elimination in urine and faeces followed over 120 hours. The greater part (81.28 %) was eliminated within 24 hours, and 97.76 % of the administered dose was accounted for. (ii) In 6 animals, a 6 % cream formulation was applied to the skin, and a metal device prevented rubbing or licking for 6 hours. At the end of that time, the area of application was washed, and the excretion of a.i. followed over 120 hours. The animals were then sacrificed. The amount excreted was very small (1.24 % in all) but some 30 % of the cutaneous application was not accounted for, even though the area of application was stripped and the strippings and the remainder of that area homogenised and counted. The deficiency is attributed to loss of epidermis over the period of the experiment. The bioavailability of the a.i. by percutaneous absorption, by comparison with intravenous injection, was found to be 1.28 %.

8. Mutagenicity

A standard Ames test was negative. A micronucleus test in the mouse was negative. A test for chromosomal aberration *in vitro* in Chinese hamster ovary cells was negative. A test for photomutagenicity in *E. coli* WP2 was negative.

10. Special investigations

Tests for capacity to produce phototoxicity and photoallergy

Rabbit. Application of a 5 % aqueous solution followed by UV irradiation was carried out daily for 2 weeks. The substance was stated to be "slightly phototoxic". No details are given.

Guinea pig. Fifteen female Dunkin-Hartley animals were used, 10 test and 5 positive controls. Each animal had areas of about 8 cm x 6 cm prepared by shaving and stripping. Three patches of filter paper were then applied to the area. In test animals, 2 of the patches were wetted with 0.3 ml of a 1 % solution; the third patch was dry. In the positive control animals, the agent used was 20 % chlorpromazine hydrochloride in petrolatum. Following 90 minutes occlusion, the dressing was removed from one of the treated areas and from the area treated with the dry patch; the second treated area remained occluded. The exposed sites were then exposed to 5 15 watt "Blacklight" tubes for 4 hours. The intensity of the irradiation was not measured; the maximum output was at 350 nm. The remaining dressing was then removed, and readings were made at once, and at 24, 48, 72 and 96 hours. Some animals showed slight reactions in the areas treated with a.i.; the positive control areas showed a brown pigmentation in the exposed areas throughout the period of observation. The test was interpreted as negative.

Guinea pig. Following pilot experiments to determine a concentration of a.i. that was not a primary irritant, patches wetted with 0.5 ml of a 6 % solution were applied for 90 minutes with occlusion to 2 groups of animals (Group 1, 3 male and 2 female animals, treated and not irradiated; group 2, 10 male and 10 female animals treated and irradiated). After removal of the patches, animals of group 2 were irradiated with a med from 2 lamps: one with an output from 400 to 310 nm, and one with an output from 350 to 285 nm. Exposure to the combined lamps was for 5 minutes, followed by 90 minutes exposure to the lamp with the longer wavelength. The sites were inspected at 6 and 24 hours, and scored with a numerical scale.

On the following day, 4 intradermal injections of FCA, 50/50 in saline, were made at each corner of the application site. On day 4 an occlusive application was made, followed by irradiation as above. This was repeated on days 7 and 9. Following a rest period, on day 22 a new site on the back was prepared, followed on day 23 by an occlusive application for 90 minutes and irradiation, from the longer wavelength lamp only, for 90 minutes. There was no evidence of phototoxic or photoallergic reactions. There were no contemporaneous positive controls, but a list is given of the results of similar experiments in the same laboratory, using known phototoxic and photoallergenic compounds; these showed strong positive reactions.

11. Conclusions

This compound has been carefully investigated. Acute toxicity was low. Tests for skin and mucous membrane irritation, and for sensitization, were negative, as were tests for

phototoxicity and photoallergenic activity. A subchronic oral study gives a No Effect Level of 179 mg/kg b.w./day. Percutaneous absorption is low: taking the worst case, and comparing it with the No Effect Level in the 3 months oral study, the safety factor may be calculated to be about 770. Tests for mutagenicity and photomutagenicity were negative.

Classification: A

S 64: *1-P-CUMENYL-3-PHENYLPROPANE-1,3-DIONE*

1. General

1.1 Primary name

1-p-cumenyl-3-phenylpropane-1,3-dione

1.2 Chemical names

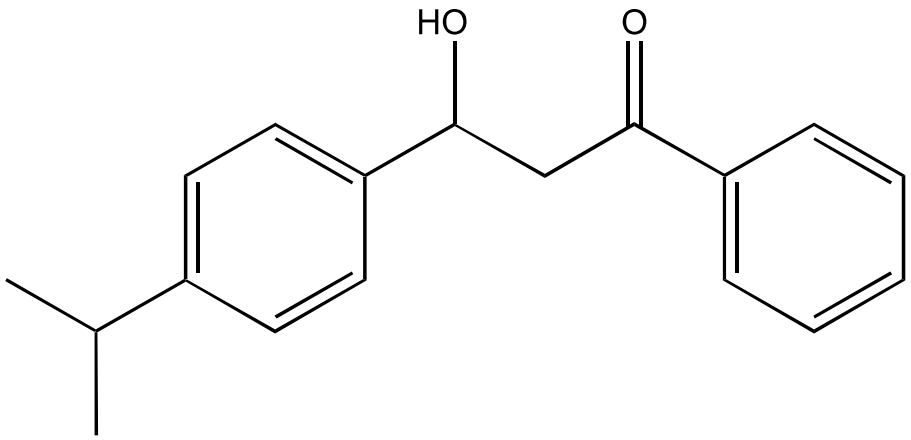
1-p-cumenyl-3-phenylpropane-1,3-dione

4-isopropyldibenzoylmethane

1.3 Trade names and abbreviations

Eusolex 8020

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{18}H_{19}O_2$

Mol weight: 266.32

1.8 Physical properties

Appearance: Yellow white crystalline substance.

1.9 Solubility

The substance is soluble in ethanol but not in water.

2. Function and uses

Proposed for use as a sunscreen in concentrations up to 5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. Oral. Greater than 10 g/kg b.w. at 1 day, and greater than 6 g/kg b.w. at 7 and 14 days.

3.2 Acute dermal toxicity

Rat. Greater than 10 g/kg b.w.

Intraperitoneal. Greater than 16 g/kg b.w. This high level is attributed to a failure to absorb the a.i. from the peritoneal cavity. Dog. No deaths at 5 g/kg b.w.

Test for capacity to produce phototoxicity

Mouse. Forty male and 40 female animals were used, in 4 groups. The test animals had 200 mg/kg b.w. in arachis oil injected intraperitoneally, followed by irradiation for 4.5 hours from a quartz lamp (no details given). The intraperitoneal a.i. was then repeated. For controls, there were: a group given the injections but no irradiation; a group given irradiation but no injections; a group given neither irradiation or injections. There was no evidence of phototoxicity (Compare, however, the intraperitoneal LD_{50}, above).

Man. One male and 4 females were tested. The a.i. was made up as a 2 % solution in ethanol. Three areas on the forearm were delineated. Two of them had applied 0.05 ml of a.i. solution; the third was not treated. The first and third areas were exposed to UV irradiation (quartz lamp, no details given); one of the treated areas was not irradiated. The test was negative.

A similar test was carried out on 3 male and 2 female subjects, except that the a.i. was made up as a 4 % solution in ethanol. The test was negative.

3.7. Subchronic oral toxicity

Rat. A 3 month study (with one month recovery) was carried out according to GLP guidelines. Groups of 15 male and 15 female animals were fed a.i. in the diet at levels equivalent to 0, 10, 30, 100 and 300 mg/kg b.w./day (respectively groups 1, 2, 3, 4 and 5). 10 male and 10 female rats in each group were sacrificed at 12 weeks; the remainder were fed normal diet and sacrificed at 17 weeks (treatment free follow up, TFFU animals).

There were no substance related deaths. Body weight gain was reduced in males of groups 3 and 5, but normal in TFFU animals. Food intake was somewhat reduced in all treated groups. Males and females of groups 4 and 5 showed significant increases in the absolute and relative weight of the liver. This was also found in males of groups 2 and 3. The absolute and relative weights of the kidney were increased in males of group 5, and the relative weights in males of group 3 and 4. Absolute and relative weights of the thyroid were increased in males of groups 4 and 5, and the relative weight in group 3; in females there was an absolute increase in thyroid weight in group 5. In TFFU animals no abnormality was found. Histological examination revealed a tendency for a dose related increase in necrosis of hepatocytes, but this was slight.

There was a suggestion of dose related increased activity of the thyroid glands, but again this was slight. In TFFU animals no abnormalities were found.

There was a significant increase of bilirubin in males of group 5, and in females of groups 3, 4 and 5. There was a significant increase of cholesterol levels in males of group 5 and females of groups 4 and 5. In TFFU animals no such changes were found. No significant changes in plasma enzymes were noted.

This seems to have been a well conducted study. The liver changes may be due to increased metabolic activity. If the rise in cholesterol is significant, the NOAEL may be between 30 and 100 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Six animals were used, of which 3 were scarified. Occlusive applications of 0.5 g of a.i. moistened with arachis oil were made for 24 hours. Another area was similarly treated except that talc replaced the a.i. There was no evidence of irritation.

Man. Six formulations were tested; 4 were cream formulations and the others were oily. The concentration of a.i. was 10 % in 3 formulations; probably less, in combination with another sunscreen, in the others. A closed epicutaneous patch test was carried out in 26 subjects suffering from various skin disorders. The amount of formulation applied was 50 mg, and the length of the test was 24 hours. No irritation was produced.

In the same group of patients, a similar experiment was carried out except that the skin was first stripped 6 times. No abnormality was produced. Subsequent exposure to a sun ray lamp (no details given) produced no abnormality.

Tests for capacity to produce irritation on repeated exposure

Man. A 5 % solution of a.i. in mineral oil was applied to the forearms of 10 female volunteers. A Duhring chamber (area 113 mm^2) was fixed to the skin. Occlusion was not used. The duration of each application is not stated. The applications were made to the same site 5 days a week for 2 weeks. There was no evidence of irritation.

A similar experiment was carried out on 2 groups of 10 female subjects. In this case, the skin was first scarified. The chambers were fixed, with occlusion, for 24 hours; this was repeated 3 times on the same area of skin. There was no evidence of irritation.

In 6 healthy volunteers, repeated application of formulations to the same areas of skin “at least once a day“ for 5 to 6 weeks gave no reaction. No details are given.

4.2 Irritation (mucous membranes)

Rabbit. A Draize test using a.i. as a powder was negative.

Chorioallantoic membrane of the chick. The membrane was exposed at 10 days gestation. Four to six eggs were used at each dose level (0.5 % and 2 %). The test was negative.

5. Sensitization

Guinea pig. Four groups of Pirbright White animals were used: treatment; positive control; vehicle control; no induction. Each group contained 5 males and 5 females, except the no induction group, which contained 6 males and 8 females.

Test animals were treated daily, 5 days a week, with 10 % a.i. in arachis oil applied to the shaved skin of the flank. Animals of the vehicle control group were treated with arachis oil only. Positive control animals received 2 % dinitrochlorobenzene in ether for the second week.

Challenge was made after a 14 day rest period. The first group received 1 % a.i. in arachis oil to a new site. The second received 0.2 % dinitrochlorobenzene similarly. The third group was treated with vehicle only. Five animals of group 4 were treated with the agents to exclude a primary irritating action. The test was negative.

Man. Twenty volunteers, 8 male and 12 female, were used. Care was taken to exclude those with a history of skin disease. Six formulations were used, but no information about them is given. They were applied on patches to the upper arm with occlusion for 24 hours, 3 days a week, for 3 weeks. After a 10 day rest period, the same applications were made once to the opposite arm. One subject, a woman, showed a clear positive response. On further questioning, it appeared she had recently begun to show contact dermatitis to some plants and perfumes.

Test for capacity to produce photosensitization

Man. Five healthy volunteers, 1 male and 4 female, were tested. Three areas of the forearm were delineated. The first 2 were treated with 0.05 ml of a 2 % solution of a.i. in ethanol. One of these areas, and an untreated area, were exposed to UV irradiation (no details given). After a 10 day rest, the same procedure was carried out, except that liquid paraffin replaced the ethanol. After a further 10 days, one area was treated with 1 ml of a 2 % solution of a.i. in soft soap. All 3 areas were then irradiated again. There was no evidence of photosensitization.

In a similar experiment, 4 % a.i. was made up in ethanol for the first application; in paraffin oil for the second; and in an 8 % hand soap solution for the third. There was no evidence of photosensitization.

6. Teratogenicity

Fertile hen's eggs. Groups of 20 fertile eggs were used. The a.i. was made up in olive oil, and amounts of 0, 0.1, 0.5, 1.0, 5.0 and 10.0 mg/egg injected on either day 1 or day 5 of development. There was a dose related increase in death rate of the embryos, which amounted to 100 % at the top doses. Some diminution of body weight, liver weight and heart weight were found with the higher doses, but there was no evidence of teratogenic activity.

Rat. Following a pilot study, doses of 0, 30, 100 and 300 mg/kg b.w./day were given by gavage to groups of 20 to 25 pregnant rats from day 6 to 15 of gestation. The a.i. was dissolved in olive oil. GLP guidelines were followed. There was some increase in early absorptions in all dosed groups, but these were not dose related or statistically significant. There was no evidence of a teratogenic effect.

This appears to have been a well conducted study with a clear negative result.

7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous Absorption

Man. Two experiments are reported.

(a) Six healthy male volunteers were used. A 5 % o/w emulsion containing ^{14}-C a.i. was applied over 200 cm^2 on the forearm of each subject, using approximately 1 g of the formulation (exact amount determined by difference). Exposure was for 6 hours. At this time, the area was washed with soap and water and then rinsed with further water. Urine was collected over 48 hours and faeces over 3 days, and the radioactivity measured. The total radioactivity in urine and faeces amounted to 2.786 % of the amount applied. However, some 39 % of the net amount of radioactivity applied was not accounted for.

(b) In a similar study, 2 male volunteers were used. The differences were, firstly, that cleaning of the skin was with ether; secondly, that 9 cm^2 of the application area was stripped 15 times at the end of the first exposure and also at the end of the experiment, and the radioactivity in the strips counted; the values were then extrapolated to 200 cm^2; and thirdly, that urine and faeces were collected over 120 hours. The mean overall recovery was now 92.9 %, which is attributed to the use of ether for cleaning, as the a.i. is insoluble in water. The mean percentage of net applied radioactivity recovered in the urine and faeces was 0.754 %.

Combining the results, and postulating the application of 25 g of formulation (one half of an average pack) the authors suggest that absorption may amount to 0.6 mg/kg b.w.

8. Mutagenicity

A standard Ames test was negative. A similar test in tryptophan dependent mutants of *E. coli* WP2 and WP2uvrA was also negative.

A chromosomal aberration test *in vitro* in Chinese hamster V79 cells was negative, although the maximum concentration which could be tested was 1.5 µg/ml, due to precipitation. Positive controls reacted as expected.

10. Special investigations

Test for capacity to produce subjective discomfort

Man. A technique devised by Frosch and Kligman was used. A solution of a.i. in 5 % mineral oil was rubbed into the nasolabial folds of 12 subjects who were sensitive to a similar application of 5 % lactic acid. No subject reported discomfort.

Test for production of photocontact allergy

Man. Twenty-five healthy volunteers were tested. The material was tested under the name "Creme W". No details are given about it. As a preliminury, a m.e.d. was determined for each subject using SSR from a xenon arc. Induction was carried out by applying 10 µl of the preparation over 4 cm^2 of the back with occlusion for 24 hours. Following removal of the patch and wiping of the skin, the area was exposed to 3 m.e.d.s from the arc. This was repeated after

24 hours, and then twice weekly for 3 weeks. After a rest period of 10 to 14 days, 2 previously untreated areas of the skin of the back were treated with a.i. in the same manner. After removal of the patches, one of the areas was exposed to 4 J/cm^2 of UVA (320 to 400 nm). The other treated area was not irradiated. A third untreated area of skin was also irradiated. There were no abnormalities.

The test was repeated with "Creme Y", about which no details are supplied. No abnormalities were produced.

11. Conclusions

Acute toxicity is low. Tests for primary irritation and for irritation on repeated insult were negative, as were tests for production of irritation of mucous membranes. Tests for production of phototoxicity were negative. A test for capacity to produce sensitization in guinea pigs was negative, but a similar test in man showed 1 positive in 20 subjects. The compound is well recognized clinically as a frequent cause of photocontact allergy and photosensitization in man. Oral subchronic testing suggests a NOAEL of 30 mg/kg b.w./day. Tests for mutagenicity were negative; tests for photomutagenicity were not carried out. Tests for percutaneous absorption in man are reported to suggest that about 0.6 mg/kg b.w. might be absorbed. Tests for teratogenic activity were negative.

It is noted that it was originally proposed to use this compound at 2 %; 5 % is now proposed. Depending on the assumptions made, the safety factor may be as low as 50. A test for photomutagenicity shoud be carried out.

Classification: B

S 72: *HOMOPOLYMER OF (.+.)-N-((4-((4,7,7-TRIMETHYL-3-OXOBICYCLO(2.2.1)HEPT-2-YLIDENE)METHYL)) PHENYL))-METHYL)-2-PROPENAMIDE*

1. General

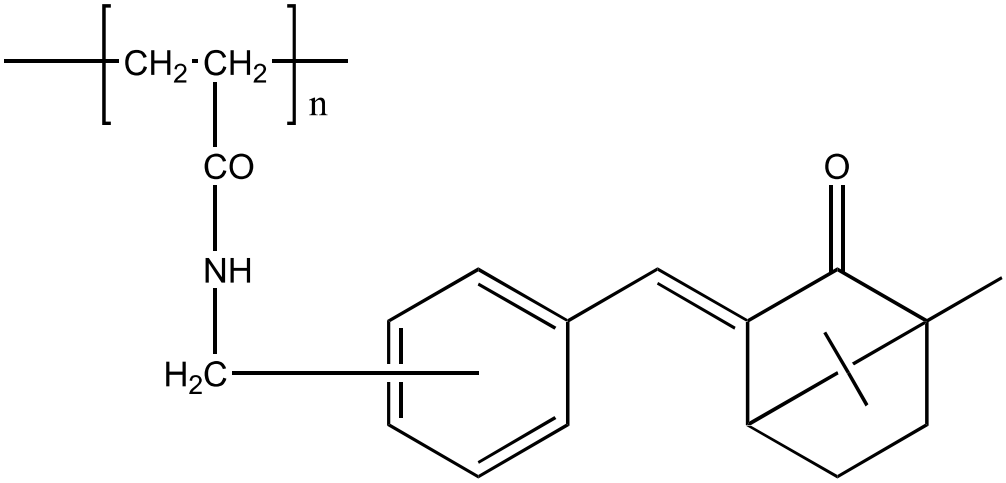
1.1 Primary name

Homopolymer of (.+.)-N-((4-((4,7,7-trimethyl-3-oxobicyclo(2.2.1)hept-2-ylidene)methyl)) phenyl))-methyl)-2-propenamide

1.2 Chemical names

Homopolymer of (.+.)-N-((4-((4,7,7-trimethyl-3-oxobicyclo(2.2.1)hept-2-ylidene)methyl)) phenyl))-methyl)-2-propenamide

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $(C_{21}H_{25}NO_2)_X$

Mol weight: highest value between 17000 and 40000; about one third has a MW less than 4000.

1.7 Purity, composition and substance codes

The substance is a mixture of isomers on positions 4' and 2' of the phenyl ring.

1.8 Physical properties

Appearance: Light brown powder.

Maximum absorbance is at 295 nm.

1.9 Solubility

Insoluble in water; soluble in organic solvents.

2. Function and uses

Proposed for use as a sunscreen at levels up to 6 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Mouse. A limit test was carried out on a group of 6 male and 6 female albino CFLP mice. The a.i. was administered once by gavage in a dose of 5000 mg/kg b.w. as a suspension in CMC 0.5 %. No abnormality was found over 14 days or at necropsy. The LD_{50} was greater than 5000 mg/kg b.w.

Rat. A similar experiment gave the same result; the LD_{50} was greater than 5000 mg/kg b.w.

3.2 Acute dermal toxicity

Test for capacity to produce phototoxicity following cutaneous application.

Guinea pig. The a.i. was used as a suspension in castor oil at 20 % w/v, and was applied under patches, with occlusion, for 90 minutes. Following preliminary testing, 5 animals were used as negative controls, being treated with a.i. but not irradiated. The test group comprised 11 male and 11 female animals. In each, 2 areas were treated with a.i., but after removal of the patches, only one of the areas was irradiated. The irradiation was from 2 lamps, with outputs at 285 to 350 and 310 to 400 nm respectively. Both lamps were used at a distance of 10 cm from the skin for 5 minutes, and then the longer wavelength lamp was placed 5 cm from the skin for 90 minutes. The total energy was 12.5 J cm^{-2}, comprising 99 % UVA and 1 % UVB. This amount of irradiation equalled 1 med under the conditions of the experiment. No contemporaneous positive control was used, but the authors give earlier results from their laboratory, using the same technique, with known phototoxic substances such as methoxypsoralens, angelica and rue extracts, etc., which yielded the expected positive results. It was concluded that the a.i. did not induce phototoxicity in this experiment.

3.7 Subchronic oral toxicity

Rat. A 3 month oral study was carried out in 4 groups of SD rats with 10 male and 10 female animals in each group. The a.i. was made up in 2 % polysorbate + 0.01 % dimethicone in water, and administered by gavage in doses of 0, 150, 450 and 1350 mg/kg b.w./day. The study was carried out in conformity with OECD guidelines. There were no deaths, and no significant abnormal findings. The NOAEL is put at 1350 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit: Six male NZW animals were used; testing was carried out according to the J.O. de la République Française of 2/2/82. Sites on either side of the dorso-lumbar spine were prepared, one abraded and one not. A 20 % suspension in carbitol was applied, in a volume of 0.5 ml, to either side, with occlusion for 24 hours. At first, slight to well defined erythema, with or without slight oedema, was noted at 4/6 intact sites and 6/6 abraded sites. At 72 hours, slight erythema was found in 2 animals only. The index of irritation was 1.2 (maximum 8). The a.i. at 20 % in carbitol was deemed to be "slightly irritant".

Three NZW animals were tested according to OECD guidelines. Areas were prepared on either side of the dorso-lumbar spine; one side was used for testing and the other as a control. On a pad moistened with 0.5 ml of water, 500 mg of a.i. was applied to the test site, and held in position with a semiocclusive dressing for 4 hours. Over the first 72 hours, there were slight erythematous changes in 2 animals, and moderate erythema in the third. All changes had disappeared by the sixth day. In accordance with 83/467/CEE, the substance was classified as "non-irritant".

4.2 Irritation (mucous membranes)

Rabbit. A Draize test was carried out in 6 NZW animals, according to the protocol of J.O. de la République Français 21/10/84. A 20 % solution of a.i. in castor oil was used in one eye, with the opposite eye acting as control. Observation was at 1 hour and then daily for 7 days. Although there was marked red colouration of the conjunctivae of 5/6 animals after 1 hour, the overall score indicated that the substance should be classified as "very slightly irritant". Three male NZW animals were tested according to OECD guidelines, using the a.i. as a powder. There was slight redness and chemosis initially, but the overall score over 72 hours indicated that the material was "non-irritant".

5. Sensitization

Test for capacity to cause delayed contact hypersensitivity.

Guinea pig. Thirty female Dunkin-Hartley albino animals were used: 20 test and 10 control. The a.i. was made up at 40 % and 20 % in carbitol; further dilutions were made by adding liquid paraffin to these suspensions. Areas of 4 x 6 cm were prepared on the upper back. Control animals were treated identically with test animals throughout, except that a.i. was omitted. In the test animals, the usual intradermal injections of Freund's complete adjuvant with or without a.i. were given. One week later, a patch saturated with a 20 % suspension of a.i. was applied to the same site for 48 hours with occlusion. Challenge applications were made 2 weeks later to 2 sites on the flank: 8 % and 4 % applications were made and occluded for 24 hours. No differences were seen between the control and test animals; the test was negative.

Test for capacity to produce contact hypersensitivity.

Man. A preliminary and a main study were carried out. In the preliminary test, the a.i. was applied as 0.5 ml of a series of dilutions in castor oil; the concentrations tested were 2.5 %, 5 %,

10 %, 15 %, and 20 %, for 48 hours with occlusion. Since no adverse reaction was seen, the concentration of 20 % was used in the main test in 30 volunteers. A dose of 0.5 ml of the solution of a.i. in castor oil was applied to a strip of material 2 x 2 cm. This was placed on the forearm and occluded for 48 hours. This application was repeated to the same site 5 times in all, over a period of 3 weeks. Following a 2 week rest period, a challenge application of the same strength was applied to 2 different areas on either forearm, again for 48 hours with occlusion. There was no evidence of hypersensitivity (There is a slight difficulty in being sure that the concentrations given above were those actually used, but this was almost certainly the case).

Test for capacity to produce photosensitization.

Guinea pig. Forty two female animals were used: 10 test, 10 negative control, 20 positive control, and 2 for range finding studies. The a.i. was made up as a 20 % suspension in carbitol. The light sources were (a) a lamp emitting from 285 to 400 nm and (b) a lamp emitting from 320 to 400 nm. Dosage was measured with Osram 'Centra' radiometers. Following a range finding test, a concentration of 20 % a.i. was chosen for testing.

Induction. 0.025 ml of the test solution was applied to a circular depilated area of 2.5 cm^2 on the back of each animal. This was allowed to remain in place for 30 minutes. The animals were then placed in a restraining cage and exposed to UVA + UVB radiation for 10 minutes: 485 and 185 mJ cm^{-2} respectively. This procedure was repeated every 48 hours, 5 times in all. Control animals were treated similarly, except that no chemical was applied to the skin. For a positive control, 0.1 % dibromosalicylanilide was used.

Challenge. After a 12 day rest, applications were made in the same manner as before, and 30 minutes later, animals were exposed to UVA only, at 10 J cm^{-2}.

There was no reaction in any group, nor any evidence of irritation. As a result, the positive control was repeated in a further 10 animals, this time using tetrabromosalicylamide in petrolatum. This gave positive reactions. It was concluded that there was no evidence for the production of photosensitization by the a.i.

6. Teratogenicity

Rat. Groups of about 20 pregnant animals were given doses of a.i. by gavage during days 6 to 15 of pregnancy. The doses used were 0, 100 and 1000 mg/kg b.w./day. There was no evidence of teratogenic activity.

7. Toxicokinetics (incl. Percutaneous Absorption)

Test for percutaneous penetration.

Hairless rat *in vivo*. Six female rats were anaesthestized and an application of 2 mg of an ointment containing 5 % a.i. was made over an area of skin of 1 cm^2, delineated by a silicon ring. Occlusion was not used. The a.i. was labelled with ^{14}C in the aromatic ring. After 4 hours, the area of application was cleaned, and the animals were transferred to individual metabolism cages which permitted the separate collection of urine and faeces. After 96 hours the animals

were sacrificed and the area of application was stripped 6 times with adhesive tape. The gastrointestinal tract and the area of application were removed. Radioactivity was estimated in the urine and faeces (24 hour collections), in the gastrointestinal tract, in the carcass, in the skin in the area of application, and in the strippings. The amounts found were (in percentages of the amount applied): urine over 96 hours, 0.052; faeces over 96 hours, 1.521; GIT at sacrifice, 0.015; skin in area of application, 0.053; stratum corneum in area of application, 0.095. Total, 1.829 % of amount applied (100 μg). If the amount in the skin and strippings at the site of application be excluded, the percentage of a.i. absorbed was 1.681 % of the applied amount. It was also shown in this experiment that the excretion halflife of the absorbed a.i. was about 24 hours.

8. Mutagenicity

A standard Ames test was carried out. There was no evidence for an increase in revertants, with or without activation.

A Chinese hamster ovary cell line was used to test for chromosomal aberrations *in vitro*. There was no evidence of clastogenic activity.

Mouse. A micronucleus test was carried out in accordance with GLP. The test was negative.

10. Special investigations

Test for photostability *in vitro*.

A 4 % o/w emulsion was studied. This was exposed in a layer 1 μm thick to SSR from a xenon arc, filtered and refracted to give UV wavelengths only. The intensity of irradiation was 0.42 mW cm^{-2} in UVB and 15 mW cm^{-2} in UVA, estimated to be about 3 times the intensity to be expected in the Mediterranean. The results showed the compound to be stable, losing only 1.35 % in 1 hour.

11. Conclusions

Acute toxicity was low, and the substance has been shown to be stable in ultraviolet light. Tests for capacity to irritate mucous membranes and skin were negative. Tests for production of hypersensitivity were negative. Tests for photosensitization and phototoxicity were accepted as being negative, despite the lack of some contemporaneous controls. Subchronic oral toxicity testing gave a NOAEL of at least 1350 mg/kg b.w./day. Percutaneous penetration was low. Tests for mutagenicity and chromosomal aberration (*in vitro* and *in vivo*) were negative. A test for teratogenesis in the rat was negative. Tests for photomutagenicity have not been carried out.

Classification: B

OPINIONS ADOPTED DURING THE 53RD PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 25 June 1993

A 43: *1-HYDROXY-3-AMINO-2,4-DICHLOROBENZENE*

1. General

1.1 Primary name

1-hydroxy-3-amino-2,4-dichlorobenzene

1.2 Chemical names

1-hydroxy-3-amino-2,4-dichlorobenzene hydrochloride
3-amino-2,4-dichlorophenol monohydrochloride
2,4-dichloro-3-aminophenol

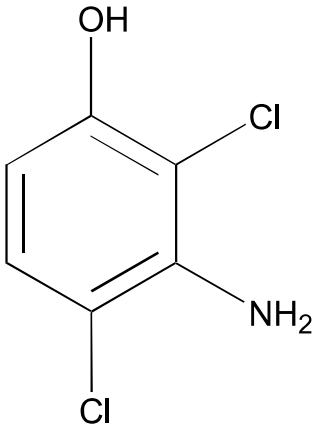
1.3 Trade names and abbreviations

Ro 151
Colipa no.: A 43.

1.4 CAS no.

CAS formula: 61693-43-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_5N_1O_1Cl_2$
Mol weight:178.02

1.7 Purity, composition and substance codes

sA: 1-hydroxy-3-amino-2,4-dichlorobenzene (purity > 97.5 %)
sB: 1-hydroxy-3-amino-2,4-dichlorobenzene (unspecified)
sC: Ro 151 (unspecified)

1.8 Physical properties

Subst. code: sA.
Appearance: grey-white powder
Melting point: 171-186 °C.

1.9 Solubility

The substance is soluble in ethanol (as hydrochloride).

2. Function and uses

The substance is included in oxidative hair dye formulations up to 2 %, since the oxidative hair dyes are mixed 1:1 with hydrogen peroxide before use, the concentration at application is only 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Route	Sub.	Species	LD_{50}/LC_{50}
oral	sB	mouse	725 mg/kg bw

Male CF 1 (Winkelmann, Borchen) mice (10/dose level) received (by stomach tube) a single dose of the test compound (using relatin as suspension agent) at dose levels of 398, 501, 631 and 1000 mg/kg b.w. The animals were observed for 7 days. The calculated LD_{50} value was 725 mg/kg b.w.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sB
Exposure: 3 m

Wistar rats (Wistar Mu Ra Han 67 SPF) (20/sex/group) received (5 days/week) 0 or 100 mg 2,4-dichloro-3-aminophenol/kg body weight by gavage for a period of 3 months. 20 animals/sex served as controls and another 10 animals/sex served as reserve animals. The age at the start of the study was approximately 8 weeks and the average body weight was 131 g for males and 127 g for females. Food and drinking water ad libitum. After 12-13 weeks the animals were sacrificed.

Examinations:

Mortality and clinical signs daily; body weights weekly.

Urinalysis (pH, vol, prot, glu, urobil, ketones, sg, blood, sed), haematology (Hb, ery, leuco) and clinical chemistry (SAP, SGOT, SGPT, urea, prot, glu, Ca) of all animals at the beginning and end of the study. Relative and absolute organ weights (all animals), gross pathology (all animals) and histology (23 tissues; 5 animals/sex/group).

Results: No mortalities during the study. No differences in body weights, organ weights, urinalysis, haematology or clinical chemistry between control and experimental animals. Also gross pathology and histology did not reveal a difference between the experimental and control animals.

Remark: - only 1 dose level (100 mg/kg b.w.) tested!
- no statistical analyses were performed (only averages with standard deviations).

4. Irritation & corrosivity

4.1 Irritation (skin)

(1) Route: skin
Species: rabbit
Subst.: sB
Exposure: 24 hr
Concentr.: 10 %

To test the primary dermal irritation of 2,4-dichloro-3-aminophenol, patches with 1 to 2 drops of a 10 % solution of the test substance in relatin were applied to the shaved skin of two male rabbits (albino New Zealand) for 24 hours. Animals were examined 24, 48 and 72 hours after application.

Result: 2,4-dichloro-3-aminophenol showed no irritating effects.

Remark: - only two rabbits used.

(2) Route: skin
Species: mouse
Subst.: sB
Exposure: 5 d
Concentr.: 10 %

Two drops of a 10 % solution of 2,4-dichloro-3-aminophenol in relatin were applied twice daily onto the hairless skin of 5 mice (hr hr) during 5 days.

Result: No irritating effects were seen during the experiment.

(3) Route: skin
Species: man
Subst.: sB
Exposure: 8 hr
Concentr.: 10 %

Patches loaded with small amounts of a 10 % solution of 2,4-dichloro-3-aminophenol in relatin were applied to the skin of 5 volunteers for 8 hours to test the primary dermal irritation.

Result: No skin irritations were seen 8 hours after removal of the patches.

4.2 Irritation (mucous membranes)

Route: eye
Species: rabbit
Subst.: sB
Dose: 0.1 ml
Concentr.: 5 %

0.1 ml of the test compound (5 % in CMC) was instilled into the conjunctival sac of the right eyes of 5 male albino rabbits (New Zealand). The untreated left eyes served as controls. The eye reactions were examined 2, 6, 24, 48 and 72 hours after installation and evaluated by the Draize method.

Result: 3 animals showed a minor opacity of the cornea up to 48 hours after instillation. The conjunctivae of all rabbits showed a weak redness, slight to moderate chemosis and exsudation.

5. Sensitization

Subst.: sB
Species: guinea pig
Method: Magnusson Kligman
Conc. induc.: 5 %/ 5 %
Conc. chall.: 1 %

20 female guinea pigs (Pirbright white strain) were induced by intradermal injection on both clipped sites with a 5 % solution of 2,4-dichloro-3-aminophenol in propyleneglycol followed after a week by topical application of 5 % of the test compound in vaseline under occlusion for 48 hours. Two weeks after the last induction the animals were challenged on the right clipped sites with 1 ml of a 1 % solution of the test compound in propyleneglycol and with 1 ml of the vehicle alone on the left clipped sites, both under occlusion for 24 hours. 20 animals served as controls. The animals were examined after removal of the patch and after a further 24 and 48 hours.

Results: No signs of sensitization were observed.

Remark: Test is inadequate; the concentrations used did not induce irritation, and no sodium lauryl sulphate was used prior to topical induction to increase the assay sensitivity.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sC
Admin. Days: 6-19

Ro 151 was orally administered to 47 pregnant female Wistar rats (TNO) from day 6 to 19 of gestation at a dose level of 100 mg/kg b.w. (dissolved in 2 % carboxymethylcellulose).

44 pregnant female Wistar rats (TNO) served as control animals. The body weights at the start of the experiment were 200 to 240 g. At day 20 of gestation the females were sacrificed.

Examinations:

Clinical signs daily. Body weights on day 1, 3, 6, 10, 14, 17 and 20 of gestation and macroscopic examination after sacrifice. Furthermore the number of corpora lutei, the number and distribution of living fetuses and dead embryo's/fetuses, the body weights and sex of the fetuses and the external visible anomalies were examined. About one third of each litter was prepared for soft tissue examination and the remaining fetuses were examined for skeletal abnormalities (after staining with alizarin red S3).

Results: No mortalities and no signs of maternal toxicity were observed. In the experimental group the body weight of 63 from the 348 fetuses examined (= 18 %) was less than 3 g, whereas in the control group the body weight of 37 of the 354 fetuses examined (= 11 %) was less than 3 g. No other differences were seen between both groups. The number of skeletal variations and signs of retardation was higher than expected in both groups (highest in the control group), so this was not considered to be substance related.

Remark: - only the dose level tested!

7. Toxicokinetics (incl. Percutaneous Absorption)

Cutaneous absorption ***in vivo:***
To determine the cutaneous absorption of 2,4-dichloro-3-aminophenol (sB), ^{14}C-labelled 2,4-dichloro-3-aminophenol was applied as a hair dye formulation onto the clipped skin (8 cm^2) of 9 male Wistar rats for 30 minutes. The animals were kept in metabolism cages.

This formulation consisted of 10 mg radio-labelled test compound x HCl dissolved in 0.4 ml ammonia with 9 g of a colouring cream, which was mixed 1:1 with 6 % hydrogen peroxide.

The cream consisted of:	Lorol	2.0 %
	Texapon N25	25.5 %
	ammonia	4.6 %
	tallow alcohol	8.5 %
	p-toluylendiaminsulphate	0.414 %
	Resorcin	0.048 %
	ammoniumsulphate	1.0 %
	sodiumsulphate	1.0 %
	Turpinal SL	0.2 %
	Water	56.738 %

After 30 minutes the skin was rinsed with a shampoo and water. 48 hours after application the absorption was determined by measuring the radioactivity. Urine and feces were collected in two intervals (0-24 hours and 24-48 hours).

Result: A total percentage of 85.1 % of the total applied radioactivity was found after in 48 hours. The percentages of radioactivity found were 1.10 % in urine (1.04 % in the first 24 hours), 0.329 % in feces (0.274 % in the first 24 hours), 0.179 % in the body, and 0.149 % in the rinsing water of the cage. 14.7 % of the applied radioactivity was found in the skin. So,

1.76 % of the total applied amount of radioactivity was absorbed during the 48 hour period. The radioactivity was excreted most widely with the urine.

Excretion after subcutaneous administration:
To determine the excretion of ^{14}C-labelled 2,4-dichloro-3-aminophenol 4.7 mg of this test compound/kg b.w. was administered subcutaneously to 5 male Wistar rats (SPF-TNO, body weights 264-321 g). The animals were kept in metabolism cages. The excretion was studied after 6 days by analysis of the amount of radioactivity in the urine, feces, rinse water of the cage, expired air, liver and kidneys. Also the radioactivity in body and skin was determined.

Result: More than 90 % of the administered radioactivity was excreted within 24 hours via urine (78.2 %) and feces (15.9 %). No (or a very low amount of) radioactivity was found in the expired air. The radioactivity found in skin, body, liver and kidneys was low (0.0023 % to 0.26 %).

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sB	*Salm typh*	TA1535	base-pair subst	4-2500 µg/plate; solvent DMSO; toxic conc 2500 µg/plate	-	-	R	AR
* sB	*Salm typh*	TA1537	frameshift mut	4-2500 µg/plate; solvent DMSO; toxic conc 2500 µg/plate	-	-	R	AR
* sB	*Salm typh*	TA1538	frameshift mut	4-2500 µg/plate; solvent DMSO; toxic conc 2500 µg/plate	-	-	R	AR
* sB	*Salm typh*	TA100	base-pair subst	4-2500 µg/plate; solvent DMSO; toxic conc 2500 µg/plate	-	-	R	AR
* sB	*Salm typh*	TA98	frameshift mut	4-2500 µg/plate; solvent DMSO; toxic conc 2500 µg/plate	-	-	R	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r=rat, m=mouse, h=hamster)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR=Aroclor, PH=Phenobarbital, MC=Methylcholantrene)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sC	mouse bone marrow		micronucleated polychromatic erythrocytes	60-600 mg/kg bw in methylcellulose by gavage	-

In vivo *mammalian test:*

Ro 151 was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of CD1 mice (5 animals/sex/group). Based on a preliminary test to determine the maximum tolerated dose, the dose levels administered were 60, 300 and 600 mg/kg b.w. (by gavage; suspended in 1 % methylcellulose) in two equal dosages, seperated by an interval of 24 hours. A negative (receiving the vehicle) and a positive (receiving mitomycin C) control group were included. The mice were killed 6 hours after the second administration and the bone marrow smears were examined (incidence of micronucleated cells per 2000 polychromatic erythrocytes).

Result: No evidence of mutagenic potential or bone marrow toxicity after oral administration up to 600 mg/kg b.w. At 600 mg/kg b.w. 3 animals died. Also hypnoea and lethargy was seen 30 minutes after dosing at this dose level. After 6 hours these symptoms disappeared.

Remark: All animals were killed 6 hours after second administration. No samples after 24, 48 and 72 hours! The test is performed according to the usual procedures in 1980. However, it is not likely that under the experimental conditions of the test, more sampling times would lead to positive effects of the compound.

11. Conclusions

General

1-hydroxy-3-amino-2,4-dichlorobenzene is used in oxidative hair dye formulations at a maximum concentration of 2 %, but because the oxidation dyes are mixed (1:1) with hydrogen peroxide before application the final concentration at usage is 1%.

Acute toxicity

In a mouse study an oral LD_{50}-value of 725 mg/kg b.w. was found, so the compound is moderately toxic.

Irritation / sensitization

The sensitization test was inadequate according to the prevailing guidelines. Skin irritation tests in mice and humans indicated that a 10 % solution of the test compound was not irritating to the skin. The eye irritation test in rabbits indicated that a 5 % solution of the test compound was irritating to the eye.

Semichronic toxicity

In a 3-month oral study Wistar rats (m+f) were daily given 0 or 100 mg 1-hydroxy-3-amino-2,3-dichlorobenzene/kg b.w. by gavage. No animals died during the study and no effects were seen at 100 mg/kg b.w.

Teratogenicity
In a teratogenicity study one dose level of 100 mg/kg b.w. was tested (a control group was included). In the experimental group 18 % of the fetuses examined was less than 3 g, whereas in the control group 11 % was less than 3 g. No other differences were seen between the two groups.

Mutagenicity
1-hydroxy-3-amino-2,3-dichlorobenzene was negative in a bacterial assay. Although the micronucleus test was not carried out according to the prevailing guidelines the negative result in this test is acceptable because a) there is no obvious structural alert for genotoxicity and b) it is not likely that more sampling times would lead to positive results. So, it can be concluded that the compound has no mutagenic potential.

Absorption
^{14}C-labelled 1-hydroxy-3-amino-2,4-dichlorobenzene was applied to the skin of rats as a hair dye formulation (containing (1:1) hydrogen peroxide). After 48 hours 1.76 % of the total applied amount of radioactivity was absorbed. 14.7 % of the applied radioactivity was found in the skin.

Conclusion
A 10 % solution of the test compound was not irritating to the skin of mice and humans. A 5 % solution of the test compound was irritating to the eye of rabbits. The sensitization test was inadequate. The dermal absorption was 1.76 %. It was concluded that the compound had no mutagenic properties in the systems tested.

The only dose level tested in a 3-month oral rat study and in a teratogenicity study was 100 mg 1-hydroxy-3-amino-2,4-dichlorobenzene/kg b.w. and this dose did not result in a toxic effect.

Despite the shortcomings in several tests, for normal use of 1-hydroxy-3-amino-2,4-dichlorobenzene, the following calculation can be made:

1 g of the test compound comes in contact with the human skin in permanent hair dye condition (based on a maximum usage volume of 100 ml hair dye containing 1 % of the compound). With a maximal percutaneous absorption of 1.76 % this results in a dermal absorption of 17.6 mg per treatment, which is 0.29 mg/kg b.w. (assuming a body weight of 60 kg). So a safety margin of 346 can be calculated between the figure for human exposure to permanent hair dye and the dose of 100 mg/kg b.w., the only dose tested, causing no effects in the 12-week oral rat study.

It should be noted that the rats were exposed daily for 3 months, while human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Classification: B.

Industry should provide data to skin sensitization potential from in-use data in the context of the volume used, together with any available information on the toxicological profile of the compound, e.g. from animal studies and/or, from experience in use in either the consumer or occupational context.

A 113: *ETHOXYBLAU.*

1. General

1.1 Primary name

Ethoxyblau.

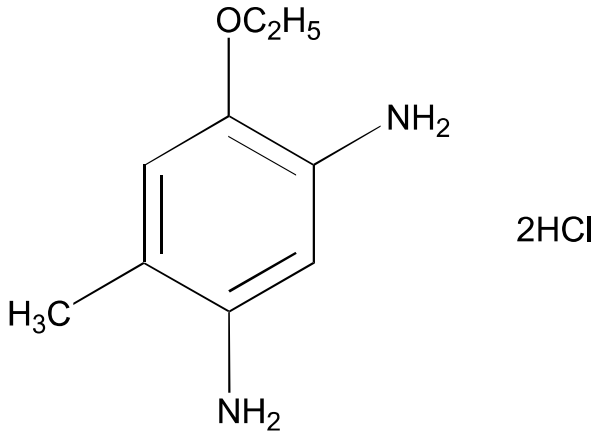
1.2 Chemical names

2,4-diamino-5-methyl-phenetol, dihydrochloride.

1.3 Trade names and abbreviations

GHS 231086
Colipa no.: A 113

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{14}N_2O_1.2HCl$
Mol weight: 239

1.7 Purity, composition and substance codes

sA: Ethoxyblau (purity 99 %)
sB: Ethoxyblau (unspecified)
sC: GHS 231086
sD: 2,4-diamino-5-ethoxytoluene-dihydrochloride ^{14}C-labelled (lot 099F9246; purity > 98 %)

1.8 Physical properties

Subst. code: sA
Appearance: pink-violet crystals

1.9 Solubility

Solubility in water: 10-20 %.

2. Function and uses

The substance will be used in oxidative hair dye formulations at a maximum concentration of 2 %. The final concentration at usage (when mixed 1:1 with hydrogen peroxide) is 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute toxicity.
2,4-amino-5-methyl-phenetol, dihydrochloride (sB) was administered once by stomach tube to CF 1 mice (10/sex/group) and Wistar rats (6/sex/group) as a 10 % solution in aqua dest. The dose levels were 350, 800 and 200, 600 mg/kg b.w. for female and male mice, respectively; and 500, 1000 and 500, 1250 mg/kg b.w. for female and male rats, respectively. The animals were sacrificed after 14 days, and post-mortem examination was carried out on all animals.

Result: The 'approximative' LD_{50} values (in mg/kg b.w.) appeared to be between the two dose levels administered per group (between 200 and 2000 mg/kg b.w.). No actual value was given.

The test substance caused reduced activity and abnormal position.

Alterations of the organs were not seen.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 14 w
Recov.p.: 4 w
DEW: 20 mg/kg b.w.

Ethoxyblau (dissolved in water) was administered 5 days/week by stomach tube to male and female Wistar rats (Bor. Wis. W.) for 90 days. The substance was administered as follows:

Group I	22 m + 22 f	5 mg	water/kg b.w. (controls)
Group II	15 m + 15 f	5 mg	ethoxyblau/kg b.w.
Group III	15 m + 15 f	10 mg	ethoxyblau/kg b.w.
Group IV	15 m + 15 f	20 mg	ethoxyblau/kg b.w.
Group V	15 m + 15 f	20 mg	ethoxyblau/kg b.w. (recovery)

At the start of the study the rats were 6-8 weeks old and the mean body weights were 142.42 and 171.2 g for females and males, respectively. Food and water ad libitum. After 14 weeks the animals of group II-IV and 10/sex/group I were sacrificed. After 18 weeks (4 weeks recovery) 12/sex/group I and the animals of group V were sacrificed.

Examinations:
Clinical signs and mortality daily. Ophthalmoscopic examinations at the start and end of the study of group I, IV and V. Body weights, food and water consumption weekly. Haematology (ery, leuco, Hb, Ht, MCV, MCH, MCHC, Diff) and biochemistry (bil, glu, total prot, SGOT, SGPT, SAP, Fe, Ca, ureum, triglyceride, chol, creat) at start of study and after 6 and 13 weeks (10/sex/group I-V) and after 17, 18 weeks (10/sex/group I and V, respectively). Urinalysis (nitrite, leuco, pH, prot, glu, ketones, urobil, bil, blood, sed) at start of the study and after 6 and 13 weeks (all groups; 5/sex) and after 18 weeks (5/sex/group I and V). Relative and absolute organ weights (8 organs; 10/sex/group I-V), gross pathology and histopathology (10/sex/group I, IV and V; 36 tissues).

Results: One male (group IV) and one female (group V) died during the study, but not due to the treatment.

The only treatment related effect observed was a dark discolouration of the thyroid glands in all animals of the highest dose group (Group IV and V). At histopathology no treatment related effects could be found. The fact that haematopoesis in the spleen was seen more seldom in group V is considered to be due to the time difference between blood collection and sacrifice (7-9 days for controls and group IV animals and same day for group V animals).

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin
Species: guinea pig
Subst.: sB
Exposure: 4 hr
Dose: 1 ml
Concentr.: 3 %

1 ml of the test compound (3 % in water) was applied onto the clipped skin (flank; 3 x 2 cm) of 5 female Pirbright white guinea pigs for 4 hours under a closed patch. After 4 hours the test compound was washed off. The animals were examined 1 hour after removal of the patch and once daily thereafter for 4 consecutive days.

Result: None of the animals showed any response to the treatment.

4.2 Irritation (mucous membranes)

Route: eye
Species: guinea pig
Subst.: sB
Exposure: 24 hr
Dose: 0.1 ml
Concentr.: 3 %

0.1 ml of the test compound (3 % in water; pH 2.1) was instilled into the right conjunctival sac of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls. The eye reactions were read after 0.5, 1, 2, 3, 4, 5, 6 and 7 hours after application. After 24 hours and

daily thereafter until the effects disappeared the reactions were read after 1 drop of 0.1 % fluorescein-sodium solution.

Result: No effects were seen.

5. Sensitization

Subst.: sB
Species: guinea pig
Method: Magnusson Kligman
Conc.induc.: 0.1 % / 40 %
Conc.chall.: 30 %

10 female Pirbright white guinea pigs were induced by intradermal injections on the clipped shoulder region with a 0.1 % solution of the test compound in Ringer solution, followed after a week by topical application (patch for 48 hours) of a 40 % solution of the test compound in distilled water. Two weeks after the last induction the animals were challenged on the left flank with a 30 % solution of the test compound in distilled water (24-hour closed patch) and with the vehicle alone on the right flank. 10 animals served as controls. 24 and 48 hours after removal of the patch the skin reactions were examined.

Result: 2 of the 10 test animals showed a slight to moderate (stained) erythema.

Remark:
- Normally 20 animals are used in a test group.
- The validity of the test is dubious because the concentrations used are too low and because no sodium lauryl sulphate was used prior to topical induction to increase the assay sensitivity.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sC
Admin.Days: 5-15

Ethoxyblau (solvent aqua deion.) was administered by gavage to pregnant Wistar rats (Crl: Wi/Br; 20/group) at dose levels of 0, 5, 15 and 30 mg/kg b.w. during day 5 to 15 of gestation. The body weight at the start of the study was 234-311 g. Food and drinking water ad libitum. After 20 days the females were sacrificed.

Examinations:

Clinical signs daily. Body weight and food consumption at the start of the study and at day 5, 10, 15 and 20. A complete autopsy and macroscopic examination of organs of the dams. Furthermore the number of corpora lutei, the number and distribution of the living and dead fetuses, placentae, sex of the fetuses and the externally visible deviations in fetuses were examined. About one third of each litter was prepared in Bouin´s solution to examine soft tissues and the remaining fetuses were eviscerated and prepared for examination of skeletal abnormalities (after staining with Alizarin Red).

Result: No maternal mortalities and no signs of maternal toxicity.

No differences or abnormalities were found in the fetuses.

7. Toxicokinetics (incl. Percutaneous Absorption)

Cutaneous absorption in vivo:

To determine the cutaneous absorption of ethoxyblau (sD), ^{14}C-labelled ethoxyblau was applied on the dorsal clipped skin (3 x 3 cm) of Sprague Dawley rats (3/sex/group; Him:OFA, SPF) for 30 minutes. The animals were kept in metabolism cages. The compound was integrated in two different hairdye formulations I and II (II containing hydrogen peroxide) or was applied as a solution in water. An oral application of the test article (2 % solution; 6/sex) was used as a reference. After 30 minutes the skin was rinsed with shampoo and water. The rinsings were collected. Urine, faeces and rinsing water from the cages were collected. The animals were killed 72 hours after dermal application of the formulations I and II and the solution and 24 (3/sex) and 72 hours (3/sex) after oral application.

Examinations:

Rinsing water, urine and faeces, treated and untreated skin, organs/tissues (13), blood, carcass. In the oral study with sacrifice after 72 hours the gastro-intestinal tract was removed also.

Result: The majority of the applied ^{14}C was removed from the skin by rinsing after 30 minutes (98.4 - 99.1 %). The mean percutaneous absorption was 0.46 % for formulation I, 0.078 % for formulation II and 0.58 % for water solution. The application sites contained 0.44 %, 0.70 % and 0.35 % after application of formulation I, II and the solution, respectively. After cutaneous application the ^{14}C amounts in carcass and organs were below or near the detection limit (0.0004 % for small and 0.0002 % for large organs). Excretion after cutaneous application was 0.451 %, 0.076 % and 0.57 % for formulation I, II and the solution, respectively (urine 56-58 %, faeces 42-44%). After oral administration 50 % of the administered ^{14}C was excreted in urine and 45 % in faeces. Most of the ^{14}C is excreted during the first 24 hours after application.

The formulations consisted of:	I	II
ethoxyblau (^{14}C)	2.00 %	2.00 %
p-toluylendiamine-sulfate	-	1.75 %
mix of resorcinol & m-aminophenol	-	0.68 %
mix of salts	0.70 %	0.35 %
ammonia 25 %	1.14 %	4.08 %
isopropane	3.90 %	1.95 %
WAS	2.00 %	1.00 %
deionised water	42.65 %	14.40 %
formulation base	47.60 %	23.80 %
Welloxon (containing 9 % hydrogenperoxide)	-	50.00 %

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sC	*Salm typh*	TA 97	frameshift mut	1-6000 µg/plate toxic conc>= 3000 µg/plate solvent water	-	-	r	AR
* sC	*Salm typh*	TA 98	frameshift mut	1-6000 µg/plate toxic conc >= 3000 µg/plate solvent water	-	-	r	AR
* sC	*Salm typh*	TA 100	base-pair subst	1-6000 µg/plate toxic conc >= 3000 µg/plate solvent water	-	-	r	AR
* sC	mouse lymp L5178Y		mutat. HGPRT	1.58-5000 µg/ml toxic 158 µg/ml -S9; toxic 1580 µg/ml +S9	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r=rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sC	mouse	NMRI	micronucleated polychromatic erythocytes	1000 mg/kg b.w. solvent water; sampl. at 24, 48, 72 hrs.	-

Ames test
Only 3 strains were used in this bacterial assay. At least 4 strains are prescribed in the OECD guidelines.

Mouse lymphoma fluctuation assay
2,4-diamino-5-methyl-phenetol, dihydrochloride was tested in duplo for mutagenicity in a mouse lymphoma fluctuation assay at the HGPRT locus of L5178Y cells (6-thioguanine resistance), both in the absence and the presence of metabolic activation (S9-mix of Aroclor 1254-induced male Wistar rats). The dose levels used were 1.58 to 5000 µg/ml. 7 days after treatment the cultures without metabolic activation in the concentration range 1.58 to 50 µg/ml

were plated for 6-thioguanine resistance. Those with metabolic activation were likewise plated in the concentration range 15.8 to 500 µg/ml. Positive and negative controls were included.

Result: At 5000 µg/ml extensive precipitation occurred in the presence of S9.

Cells treated with 158 µg/ml or more in the absence of S9 and cells with 1580 µg/ml or more in the presence of S9 failed to grow during the seven day expression period. It was concluded that the compound has no mutagenic activity at the HGPRT locus of L5178Y mouse lymphoma cells.

Micronucleus test

Ethoxyblau was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of 3 groups of mice (5/sex/group; Crl: NMRI BR-mice). The solvent and 40 mg cyclophosphamide served as a negative and positive control, respectively. 24, 48 and 72 hours after application a group of 5 males and 5 females was sacrificed and 1000 PCE´s/animal were analysed. The negative controls were sacrificed after 48 hours, the positive controls after 24 hours. Dosage of 1000 mg/kg b.w. was derived from a preliminary range finding study.

Result: Ethoxyblau was not mutagenic in the micronucleus test at a dose of 1000 mg/kg b.w. It had a cytotoxic effect 24 hours after application of the dose, after 72 hours a toxic effect was not seen.

Remark: Although only one dose level was used, the study can be evaluated because there is a clear indication that the substance reached the bone marrow, as shown by the toxic effect in the cells.

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sC	Chin hamst ovary cell		sister chr exchanges	2.39-2390 µg/ml in water; toxic >= 239 µg/ml	-	-	R	

Abbreviations:

meas.endp. = measured endpoint
sp = species used for activation (r=rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR=Aroclor, PH=Phenobarbital, MC=Methylcholantrene)

Ethoxyblau was tested for sister chromatid exchanges in Chinese hamster ovary cells (Kl-cells). Concentrations tested were 10-10000 µM (2.39-2390 µg/ml), positive and vehicle controls were included. The test was done in triplo and positive and negative controls were included. At each concentration 100 metaphases were evaluated at most.

Result: The toxic concentration in the presence of S9-mix was $\geq$ 717 µg/ml, in the absence of S9-mix $\geq$ 239 µg/ml. In the first test a slight increase in SCE´s was seen at the highest dose level tested. Such increase was not seen in the other two tests so it can be concluded that ethoxyblau did not induce sister chromatid exchanges.

11. Conclusions

Quality Assurance was included at the sensitization test, the mouse lymphoma and micronucleus test and the 90-day oral rat study.

General
Ethoxyblau is used in oxidative hair dye formulations at a maximum concentration of 2 %, but because the oxidation dyes are mixed (1:1) with hydrogen peroxide before application the final concentration at usage is 1 %.

Acute toxicity
Based on the oral LD_{50}-value for the mouse and rat (between 200 and 2000 mg/kg/b.w.) ethoxyblau can be classified as moderately toxic.

Irritation/sensitization
The eye and skin irritation tests were carried out with guinea pigs and not with rabbits, the species normally used and for which the Draize scorings system is applicable. A 3 % solution of the test compound was not irritating to the skin and eye. The sensitization test was carried out inadequately.

Semichronic toxicity
In a 90-day feeding study Wistar rats (m+f) were daily given 0, 5, 10 or 20 mg ethoxyblau/kg b.w. by stomach tube. A part of the control animals and an additional highest dose group were observed for another 5 weeks without treatment. Two animals died during the study but not due to treatment. The only treatment related effect was the macroscopically observed dark colouration of the thyroid glands in all highest dose animals. In histopathology no treatment related effect could be found. Based on these results a NOAEL of 20 mg/kg b.w. can be established.

Teratogenicity
In a teratogenicity study with rats at dose levels of 0, 5, 15 and 30 mg/kg b.w., no indications were found for a teratogenic or foetotoxic effect and no maternal toxicity was observed.

Mutagenicity
Ethoxyblau was tested for gene mutations in bacterial cells as well as in mammalian cells *in vitro* and in a micronucleus test. Furthermore ethoxyblau did not induce SCE´s in CHO cells. The negative results of these tests are acceptable so it can be concluded that ethoxyblau does not have a mutagenic potential.

Absorption
^{14}C-labelled ethoxyblau was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) and as a solution in water. An oral application was used as a reference. The majority of the applied radioactivity was removed by rinsing (98.4 - 99.1 %). The application sites contained 0.44 %, 0.70 % and 0.35 % for the formulation without hydrogen peroxide, with hydrogen peroxide and the solution, respectively. The mean percutaneous absorption was 0.46 % for the formulation without and 0.078 % for the formulation with hydrogen peroxide. It was 0.58 % for the solution.

Conclusion
A 3 % solution of the test compound did not show any irritation. The sensitization test was carried out inadequately. The highest dermal absorption for the formulations was 0.46 % (formulation without hydrogen peroxide).

It was concluded that the compound had no mutagenic properties when tested in various systems.

No indications for a teratogenic or foetotoxic effect were found after administration of 30 mg ethoxyblau/kg b.w. In a 90-day oral rat study 20 mg/kg b.w. was considered to be the NOAEL.

Despite the shortcomings in the sensitization test, for normal use of ethoxyblau, the following calculation can be made:

1 g of ethoxyblau comes in contact with the human skin in permanent hair dye condition (based on a maximum usage volume of 100 ml hair dye containing 1 % ethoxyblau). With a maximal percutaneous absorption of 0.46 % this results in a dermal absorption of 4.6 mg per treatment, which is 0.08 mg/kg b.w. (assuming a body weight of 60 kg). So a safety margin of 250 can be calculated between the figure for human exposure to permanent hair dye and the NOAEL found in the 90-day oral rat study.

It should be noted that the NOAEL comes from a daily exposure for 90 days, while human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Classification: B.

Industry should provide data on skin sensitization potential from in-use data in the context of the volume used, together with any available information on the toxicological profile of the compound, e.g. from animal studies and/or, from experience in use in either the consumer or occupational context.

A 116: 2(*2,4-DIAMINO-5-METHYL-PHENOXY) ETHANOL*

1. General

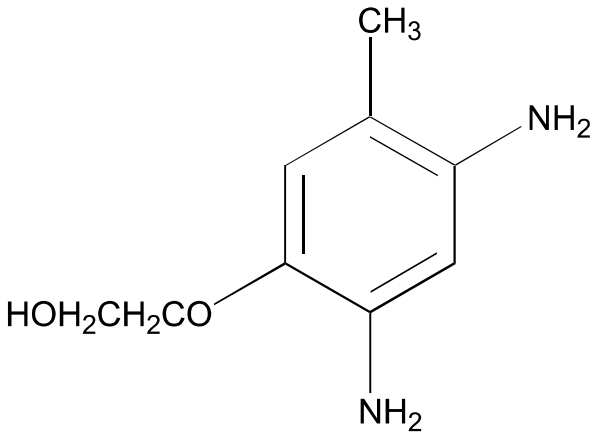
1.1 Primary name

HB-Blau.

1.2 Chemical names

2,4-diamino-5-(2´-hydroxyethyloxy)-toluol-dihydrochloride
1-methyl-2,4-diamino-5-(2´-hydroxyethyloxy)-benzene-dihydrochloride

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{14}N_2O_2x2HCl$
Mol weight: 255

1.7 Purity, composition and substance codes

sA: HB-Blau (GHS 280786); purity ca. 99 %.

Formulation(s) in which substance is used:

Code	Formulation	Quantity
fA	L5/76/1 + Welloxon 9%	1.5 %
fB	HB-Blau without Welloxon 9 %	3 %
fC	HB-Blau plus Welloxon 9 %	3 %

Composition of formulation(s):

fA: 50 % Koleston 2000 (=L5/76/1) cont. 3 % sA (final sA conc.: 1.5 %)
50 % Welloxon 9 % cont. 9 % hydrogen peroxide

fB: 3 % sA
0.7 % mixture of salts
1.6 % ammonia, 25 %
3.9 % isopropanol
2 % WAS
41.2 % deionised water
47.6 % formulation base

fC: 3 % sA
1.75 % p-toluylendiamine-sulfate
0.68 % mixture of resorcinol and m-aminophenol
0.35 % mixture of salts
4.52 % ammonia, 25 %
1.95 % isopropanol
1 % WAS
12.96 % deionised water
23.8 % formulation base
50 % Welloxon 9 % (cont. 9 % hydrogen peroxide)

1.8 Physical properties

Subst. code: sA
Appearance: fine, white-pink chrystalline powder

1.9 Solubility

sA is soluble in water and in dimethylsulphoxide.

2. Function and uses

sA exists as a dihydrochloride. It is included in oxidative hair dye formulations at a maximum concentration of 3 %.

As the oxidation dye is mixed with hydrogen peroxide before application, the concentration at use is only 1.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	rat (f)	875 mg/kg b.w.
sA	oral	rat (m)	725 mg/kg b.w.
sA	oral	mouse (f)	1100 mg/kg b.w.
sA	oral	mouse (m)	1040 mg/kg b.w.

Groups of 5 male and 5 female rats received a single dose of 500, 750 or 1000 mg/kg, and 750, 1000 and 1250 mg/kg, respectively. Groups of 10 male and 10 female mice received a single dose of 800, 1100 or 1400 mg/kg, and 750, 1000 or 1200 mg/kg. A 10 % suspension of sA in aqua dest. was administered via stomach tube. sA caused an initially increased activity, decreasing after 10 minutes, spasms and side position.

3.7 Subchronic oral toxicity

Route: oral

Species: rat

Subst.: sA

Exposure: 3 m

DWE: 30 mg/kg b.w.

LED: 60 mg/kg b.w.

Method: sA, dissolved in water, was administered to four groups of 15 male and 15 female Wistar rats via stomach tube, once daily for 3 months (5d/w). Test doses were 0, 15, 30 and 60 mg/kg b.w., respectively.

Observations: Clinical signs (daily), mortality (daily), ophtalmoscopic changes, body weight (weekly), food and water consumption (weekly), hematology (week 0, 7, 13 and 17: ery, leu, HB, HCT, MCV, MCH, MCHC), clinical chemistry (week 0, 7, 13 and 17: bil, glu, total prot, GOT, GPT, AP, uric, acid, urea, Fe, Ca, TG, chol, creat), urinalysis (week 0, 5, 11, 15: leu, nitrite, pH, prot, glu, ketones, urobil, bil, blood, sed), organ weights (8 organs), macroscopic changes and histopathology (about 30 organs).

Results: No toxic signs or mortality occurred. Food consumption, water consumption and body weight were normal in all groups. Hematological chemistry data were within the physiological range and did not show dose-related changes. Urine investigations did not reveal treatment-related alterations.

After 7 weeks, a slight but dose related decrease in creatinin was observed in all dose groups, which had disappeared after 13 weeks. At week 13 glucose levels in females were dose relatedly increased in female rats, while for male rats urea had decreased dose relatedly.

These slight biochemical changes were considered not toxicologically relevant. Absolute and relative spleen weights were slightly but significantly increased in females at 60 mg/kg b.w. Histopathological observations of the spleen showed a decreased hematopoiesis in rats exposed to 60 mg/kg b.w.

No other substance-related macroscopic changes or histopathological deviations of the spleen or any other organ were noticed in any group.

The no-observed-adverse-effect-level was determined at 30 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin
Species: guinea pig
Subst. sA
Exposure: 4 h
Dose: 1 ml
Concentr.: 3 %

Method: 1 ml of a 3 % solution of sA in distilled water was applied on patches to the clipped dorsal skin of five Pirbright white guinea pigs. After 4 h exposure, the patches were removed and the treated skin was washed. Observations were made according to Draize (OECD 404), up to one week after application. Possible behavioural disturbances were noted.

Results: No irritation was observed at any time. No changes in behaviour were found.

Remark: Guinea pigs were used instead of the rabbits recommended according to OECD guideline 404.

4.2 Irritation (mucous membranes)

Route: eye
Species: guinea pig
Subst.: sA
Exposure: 24 h
Dose: 0.1 ml
Concentr.: 3 %

Method: 0.1 ml of a 3 % solution of sA in aqua dest. was instilled into the right eye of five female Pirbright white guinea pigs. The left eye remained untreated and served as a control. Eye irritation was scored, according to the scale of Draize, at 0.5, 1, 2, 3, 4, 6 and 7 h after application. Further readings, at 24 h and once each following day, were carried out after the instillation of one drop of a 0.1 % fluorescein-sodium solution.

Results: None of the animals showed any reaction to the treatment.

Remark: Guinea pigs were used instead of the rabbits recommended in the method according to Draize (OECD 405).

5. Sensitization

Test I

Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 0.1 - 40 %
Conc.chall.: 40 %
Result: positive

Method: The study was performed according to OECD 406. Ten female Pirbright guinea pigs were induced by three intradermal injections of 0.05 ml of a 0.1 % sA solution on both the right and left clipped shoulder region. The three injected solutions were 0.1 % sA in Ringer solution, 0.1 % sA in Freund´s complete adjuvant and Freund's complete adjuvant plus Ringer solution at 1:1, respectively. After one week a 40 % sA solution in distilled water was applied on a patch to the shoulder regions for 48 h. A second group of 10 animals served as a control.

Two weeks after the last induction the animals were challenged on the left flank with a 40 % sA solution in distilled water on a patch. The right shoulder served as a control. After 24 and 48 h skin reactions were read.

Results: Skin reactions (erythema) were observed in four animals of the test group. According to the method of Magnusson and Kligman, sA can be classified as moderately sensitizing to the skin of guinea pigs.

Test II

Subst.: fA
Species: guinea pig
Method: Buehler
Conc.induc.: 25 %
Conc.chall.: 1 %
Result: negative

Method: A Buehler test was performed according to OECD 406. During the induction period of three weeks 0.5 ml of a 25 % dilution of fA in deionized water was applied to the shaven left flank of 20 Pirbright guinea pigs once weekly. 10 animals served as a control. After 6 h occlusion the test formulation was washed off with water.

Two weeks after the last treatment, the challenge was carried out in all animals, using 0.5 ml of a 1 % dilution of fA in deionized water. 24 h after application the test area was depilated. Skin reactions were examined at 2, 24 and 48 h after depilation.

Results: During the induction period no skin reactions could be observed due to oxidative staining of the skin by the 25 % fA solution. After the challenge no signs of sensitization were observed.

Remark: Both the induction and the challenge concentrations were based on a range finding test. At the induction concentration (25 % fA, containing 0.4 % sA) erythema was observed while at the challenge concentration (1 % fA = 0.015 % sA) no skin irritation was found.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 6-15
DWE: 180 mg/kg b.w.

Method: Doses sA (dissolved in aqua bidest.) of 0, 30, 60 or 180 mg/kg b.w. were administered by oral gavage to four groups of 20 pregnant Wistar rats. On day 20 of gestation the animals were sacrificed.

Observations: Until day 20 the dams were clinically observed and body weights and food consumption were measured. On day 20 complete autopsy and macroscopic examination of the organs (not specified) were carried out. Viable fetuses, fetal sex ratio, fetal body weights, birth position, number of runts, implantations, resorptions, post-implantation losses, corpora lutea, uteri weights and placenta weights were determined. Fetuses were examined for external, skeletal and visceral deviations.

Results: Maternal body weight gain and food consumption were both dose relatedly reduced during the treatment period in animals exposed to sA. Laparotomy: The number of early resorptions was slightly increased in the 180 mg/kg b.w. group. One runt was found in the 30 and 180 mg/kg b.w. groups. Fetal examination: In the 30 mg/kg b.w. group one fetus was found with a shortened spine and missing coccygeal vertebrae and one with a head-neck edema. In the 180 mg/kg b.w. group one fetus revealed an umbilical hernia with extruded liver. However, no treatment related effect on the fetuses was observed. Up to 180 mg/kg b.w. sA did not show embryotoxic or structural irreversible effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption *in vivo*:

About 30 mg ^{14}C-labelled sA, included in two different hair dye formulations (fB and fC) or dissolved in water at 9.99 %, was applied to the clipped dorsal skin of three male and three female Sprague Dawley rats. After 30 min. the substance was washed off with shampoo, water and absorbent cellulose tissue. Rinsing was continued until the rinsing water and tissues were free of colour. Thereafter the skin was covered with gauze for 72 h, after which the animals were killed. Radioactivity of rinsings, application site, urine, faeces, blood, organs (15) and carcass was estimated by liquid scintillation counting.

Oral application of 30 mg sA in water was used as a reference.

Results cutaneous application:

The majority of the applied ^{14}C (98.9 % to 99.2 %) was removed from the skin by rinsing after the cutaneous treatment. The mean ^{14}C content of the skin at the application site was 0.32 % (fB), 1.09 % (fC) and 0.20 % (sA solution in water).The mean percutaneous absorptions were 0.15 % for fB, 0.06 % for fC and 0.2 % for the sA solution in water.

Excretion: After cutaneous application means of 0.147 % (fB), 0.054 % (fC) and 0.21 % (sA in water) of the applied ^{14}C were recovered in urine and faeces within 72 h. Of the eliminated amount of sA, 54 % to 68 % was excreted in urine and 31 to 44 % in faeces. 74 to 91 % of the totally eliminated amount was excreted in the first 24 h after application.

The mean total recovery of the applied radioactivity varied from 96.5 to 100.1 %.

Carcass: The remaining mean amounts of ^{14}C in the carcass at 72 h after application were below or near the detection limit and varied from 0.0024 to 0.0029 % of the administered dose, which corresponds with 1.5 to 4.4 % of the absorbed amount of sA.

Organs: 72 h after application mean concentrations of ^{14}C were near or below the detection limits in all organs. The detection limits were about 0.00002 %/g for large organs, 0.0001 %/g for adrenals and 0.005 %/g for thyroids. Highest concentrations were noted in thyroids, liver and adrenals although below the detection limit. Lowest concentrations were detected in testes, brain and muscles.

Results oral application:
Excretion: 61 % of the adminitered ^{14}C dose was excreted in urine, 35 % in faeces. 73 % of the totally eliminated sA was excreted within the first 24 h.

Carcass: The remaining mean radioactivity in the carcass after 72 h was 0.71 % of the administered dose.

Organs: The blood level was highest 2 h post application: 0.25 % of the applied dose. An initial half-life of 5 h was determined. 72 h after application mean concentrations of ^{14}C were highest in thyroid glands (25 times higher than in blood), liver (4 times higher than in blood) and kidneys, and lowest in fat (9 times lower than in blood), testes and brain (both 5 times lower than in blood).

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	*Salm.typh.*	TA97	frameshift mut	3-6.000 µg/plate; solvent water; toxic at ≥3000;[1]	-	-	r	
* sA	*Salm.typh.*	TA98	frameshift mut	3-6000 µg/plate; solvent water; toxic at ≥1000;[1]	-	-	r	
* sA	*Salm.typh.*	TA100	base-pair subst	3-6000 µg/plate; solvent water; toxic at ≥3000;[1]	-	-	r	
* sA	mouse lymp cel L5178Y		mutation HGPRT-loc.	+act: 78-1250 µg/ml -act: 1.58-158 µg/ml;[2]	-	-	r	AR
* sA	Chin hamst ovary cell		chrom. aber.	+act.: 158-1580 µg/ml; -act.: 15.8-158 µg/ml;[3]	+	+	r	AR

Abbreviations:
meas.endp.: = measured endpoint
sp = species used for activation (r=rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	Mouse, bone marrow	NMRI	micronucleated polychromatic erythrocytes	600 mg/kg b.w. solvent DMSO; samples at 24, 48 and 72 h;[4]	-

1. From the at least four *Salmonella typhimurium* strains recommended by OECD guideline no. 471, only TA 97, TA 98 and TA 100 are tested here.

2. sA was tested in concentrations from 1.58 to 158 µg/ml, in absence of S9, and in dose levels of 78 to 1250 µg/ml, in presence of S9. 1250 µg/ml without S9 caused 91 % toxicity and 158 µg/ml with S9 98 % toxicity. sA failed to induce mutation at the HGPRT-locus of mouse cells.

3. sA caused complete mitotic inhibition at concentrations of 500 µg/ml (without S9) and 5000 (with S9). sA caused a statistically significant increase in the number of aberrations at 158 µg/ml, without S9, and at 1580 µg/ml in presence of S9. It is concluded that sA is able to induce chromosome aberrations in cultured CHO cells in the absence and in the presence of metabolic activating enzymes.

4. Only one concentration (600 mg/kg b.w.) is tested, based on the oral LD_{50} for mice and rats. No toxic effects were observed. In the dosed groups micronuclei rates were slightly higher than in the negative control group. However, the amounts of micronucleated polychromatic erythrocytes in the dosed groups were far below the amounts in the positive control group and comparable to historical negative controls.

The amount of micronucleated nomochromatic erythrocytes was comparable in all groups.

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	rat		UDS in hepatocytes	70, 200 and 600 mg/kg b.w. in water; samples at 24 h;[1]	-

1. In rat liver cells no adverse sA related effects were noted. Mean silvergrain counts per nucleus were significantly and dose-relatedly increased in groups exposed to concentrations of 200 and 600 mg/kg b.w. This increase was mainly due to an enhanced amount of cells with one or two silvergrains per nucleus. Values were within the range of former negative control data and far below the values obtained from the positive control group.

11. Conclusions

In all provided reports a Quality Assurance for the performance of the study was included, except for the acute toxicity study, the irritation tests, the sensitization test, and the Ames test.

General

HB-Blau is used in oxidative hair dyes up to a concentration of 3 %. The final concentration at application when mixed with peroxide will be 1.5 %.

Acute toxicity

The substance is moderately toxic, based on acute oral toxicity tests with LD_{50} values of 725 (rat) and 1040 mg/kg b.w. (mouse).

Irritation

A 3 % solution of HB-Blau in distilled water was neither irritating for the skin nor for the eyes of guinea pigs.

Sensitization

HB-Blau caused skin erythema in the maximization test of Magnusson and Kligman, and was classified as moderately sensitizing. For a hair dye formulation (L5/76/1 + Welloxon 9 %), containing 1.5 % HB-Blau, no signs of sensitization were observed in a Buehlertest.

Semichronic toxicity

In a 90-day study, Wistar rats were daily given HB-Blau by stomach tube, at doses of 0, 15, 30 or 60 mg/kg b.w. No mortality or clinical signs were observed in any dose group. Slight but significant changes in clinical chemistry were seen in all treated groups. After 7 weeks, a slight but dose related decrease in creatinin was observed in all dose groups, which had disappeared after 13 weeks. At week 13 glucose levels in females were dose relatedly increased in female rats, while for male rats urea had decreased dose relatedly. These slight biochemical changes are considered not toxicologically relevant. Absolute and relative spleen weights were slightly but significantly increased in females at 60 mg/kg b.w. Histopathological observations of the spleen showed a decreased hematopoiesis in rats exposed to 60 mg/kg b.w. This could be due to the difference in time between the last blood sample was taken and the time the animals of the different groups were sacrificed. No further substance-related macroscopic changes or histopathological deviations of the spleen or any other organ were noticed in any group.

30 mg/kg b.w. is considered to be a no-observed-adverse-effect-level.

Teratogenicity

A teratogenicity study in rats showed that HB-Blau in concentrations up to 180 mg/kg b.w. does not cause embryotoxic or structural irreversible effects.

Genotoxicity

An Ames test with HB-Blau in bacterial cells was not performed according to the OECD guidelines: only 3 instead of the recommended 4 strains were used. HB-Blau was not mutagenic in a mouse lymphoma assay for HGPRT gene mutations. From a test in Chinese hamster ovary cells it is concluded that HB-Blau is able to induce chromosome aberrations in absence and in the presence of metabolic activating enzymes. An *in vivo* DNA repair test and a micronucleus test were negative.

Absorption
In an in vivo skin absorption test in rats mean percutaneous absorptions were determined of 0.2 % for sA dissolved in water, 0.15 % for fB and 0.06 % for fC (mixed 1:1 with 9 % hydrogen peroxide).

Conclusion
HB-Blau is moderately toxic after oral administration.

A testsubstance containing 3 % HB-Blau did not cause skin and eye irritation.

Tested according to the method of Magnusson & Kligman, HB-Blau could be classified as moderately sensitizing to the skin of guinea pigs.

The mean dermal absorptions were 0.2 % for a HB-Blau solution in water, 0.15 % for the formulation without hydrogen peroxide (fB), and 0.06 % for the formulation with hydrogen peroxide (fC).

In a teratogenicity test in rats, HB-Blau did not cause embryotoxic or structural irreversible effects up to a concentration of 180 mg/kg b.w. HB-Blau appeared to be non-mutagenic in a mouse lymphoma assay. An unscheduled DNA synthesis assay and a micronucleus test were both negative. A chromosome aberration test in hamster ovary cells was positive with and without metabolic activation. In an Ames test only three of the four required bacterial strains were used. Although this compound had been shown in *in vitro* studies to have some mutagenic potential, the negative results obtained *in vivo* in both the bone marrow and the liver provided adequate reassurance that this activity was not exposed *in vivo*.

In the 90 day feeding study effects were particularly found at the 60 mg/kg b.w. level, although some slight changes in clinical chemistry (glucose, creatinin, urea) were seen in all treated groups. The no-observed-adverse-effect-level was considered to be 30 mg/kg b.w.

Despite the inadequate data on irritation and mutagenicity, the following human risk calculation for normal use can be made: A maximum amount of 100 ml of the hair dye formulation fA, containing 1.5 % HB-Blau, comes in contact with the human skin. This corresponds to 1500 mg HB-Blau. As skin penetration for the formulation containing 50 % hydrogen peroxide was 0.06 %, dermal absorption will be 0.9 mg HB-Blau per treatment. Assuming a body weight of 60 kg, the exposure of a human per kg body weight will be 15 µg/kg b.w.

So a safety margin of 2000 can be calculated between the figure for human exposure to permanent hair dye and the NOAEL of 30 mg/kg b.w. found in the 90d oral rat study. It should be noted that the NOAEL found in rats is based on daily exposure for 90d, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Classification: B.

Industry should provide data on the incidence of skin sensitization in use, to ensure that this compound was not unacceptable in this regard.

B 54: 2-(*4-HYDROXY-2NITROANILINO) ETHANOL*

1. General

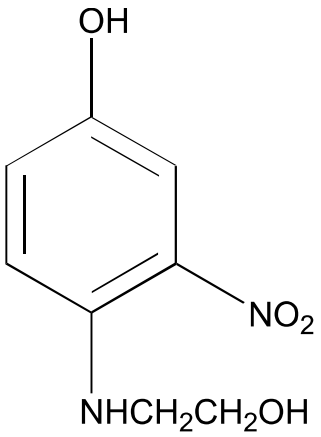
1.1 Primary name

Imexine FH.

1.2 Chemical names

1-hydroxy-3-nitro-4-(beta-hydroxyethyl)amino-benzene
3-nitro-beta-hydroxyethylamino-phenol
N-beta-hydroxy ethyl amino-4 nitro-3 hydroxy benzene

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{10}N_2O_4$
Mol weight: 198

1.7 Purity, composition and substance codes

sA: 1-hydroxy-3-nitro-4-(beta-hydroxyethyl)amino-benzene (purity: >98 %)

1.8 Physical properties

Subst. code: sA
Appearance: odourless greenish brown powder
Melting point: 142-145 °C

1.9 Solubility

The solubility of the substance is 0.10 g in 100 ml water at 70°C and 0.10 g in 100 ml ethanol at 25°C.

2. Function and uses

1-hydroxy-3-nitro-4-(beta-hydroxyethyl)amino-benzene is included in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 6 %.

In permanent hair dyes the maximum concentration is 6 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 3 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	mouse (f)	>3000 mg/kg b.w.
sA	oral	mouse (m)	>3000 mg/kg b.w.
sA	oral	rat (f)	>3000 mg/kg b.w.
sA	oral	rat (m)	>3000 mg/kg b.w.

The test compound (a 10 % dilution in propylene glycol) was given once by stomach tube to Swiss mice (5/sex/group) and Albino Wistar rats (5/sex/group) at increasing concentrations up to 3000 mg/kg b.w.

The animals were observed for 8 days and at the end of the 8-day observation period all surviving animals were sacrificed and gross necropsies were performed.

No mortalities were observed in the mice and 10 % mortality in the rats. No abnormalities were found in the animals necropsied on day 8.

The test substance is slightly toxic (LD_{50}>2000 mg/kg b.w.).

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 96 d
DWE: 1000 mg/kg b.w.

Imexine FH was administered, by gavage, once daily to 4 groups Sprague-Dawley (Ico: OFA SD) rats (10/sex) for 96 days. The test substance was administered at dosage levels of 40, 200 and 1000 mg/kg b.w. The control group received the vehicle (polysorbate + carboxy-methylcellulose) only. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded individually, twice a week. An ophthalmoscopic examination was performed. Haematological and clinical chemical investigations were carried out on week 4

and 13. Urine samples were collected at week 4 and 13. Organ weights (c. 6) were measured and macroscopy and histopathology (c. 35 organs/tissues) was performed.

No animal died during the study. In the high dose group, the following effects were observed: red discolouration of urines and haircoat, immediately after dosing and an increased salivation from week 7.

The dose level without adverse effect was 1000 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin
Species: rabbit
Subst.: sA
Exposure: 23 h
Dose: 0.5 ml
Concentr.: 4 %

0.5 ml of the test substance was applied occlusively on the clipped, right (abraded skin) and left flank (intact skin) of 6 male NZW rabbits. After 23 hours the patches were removed.

Observations for signs of dermal irritation were recorded at 1, 24, 48 and 72 hours after removal of the patches. Biopsies were carried out for histological examinations on 3 rabbits 1 hour after patch removal (from the right flank) and on the 3 remaining rabbits 48 hours after patch removal (from the left flank). No erythema could be recognized, because the skin was coloured red by the test substance. No irritation was observed. The histological examination did not reveal any pathological aspect.

Remark: The exposure time should be 4 hours instead of 23 hours. The Draize score could not be calculated, because the value of erythema is unknown.

4.2 Irritation (mucous membranes)

Route: eye
Species: rabbit
Subst.: sA
Dose: 0.1 ml
Concentr.: 4 %

0.1 ml of the test substance was instilled in the conjunctival sac of the right eye of 6 albino NZW rabbits. The untreated left eyes served as controls. The eyes were examined 1, 24, 48 and 72 hours and 4 and 7 days after application. Additional examinations were carried out upon the instillation of one drop of 2 % fluorescein-solution.

1 hour after application, chemosis and redness of the conjunctivae was observed in 5 and 3 animals, respectively and congestion of the iris in 4 animals. 24 hours after application, chemosis and redness of the conjunctivae was observed in 2 animals and congestion of the iris

in 1 animal. After 1 day these effects were disappeared. No other irritating effects were observed. The Draize score was 2.46 (not irritating).

5. Sensitization

Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 50 %
Conc.chall.: 100 %
Result: negative

20 Albino Hartley guinea pigs were used in this skin sensitization study. The induction phase consisted of 10 topical applications of the test substance using occlusive patches (just above the injection site) and 2 intradermal injections of FCA. On day 1 and 10 the guinea pigs received an intradermal injection of 0.1 ml of FCA diluted to 50 % in sterile isotonic saline. The test substance is applied 3 times per week, with a two days interval, for 3 weeks and once at the start of the fourth week. The 10 and final patch is removed on day 24, after 48 hours of contact with the skin. Day 24-35: rest period. On day 35, the animals were challenged by closed patch test, using 0.5 ml undiluted test substance. After 48 hours the patches were removed.

Any sign of erythema and oedema was recorded 1, 6, 24 and 48 hours after the challenge.

1 guinea pig died during the study. No erythema could be recognized because the skin was coloured red by the test substance. The histological examination did not show any sensitization aspect. The test substance did not produce dermal sensitization.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 6-15
DWE: 100 mg/kg b.w.
LED: 1000 mg/kg b.w.

Imexine FH, suspended in carboxymethylcellulose, was administered orally to 2 groups of 25 pregnant rats (Sprague-Dawley). The test substance was daily administered at dosage levels of 100 and 1000 mg/kg b.w. The control group received the vehicle only. All dams were sacrificed at day 20 of gestation. All animals were observed daily for mortality and clinical signs. Individual body weights were recorded on days 0, 6, 9, 12, 15 and 20. Food consumption was measured for the phases 0-6, 6-9, 9-12, 12-15 and 15-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were examined for skeletal defects and variations of the ossification process by Alizarin Red staining and for organic defects.

No animal died during the study. In the treated groups, all females showed reddish coloured urine from day 7 till day 16 of pregnancy. In the high dose group was the number of post-implantation loss slightly increased. This resulted in a decrease in the number of live foetuses. In the high dose group, two malformed foetuses were observed, that is one with soft tissue malformation of the face and another showed a polydactyly (skeletal malformation). These malformations were incidental and so not considered as an effect of the substance.

The dose level without maternal toxicity was 1000 mg/kg b.w. and without foetotoxicity was 100 mg/kg b.w.

Remark: No maternal toxicity was observed. However, foetotoxicity was found in the high dose level, so the necessity of maternal toxicity has been cancelled.

7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption of Imexine FH
The method used is: *in vitro*, diffusion cell (Franz cell) using human breast epidermis.

4 % Imexine FH* was applied 8 times, in absence and in presence of hair (adding 10 mg of finely cut tinted hair), using human breast epidermis, for 30 minutes.

Then the skin was washed and dried.

The formulation was left for 30 minutes and was then rinsed-off using 10 ml distilled water. Then the contact area was dried with cotton wool swabs.

The formulation content was determined in rinsing water and treated skin areas. C. 99 % of the applied amount was removed from the skin by rinsing 30 minutes after the beginning of the percutaneous treatment.

The mean percutaneous absorption was 0.003 % of the administered formulation in absence of hair, and 0.004 % in presence of hair.

*Composition of the formulation:

- imexine FH 4 g
- polyethylene glycol 60 g
- ammonia (20 %) 10 g
- deionised water 26 g

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	*Salm. typh.*	TA98	frameshift mut	0.1-500 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA100	base-pair subst	0.1-500 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1535	frameshift mut	0.1-500 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1537	frameshift mut	0.1-500 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1538	base-pair subst	0.1-500 µg/pl	-	-	r	AR

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	*Salm. typh.*	TA98	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA100	missence	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1535	missence	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1537	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1538	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA98	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA100	base-pair subst	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1535	base-pair subst	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1537	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Salm. typh.*	TA1538	frameshift mut	5-1000 µg/pl	-	-	r	AR
* sA	*Sacch.cer.*	D4	gene conv.	0.1-500 µg/pl	-	-	r	AR
* sA	Chin. hamster	CHO	chrom.aber.	0.05, 0.1, 0.2 and 0.4 mg/ml	-		r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	mouse	CD-1	micronuclei	1250, 2500 and 5000 mg/kg b.w.	-
* sA	mouse	Swiss	micronuclei	37.5, 75.0, 150 and 300 mg/kg b.w.	-

Salmonella assays
5 strains of *Salmonella typhimurium* were exposed to sA, dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose levels tested were 0.1-500 µg/plate. The negative control was DMSO and the positive control substances were N-methyl-N´-nitro-N-nitroso-guanidine, 2-nitro-fluorene in the absence of S9 mix and anthracene, 2-acetyl-amino-fluorene in the presence of S9 mix.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

5 strains of *Salmonella typhimurium* were exposed to sA, dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose levels tested were 5-1000 µg/plate. The negative control was DMSO and the positive control was 2-nitrofluorene.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

5 strains of *Salmonella typhimurium* were exposed to sA, dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose levels tested were 5-1000 µg/plate. The negative control was DMSO and the positive control substances were 1,2 diamino-4-nitrobenzene and 2-aminoanthracene.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

Saccharomyces cerevisiae assay
Cell cultures of the yeast were exposed to sA, dissolved in DMSO, at concentrations of 0.1-500 µg/plate, in the presence and absence of S9-mix. The negative control was DMSO and the positive control substances were N-methyl-N´-nitro-N-nitroso-guanidine, 2-nitro-fluorene in the absence of S9 mix and anthracene, 2-acetyl-amino-fluorene in the presence of S9 mix.

There was no genotoxic effect found, neither in the absence nor in the presence of S9 mix.

Cytogenetics assay
sA dissolved in DMSO, was tested in triplicate, in a cytogenetics assay using Chinese Hamster Ovary (CHO) cells, in the absence of S9 mix. Cells were treated with 0.05, 0.1, 0.2 and 0.4 mg/ml for 6, 12 and 16 hours, respectively. 100 metaphases per culture were analysed for chromosome aberrations. The negative control was DMSO.

Imexine FH did not induce chromosomal aberrations, in the absence of S9 mix.

Micronucleus assays
Imexine FH, suspended in 1 % methylcellulose, was administered twice orally, by gavage, separated by a 24-hour interval. 5 groups of CD-1 mice (5/sex) received two equal doses of 1250, 2500 and 5000 mg/kg b.w. The negative control group received 0.1 ml methyl-cellulose/10 g b.w. by gavage and the positive control group received intraperitoneally 0.4 mg/ml mitomycin C. The mice were killed 6 hours after the second dose. 2000 polichromatic erythrocytes were examined for each animal in two bone marrow smears and the ratio of polychromatic to normochromatic erythrocytes was estimated.

Imexine FH did not induce micronuclei in bone marrow cells of the mouse.

Imexine FH, dissolved in DMSO, was administered intraperitoneally, in duplicate, to 5 groups of 4 Swiss male mice at doses of 37.5, 75.0, 150 and 300 mg/kg b.w. The negative control group received DMSO. Bone marrow samples were taken 24 and 48 hours after treatment, respectively. 1000 polychromatic erythrocytes were analysed in each group and the ratio of polychromatic to normochromatic erythrocytes was estimated.

Imexine FH did not induce micronuclei in bone marrow cells of the mouse.

11. Conclusions

A Quality Assurance Declaration was only included by the semichronic toxicity and teratogenicity study.

General
1-Hydroxy-3-nitro-4-(beta-hydroxyethyl)amino-benzene is included in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 6 %.

In permanent hair dyes the maximum concentration is 6 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 3 % only.

Acute toxicity
The test substance is slightly toxic, based on the result of the acute oral toxicity test (LD_{50} mouse, rat >3000 mg/kg b.w.).

Irritation
A concentration of 4 % in the eye and 4 % in the skin irritation study showed no signs of irritation.

Sensitization
No signs of sensitization in guinea pigs were observed in the Magnusson Kligman test.

Semichronic toxicity
In a 96-day feeding study, Sprague Dawley rats were fed 0, 40, 200 or 1000 mg Imexine FH/kg b.w., by gavage once daily. All high dose animals showed red coloured urine and a red haircoat. The dose level without adverse effects was 1000 mg/kg b.w.

Teratogenicity
In a teratogenicity study, Sprague-Dawley rats were fed 0, 100 and 1000 mg Imexine FH/kg b.w. No animals died during the study. The urine of all animals was coloured red. In the high dose group was the number of post-implantation loss slightly increased and so the number of live foetuses decreased. No irreversible structural changes were found.

The dose level without maternal toxicity was 1000 mg/kg b.w. and without foetotoxicity 100 mg/kg b.w.

Genotoxicity
Imexine FH was tested for its mutagenic potential in *in vitro Salmonella*, *Saccharomyces cerevisiae* and cytogenetics assays and *in vivo* in micronucleus assays.

Imexine FH was negative in all tests, therefore imexine FH is considered to be not genotoxic.

Absorption
The mean percutaneous absorption was 0.003 % of the administered formulation in absence of hair, and 0.004 % in presence of hair.

Conclusions
Imexine FH was found slightly toxic in the acute oral toxicity test. A 4 % solution of the test compound did not show eye and skin irritation. Imexine FH showed no signs of sensitization. In the 96-day study with rats, 1000 mg/kg b.w. was considered to be the NOAEL.

In the teratogenicity study, no irriversible structural changes were observed in the foetuses of the rat, after administration of 100 mg/kg b.w.

Imexine FH has no genotoxic potential.

The cutaneous absorption was 0.004 % of the administered hair dyeing formulation. For normal use of hair dye, the following calculation can be made:

3 g Imexine FH comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing 3 % imexine FH). With a maximal penetration of 0.004 %, this results in a dermal absorption of 0.12 mg per treatment, which is 0.002 mg/kg b.w. (assuming a body weight of 60 kg). 2.1 g Imexine FH comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 6 % Imexine FH). With a penetration of 0.004 %, this results in a dermal absorption of 0.084 mg per treatment, which is 0.0014 mg/kg b.w.

So a margin of safety of 50000 can be calculated between the figure for human exposure to oxidative hair dye and the NOAEL for foetotoxicity found in rats in the teratogenicity study. For the semi-permanent hair dye a safety margin of 700000 can be calculated. It should be noted that the NOAEL stems from a daily exposure for 10 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week.

Classification: A.

B 71: *ROT Y*

1. General

1.1 Primary name

Rot Y

1.2 Chemical names

Red Y is a mixture of:
(1) 1-amino-2-nitro-4-(2′,3′-dihydroxypropyl)-amino-5-chloro-benzene (51 %)
(2) 1,4-bis-(2′,3′-dihydroxypropyl)-amino-2-nitro-5-chlorbenzene (46 %) and an acessory component:
(3) 1-amino-2-nitro-4-amino-5-chloro-benzene (about 2%)
IUPAC-Name:
3-(4-amino-2-chloro-5-nitroanilino)propane-1,2-diol plus 3,3′-(2-chloro-5-nitro-p-phenylenediimino)di(propane-1,2-diol)

1.6 Empirical formula

Emp. formula:(1) $C_9H_{12}N_3O_4Cl$
Mol weight:(1) 261.7

Emp. formula: (2) $C_{12}H_{18}N_3O_6Cl$
Mol weight: (2) 335.7

Emp. formula: (3) $C_6H_6N_3O_3Cl$
Mol weight: (3) 187.5

1.7 Purity, composition and substance codes

sA: Red Y (commercial product) (purity: 97.5 %)
sB: 1-amino-2-nitro-4-(2′,3′-dihydroxypropyl)-amino-5-chloro-benzene (component of Red Y)
sC: 1,4-bis-(2′,3′-dihydroxypropyl)-amino-2-nitro-5-chlorobenzene (component of Red Y)

1.8 Physical properties

Subst. code: sA
Appearance: dark brown to violet powder
Melting point: 135 °C

1.9 Solubility

The test substance exists as a free base or as a hydrochloride. The test substance is soluble in ethanol and water and insoluble in petrolether.

2. Function and uses

The compound is included in hair tinting products and colouring setting lotions at a maximum concentration of 1 %.

In oxidative hair dye formulations the maximum concentration included is 2 %. Since the oxidative hair dyes are mixed with hydrogen peroxide before use, the concentration at application is 1 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	mouse (f)	1875 mg/kg b.w.
sA	oral	mouse (m)	1860 mg/kg b.w.
sA	oral	rat (f)	1830 mg/kg b.w.
sA	oral	rat (m)	2196 mg/kg b.w.

The test compound (a 10 % dilution in distilled water) was administered orally (by gavage) at single doses of 1200 (1050), 1800 (1800), 2400 (2550) or 3000 (3300) mg/kg b.w. to male and (female) CF1 mice, respectively. 1200 (1000), 2400 (2000) or 3400 (3000) mg/kg b.w. to male and (female) Wistar rats, respectively. During an observation period of 14 days, mortalities were recorded daily, body weights weekly and clinical toxicological observations of animals of the two highest dose levels were recorded 1, 2, 4 and 24 hours and 7 and 14 days after the administration.

During the observation period, lateral position, tonoclonic spasms, exitus and a red coloured urine was observed at all dose levels. The test substance is moderately to slightly toxic.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	derm	rabbit	>2000 mg/kg b.w.

The moistened test substance was once dermally, occlusively administered to the clipped skin of NZW rabbits (5/sex). The applied dose was 2000 mg/kg b.w. Clinical toxicological observations were carried out at 1, 2, 3, 6, 24 and 48 hours and once a day for 14 days. Observation of skin alterations was done once daily. A post mortem examination was carried out on all animals.

All animals survived the 14-day observation period. Macroscopic and clinical toxicological alterations were not observed.

The test substance is slightly toxic.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 90 d
Recov.p.: 4 w
DWE: 40 mg/kg b.w.
LED: 60 mg/kg b.w.

Red Y was administered, by gavage, once daily to 3 groups albino Wistar rats (20/sex) for 90 days. The control and high dose group included additionally 5 animals/sex, which were deprived from treatment after 13 weeks and remained for 4 further weeks for recovery observations. The test substance, diluted in deionized water, was administered at dosage levels of 20, 40 or 60 mg/kg b.w. The control group received deionized water only. All animals were sacrificed at the end of the study.

All animals were observed twice daily for mortality and daily for clinical signs. Body weights and food consumption were recorded weekly. On day 0 and after 6 and 13 weeks on 10 male and 10 female rats of the control and high dose group hearing tests, ophtalmological and reflex-examinations were carried out. Haematological and clinical chemical investigations were carried out on day 0 and at week 6, 13 and 17. Urine samples were collected from 5 male and 5 female rats of each group at week 6, 13 and 17. Organ weights (c. 10) were measured. Macroscopy and histopathology (c. 35 organs/tissues) was performed.

1 animal of the 20 mg/kg b.w. group was found dead, due to an intubation error (intratracheal). The animals of all dose groups showed red coloured urines and red stained perigenital haircoat from the start of treatment, which disappeared during the recovery period. High dose females showed an increase of liver weights and protein and potassium level in blood. High dose males showed a reduced sodium/potassium rate in blood. At the end of the recovery period no differences between control and test groups were found.

The dose level without adverse effects was 40 mg/kg b.w.

4. Irritation & corrosivity

4.1. Irritation (skin)

1.
Route: skin
Species: guinea pig
Subst.: sA
Exposure: 4 h
Dose: 0.5 ml
Concentr.: 1 %

0.5 ml of a 1 % suspension in propylene glycol, was occlusively applied to the clipped back of 5 female Pirbright guinea pigs. After 4 hours, the patches were removed and the test substance was rinsed.

Observations for signs of dermal irritation were recorded 1 hour and once daily for 14 days, after patch removal.

No erythema could be recognized, because the skin was coloured red by the test substance. No oedema and necrosis were observed.

2.
Route: skin
Species: rabbit
Subst.: sA
Exposure: 4 h
Dose: 0.5 g
Concentr.: 100 %
Pr.Irr.Index: 0.0
Effect: not irrit.

A cellulose patch, with 0.5 g of the test substance soaked with 1 ml distilled water, was applied to the right, clipped back of 3 female NZW rabbits. After 4 hours, the patches were removed and residual test substance was wiped off using wetted cellulose tissue (rinsing).

Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours after patch removal.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes)

1.
Route: eye
Species: guinea pig
Subst.: sA
Dose: 0.1 ml
Concentr.: 1 %

0.1 ml of the test substance was instilled into the conjunctival sac of the right eye of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls.

The eyes were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after application. At 24 hours, additional examinations were carried out upon the instillation of 0.1 % fluorescein-solution.

Erythema was observed in 3 animals and fluid output in 4 animals, for 4 hours after application. No pathological alterations were found on conjunctivae, iris, cornea and the fundus of the eyes.

2.
Route: eye
Species: rabbit
Subst.: sA
Dose: 0.1 ml
concentr.: 100 %
Pr.Irr.Index: 1.6
Effect: not irrit.

0.1 ml (about 57-62 mg) of the undiluted test substance was instilled into the conjunctival sac of the right eye of 3 female NZW rabbits. The untreated left eyes served as controls.

The eyes were examined 1, 24, 48, and 72 hours after application.

In all animals redness of the conjunctivae was observed, 1, 24 and 72 hours after application, respectively. Minimal oedema of the conjunctivae was noted in 2 animals, 1 and 24 hours after application, respectively. The Draize score was 1.6 (not irritating).

5. Sensitization

Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc: 5/50 %
Conc.chall.: 50 %
Result: negative

20 Albino Dunkin-Hartley guinea pigs were used in this skin sensitization study and 10 guinea pigs were used as irritation controls.

The induction phase consisted of 3 series of 2 intradermal injections (0.1 ml) in the shoulders of the treatment and the control group. The intradermal injections were divided as follows: 2 injections of FCA/distilled water 1:1, 2 injections of test substance (5 %) in FCA. The control group received 2 injections of FCA/distilled water 1:1, 2 injections of FCA and arachis oil and 2 injections of arachis oil.

Day 1-7: examination on local tolerance. Day 8, dermal induction of 0.1 ml (50 %) test substance. The occlusive patch application lasted for 48 hours on the surface corresponding to the injections. Day 12-21: rest period. On day 22, the challenge phase started. 0.1-0.2 ml (50 %) test substance was occlusively applied to the clipped left flank. After 24 hours the patches were removed.

Any sign of erythema and oedema was recorded 24 and 48 hours after the challenge.

Faint pink stains were observed at the test material sites of all test and control animals. This staining did not affect the assessment of the skin responses. No adverse skin reaction was observed at the test material or vehicle control sites of any of the test or control animals. The test substance did not produce dermal sensitization.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 5-15
DWE: 30 mg/kg b.w.

Red Y, dissolved in deionized water, was administered, by gavage, to 4 groups of 24 pregnant rats (BOR:WISR-SPF TNO). The test substance was daily administered at dosage levels of 10, 15 or 30 mg/kg b.w. The control group received the vehicle only. All dams were sacrificed on

day 20 of gestation. The animals were observed daily for clinical signs. Individual body weights were recorded at day 0, 5, 10, 15 and 20. Food consumption was measured for the phases 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the utureus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were examined for skeletal defects and variations of the ossification process by Alizarin Red staining and for organic defects.

No animal died during the study. The haircoat of all females appared smooth and brightly. The urine of the 30 mg/kg b.w. group was coloured red. No irreversible structural changes were found.

The dose level without maternal and foetotoxicity was 30 mg/kg b.w.

Remark: no maternal toxicity was observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C labelled Red Y was applied to the clipped dorsal skin of Sprague Dawley rats (Him: OFA, SPF) for 30 minutes and then washed off. In the 3 studies, 3 rats/sex were used. The test substance was integrated in 2 different hair dyeing formulations* or was used as a solution in water/DMSO 3:1.

Hair dyeing formulation II was mixed with hydrogen peroxide before application. The amount of the test substance applied per animal was 10.0 mg of formulation I (1 %), 5.0 mg of formulation II (0.5 %) and 10.3 mg of the 3.33 % solution of the test substance in DMSO/water 3:1. The content of radioactivity was determined in rinsing water, treated skin areas, urines, faeces, organs and carcass. The formulation or the solution was left for 30 minutes and was then scraped off using a spatula, followed by a rinse-off using about 100 ml of shampoo-solution and water of about 37 °C. Rinsing was continued until the rinsing water and the absorbent tissue, which was used to dab the skin dry, were free of colour.

98.2-99.8 % of the applied ^{14}C-amount was removed from the skin by rinsing 30 minutes after the beginning of the percutaneous treatment. The absorbed amount of ^{14}C-labelled test substance was rapidly excreted via urine (60 %) and via faeces (40 %).

Very small ^{14}C-concentrations that were close to or below the detection limit, were found in the organs after 72 hours (0.00002 % for the large organs and 0.0003 % for small organs, especially the thyroid). Relatively high ^{14}C concentrations were detected in thyroid, liver, adrenals and fat and relatively low concentrations were observed in muscle, brain, gonads and heart.

The treated area of skin, still contained a small ^{14}C-activity of 0.85% of the administered ^{14}C-activity for formulation I, 1.68 % for formulation II and hydrogen peroxide and 0.10 % for the solution of the test substance. The mean percutaneous absorption was 0.037 % of the administered ^{14}C for hair dyeing formulation I, 0.061 % for formulation II and 0.066 % for the solution.

* Composition of the formulations I and II:

	I (%)	II (%)
- ^{14}C labelled red Y	1.00	0.50
- p-toluylendiamine, sulfate	—	1.75
- mixture of resorcinol and m-aminophenol	—	0.68
- mixture of salts	0.70	0.35
- ammonia (25%)	0.36	1.00
- isopropanol	3.90	1.95
- WAS	2.00	1.00
- deionised water	44.44	17.95
- formulation base	47.60	23.80
- ammonia (25%)	—	1.83
- Welloxon (containing 9% H_2O_2)	—	50.00

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sB	*Salm typh*	TA97	frameshift mut	10-3000 µg/pl	+	+	r	AR
* sB	*Salm typh*	TA98	frameshift mut	10-3000 µg/pl	+	+	r	AR
* sB	*Salm typh*	TA100	base-pair subst	10-3000 µg/pl	+	+	r	AR
* sB	*Salm typh*	TA1535	frameshift mut	10-3000 µg/pl	-	-	r	AR
* sB	*Salm typh*	TA1538	base-pair subst	10-3000 µg/pl	+	+	r	AR
* sB	mouse lymph.	L5178Y	HGPRT and NA+/K+ ATPase locus	12.5-200 µg/ml	-	-	r	AR
* sB	Chin. hamster	CHO	chrom. aber	50-500 µg/ml	-	-	r	AR
* sC	*Salm typh*	TA97	frameshift mut	1-6000 µg/pl	+	+	r	AR
* sC	*Salm typh*	TA98	frameshift mut	1-6000 µg/pl	+	+	r	AR
* sC	*Salm typh*	TA100	base-pair subst	1-6000 µg/pl	+	+	r	AR
* sC	*Salm typh*	TA1535	frameshift mut	1-6000 µg/pl	-	-	r	AR
* sC	*Salm typh*	TA1538	base-pair subst	1-6000 µg/pl	-	-	r	AR
* sC	mouse lymph.	L5178Y	HGPRT and Na+/K+ ATPase locus	12.5-200 µg/ml	-	-	r	AR
* sC	Chin. hamster	CHO	chrom.aber	50-500 µg/ml	+	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	mouse	NMRI	micronuclei	200, 760 and 2000 mg/kg b.w.	-
* sA	mouse	NMRI	spot test	250, 830 and 2500 mg/kg b.w.	-

Salmonella assays
5 strains of *Salmonella typhimurium* were exposed to sB, dissolved in dimethylsulfoxid and diluted with water, in the presence and absence of rat liver S9 mix. The dose levels tested were 10-3000 µg/plate. The negative control was DMSO and the positive control substances were amino-anthracene, benzopyrene-oxide and sodium-azid.

sB showed a positive result in the strains TA97, TA98 and TA1538 at 300 µg/pl, in the presence of S9. sB showed also a positive result from 1000 µg/pl in the strains TA97, TA98, TA100 and TA 1538, in the absence and presence of S9. There was no mutagenic effect found in the strain TA1535. The concentration of 3000 µg/pl was toxic to the bacteria.

5 strains of *Salmonella typhimurium* were exposed to sC, dissolved in dimethylsulfoxid and diluted with water, in the presence and absence of rat liver S9 mix. The dose levels tested were 1-3000 µg/plate. The negative control was DMSO and the positive control substances were amino-anthracene, benzopyrene-oxide and sodium-azid.

sC showed a positive result in the strains TA98 and TA100 at 3000 µg/pl, in the absence of S9. sC showed also a positive result at 6000 µg/pl in the strains TA97, TA98 and TA100, in the absence and presence of S9. There was no mutagenic effect found in the strains TA1535 and TA1538.

Mouse lymphoma fluctuation assays
sB and sC were tested for mutagenicity in the mouse lymphoma fluctuation assay for mutations to 6-thioguanine (HGPRT-locus) and ouabain resistance (Na+/K+ ATPase-locus), both in the absence and presence of a rat liver post mitochondrial fraction. Cells were treated with sB and sC, dissolved in DMSO, in duplicate at 12.5, 25, 50, 100 and 200 µg/ml. Benzo(a)pyrene and 4-nitroquinoline-1-oxide were included as positive controls in the presence and absence of S9-mix, respectively. (200 µg/ml is a toxic concentration). sB and sC have no activity at the HGPRT and Na+/K+ ATPase locus of mouse lymphoma cells, neither in the absence nor in the presence of S9-mix.

Cytogenetics Assay
sB and sC were tested in a cytogenetics assay using duplicate cultures of Chinese hamster ovary (CHO) cells, both in the absence and presence of metabolic activation. Cells were treated

with sB and sC, dissolved in DMSO, at 50, 250 and 500 µg/ml. 100 metaphases from each culture were analysed for chromosome aberrations. Ethylmethanesulphonate without S9- mix and cyclophosphamide with S9-mix were used as positive controls (500 µg/ml is toxic concentration). sB did not show any aberrations, neither in the absence nor in the presence of S9-mix.

sC caused an increase in aberrations, at 500 µg/ml, in the absence of S9- mix; sC did not show any aberrations in the presence of S9- mix.

Micronucleus assay

Red Y, dissolved in DMSO, was administered by gavage at single doses of 200, 760 or 2000 mg/kg b.w. to groups of NMRI mice (6/sex). The positive control group was treated with 30 mg/kg b.w. cyclophosphamide. The bone marrow smears were prepared 24 hours after the administration of the test substance and in the highest dose group 48 and 72 hours after the administration. 1000 polychromatic erythrocytes were analysed in each group (5/sex) and the ratio between polychromatic to nonchromatic erythrocytes was calculated. sA did not induce micronuclei in the mouse bone marrow cells. 2000 mg/kg b.w. caused a cytotoxic effect 24 hours after application, after 72 hours the toxic effect was not seen.

Spot test

sA, dissolved in DMSO, was administered orally to groups of 60 gravid female NMRI mice at single doses of 250, 830 or 2500 mg/kg b.w., on day 9 of gestation (crossing of the mouse strains NMRI (females) and DBA/2J (males)). The positive control group was treated with 20 mg/kg b.w. ENU (1-nitroso-1-ethyl urea). Mutations in embryonic pigment cells in particular gene loci for fur colour, which lead to the formation of light spots on the fur of black mice, were recorded and the hairs out of the spot regions were microscopically analysed for a changed pigmentation pattern, at a concentration of 2500 mg/kg b.w.

sA did not cause any mutations in somatic cells (melanoblasts) of the mouse.

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	hamster	syrian	cell transformation	10-1000 µg/ml	-	-	r	AR
* sB	HeLa S3 cells		UDS	0.125-250 µg/ml	-	+	r	AR
* sC	HeLa S3 cells		UDS	0.125-250 µg/ml	-	-	r	AR

Abbreviations:

meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos.; - = neg.; e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	rats	WISW	UDS	100, 300 and 1000 mg/kg b.w.	-
* sA	mouse		SCE	50, 150 and 450 mg/kg b.w.	-

Cell transformation assay

Syrian hamster embryo (SHE) cells were treated with sA, dissolved in DMSO, and assessed for its potential to transform Syrian hamster embryo cells. SHE cells were treated with concentrations of sA of 10, 100, 500 or 1000 µg/ml for 6 hours in the presence of rat liver S9-mix and with concentrations of 10, 50, 250 or 500 µg/ml for 6 and 48 hours in the absence of rat liver S9-mix. Untreated cells and cells treated with DMSO served as negative controls. The positive controls were N-methyl-N-nitro-N-nitrosoguanidine without S9-mix and benzo(a)pyrene with S9-mix. In each experimental group 1000 colonies were scored for the occurrence of transformation. sA showed toxic properties at concentrations from 250 µg/ml without S9-mix and at 1000 µg/ml with S9-mix.

sA did not induce cell transformation in the SHE-culture.

*Unscheduled DNA synthesis **(in vitro)***

sB and sC, dissolved in DMSO, were assayed with and without S9-mix at dose values from 0.125 to 250 µg/ml, in a cell culture medium containing ^{3}H-thymidine at 5 µCi/ml. The positive controls were 3,3-dichlorobenzidine with S9-mix and 4-nitroquinoline-1-oxide without S9-mix. Incorporation of ^{3}H-thymidine per mg DNA was calculated for each of triplicate tested dose values and compared with the negative control.

sB induced UDS in HeLa cells in the presence of S9-mix, at all tested dose levels. sC did not induce UDS in HeLa cells, neither in the presence nor in the absence of S9-mix.

*Unscheduled DNA synthesis **(in vivo)***

sA, dissolved in DMSO, was administered to groups of WISW rats (6/sex), by gavage, at concentrations of 100, 300 or 1000 mg/kg b.w. The positive control was methylmethane-sulfonate. 24 hours after administration the animals were sacrificed and the livers were removed. Liver preparations were incubated with ^{3}H-thymidine, washed, fixed onto slides and stained. 100 cells per animal were microscopically examined and grains/nucleus counted.

The toxic concentration is 1000 mg/kg b.w. sA did not induce UDS *in vivo*.

Sister Chromatid Exchange Assay

2 hours before the animals were treated BrdU (bromodeoxyuridine) was implanted subcutaneously, sA, dissolved in aqua bidest, was administered orally to NMRI/SPF mice (5/sex) at concentrations of 50, 150 or 450 mg/kg b.w. The positive control was treated with 10 mg/kg b.w. cyclophosphamide. 22 hours after administration, the animals were treated with colcemid to arrest bone marrow cells in the metaphase (to stop mitosis). Samples of the bone marrow were taken 24 hours after treatment and examined for SCE´s. Per animal 30 metaphase

cells were evaluated. The mitotic index was significantly reduced in all dose groups, thus indicating an inhibitory effect to the bone marrow cells. sA did not induce SCE´s.

11. Conclusions

A Quality Assurance Declaration was included by all tests, except for the acute oral toxicity test, eye and skin irritation study and the two Salmonella assays.

General
Red Y is included in hair tinting products and colouring setting lotions at a maximum concentration of 1 %.

In oxidative hair dye formulations the maximum concentration included is 2 %. Since the oxidative hair dyes are mixed with hydrogen peroxide before use, the concentration at application is 1 % only.

Acute toxicity
The test substance is moderately to slightly toxic via the oral route (LD_{50} mouse c. 1867 mg/kg b.w.; LD_{50} rat c. 2013 mg/kg b.w.) and slightly toxic after dermal administration (LD50 rabbit >2000 mg/kg b.w.).

Irritation
No signs of irritation were observed in the eye and skin irritation tests in rabbits. A concentration of 1 % in the eye and skin irritation study with guinea pigs showed no signs of irritation.

Sensitization
No signs of sensitization in guinea pigs were observed in the Magnusson Kligman test.

Semichronic toxicity
In a 90-day feeding study, Wistar rats were fed 0, 20, 40 or 60 mg Red Y/kg b.w., by gavage once daily. The animals of all dose groups showed red discoloured urines and red stained perigenital haircoat from the start of treatment, which disappeared during the recovery period. In the 60 mg/kg b.w. group the following effects were observed: an increase of liver weights and protein and potassium level in females and a reduced sodium/potassium rate in males. After the recovery period no differences between control and test groups were found.

The dose level without adverse effects was 40 mg/kg b.w.

Teratogenicity
In a teratogenicity study, Wistar rats were fed 0, 10, 15 or 30 mg Red Y/kg b.w. No animal died during the study. The urine of the high dose group was coloured red. No irreversible structural changes were found.

The dose level without maternal and foetotoxicity was 30 mg/kg b.w.

Genotoxicity
The two main components of Red Y (that is, 1-amino-2-nitro-4-(2´,3´-di-hydroxypropyl)-amino-5-chloro-benzene (sB) and 1,4-bis-(2´,3´-dihydroxypropyl)-amino-2-nitro-5-

chloroben-zene (sC) were tested for their mutagenic potential in *in vitro Salmonella*, Mouse Lymphoma, cytogenetics and UDS assays. Red Y was tested *in vitro* in a cell transformation assay. Red Y was tested *in vivo*, in micronucleus, spot, UDS and SCE assays. In the Salmonella assays sB and sC were genotoxic in the strains TA97, TA98, TA100 and sB also in TA1538, with and without rat liver metabolizing system. In the strain TA1535, both components were found not genotoxic. Both components showed no genotoxic effects in the mouse lymphoma assay, neither in the absence nor in the presence of S9-mix. sB did not cause any increase of chromosome aberrations and sC caused an increase of chromosome aberrations in Chinese hamster ovary cells in the absence of S9-mix. In HeLa cells sB induced unscheduled DNA synthesis in the presence of S9-mix and sC did not induce UDS, neither in the absence nor in the presence of S9-mix. Red Y did not reveal any genotoxic properties in a cell transformation assay with Syrian hamster embryo (SHE) cells, with and without rat liver S9-mix. Red Y was not genotoxic in the 4 *in vivo* assays.

Absorption

^{14}C-labelled Red Y was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in water/DMSO. Most of the substance was recovered by rinsing (98.2-99.8 %). The cutaneous absorption was 0.037 % of the administered ^{14}C for hair dyeing formulation I, 0.061 % for formulation II and 0.066 % for the solution.

Conclusions

Red Y was found moderately to slightly toxic in the acute oral toxicity test and slightly toxic after dermal administration to rabbits. Red Y showed no signs of irritation and sensitization. In the 90-day study with rats, 40 mg/kg b.w. was considered to be the NOAEL. In the teratogenicity study, no irreversible structural changes were observed in the foetuses of the rat, after administration of 30 mg/kg b.w. Red Y has mutagenic potential in *in vitro* assays, but has to be considered as a non-genotoxic *in vivo*.

The cutaneous absorption was 0.037 % of the administered ^{14}C for hair dyeing formulation I, 0.061 % for formulation II and 0.066 % for the solution.

For normal use of hair dye, the following calculation can be made:

1 g Red Y comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1 % Red Y). With a maximal penetration of 0.061 %, this results in a dermal absorption of 0.61 mg per treatment, which is 0.01 mg/kg b.w. (assuming a body weight of 60 kg).

0.35 g nitroblau comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 1 % Red Y). With a penetration of 0.037 %, this results in a dermal absorption of 0.129 mg per treatment, which is 0.0022 mg/kg b.w.

So a margin of safety of 3934 can be calculated between the figure for human exposure to oxidative hair dye and the no adverse level found in rats in the 90-day study. For the semi-permanent hair dye a safety margin of 18532 can be calculated.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week.

Classification: A.

B 73: *NITROBLAU*

1. General

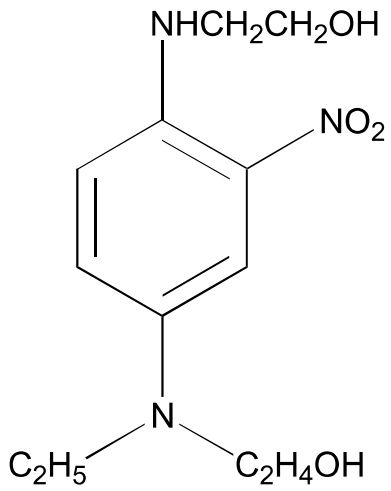
1.1 Primary name

Nitroblau.

1.2 Chemical names

1-(beta-hydroxyethyl)-amino-2-nitro-4´N-ethyl-N-(beta-hydroxyethyl)-amino-benzene.
1,4-di(beta-hydroxyethyl)-amino-4-N-ethyl-2-nitro-benzene.
4-N-ethyl,N-(beta-hydroxyethyl)-amino-1-(beta-hydroxyethyl)-amino-2-nitro-benzene.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}H_{19}N_3O_4$
Mol weight: 305.5 (HCL)

1.7 Purity, composition and substance codes

sA: 1-(beta-hydroxyethyl)-amino-2-nitro-4´ N-ethyl-N-(beta-hydroxyethyl)-amino-benzene hydrochloride (purity: 99 %)
sB: 1-(beta-hydroxyethyl)-amino-2-nitro-4´N-ethyl-N-(beta-hydroxyethyl)-amino-benzene (mol. weight 269)

1.8 Physical properties

Subst. code: sA
Appearance: dark violet, fine grained powder (free base)
Melting point: 62 °C.

1.9 Solubility

The substance exists as a free base and as a hydrochloride (commercial product; appear.: beige crystalline powder).

The substance is fully soluble in dimethylsulphoxide and ethanol and soluble in water.

2. Function and uses

1-(beta-hydroxyethyl)-amino-2-nitro-4,N-ethyl-N-(beta-hydroxyethyl)-amino-benzene is included in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 1.5 %.

In permanent hair dyes the maximum concentration is 1.5 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 0.75 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	mouse (f)	1775 mg/kg b.w.
sA	oral	mouse (m)	1770 mg/kg b.w.
sA	oral	rat (f)	1660 mg/kg b.w.

A 10 % solution of 1-(beta-hydroxyethyl)-amino-2-nitro-4′ N-ethyl-N-(beta-hydroxy-ethyl)-amino-benzene hydrochloride in distilled water, was given once by stomach tube to CF1 mice (10/sex) and Wistar rats (6 females) at several concentrations.

During an observation period of 14 days, the mortalities and clinico-toxicological findings were recorded daily and the body weights were noted weekly. At the end of the observation period all surviving animals were sacrificed and the organs of all animals were examined.

The test substance caused a limitation of the animal´s activity, abdominal position and a blue colouration of extremities, till 24 hours after administration.

The test substance is moderately toxic.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	derm	rabbit	>2000 mg/kg b.w.

The moistened test substance was once, dermally administered to the shaven back of NZW rabbits (5/sex). The applied dose was 2000 mg/kg b.w.

During an observation period of 14 days, clinico-toxicological and skin alteration observations were recorded daily. Body weights were recorded at day 0 and 14. A post mortem examination was carried out on all animals. No mortalities were observed.

The test substance is slightly toxic.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 90 d

Nitroblau, dissolved in water, was administered by gavage once daily to one group albino Wistar rats (12/sex) at a concentration of 30 mg/kg b.w. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded weekly. On day 0 and day 90 opthalmoscopic examination was carried out. Haematological and clinical chemical investigations were carried out on day 0 and at week 6 and 13. Urine samples were collected at the beginning and at week 6 and 13. Organ weights (c. 10) were measured and macroscopy and histopathology (c. 30 organs/tissues) was performed, on 10 animals/sex.

No animal died during the study. The urines of the treated animals were coloured violet. No other effects were observed. The dose level without adverse effects was 30 mg/kg b.w.

Remark: only one concentration was tested, instead of three, based on the following criteria: maximum concentration in product, maximum resorption and safety factor.

4. Irritation & corrosivity

4.1 Irritation (skin)

1.
Route: skin
Species: guinea pig
Subst.: sA
Exposure: 4 hr
Dose: 0.5 ml
Concentr.: 3 %

0.5 ml of a 3 % test solution was occlusively applied to the clipped back of 5 female Pirbright white guinea pigs. After 4 hours, the patches were removed and the test substance was rinsed.

Observations for signs of dermal irritation were recorded 1 hour and once daily for 14 days, after patch removal.

It was impossible to recognize erythemas because the skin was coloured blue, by the test substance. No oedema and necrosis were observed.

2.
Route: skin
Species: rabbit
Subst.: sA
Exposure: 4 hr
Dose: 0.5 g
Concentr.: 100 %
Pr.Irr.Ind: 0.0
Effect: not irrit.

A cellulose patch, with 0.5 g of the test substance soaked with 1 ml distilled water, was applied to the clipped back of 3 female NZW rabbits. After 4 hours, the patches were removed and residues were then wiped off using wetted cellulose tissue.

Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours after patch removal.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes)

1.
Route: eye
Species: guinea pig
Subst.: sA
Dose: 0.1 ml
Concentr.: 2 %

0.1 ml of the test substance was instilled into the conjunctival sac of the right eye of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls.

The eyes were examined 0.5, 1, 2, 3, 4, 6, 7, 24, 48 and 72 hours after application. At 48 and 72 hours, additional applications were carried out upon the instillation of one drop of 0.1 % fluorescein-solution.

Redness was observed in 1 animal and fluit output in 2 animals. These effects were disappeared 3 hours after application.

2.
Route: eye
Species: rabbit
Subst.: sA
Dose: 0.1 ml
Concentr.: 100 %
Pr.Irr.Index: 0.9
Effect: not irrit.

0.1 ml of the undiluted test substance was instilled into the conjunctival sac of the right eye of 3 female NZW rabbits. The untreated left eyes served as controls.

The eyes were examined 1, 24, 48 and 72 hours after application.

Minimal redness was observed in 2 animals for 48 hours and minimal oedema of the conjunctivae in 2 animals 1 hour after application. The Draize score was 0.9 (not irritating).

5. Sensitization

Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 3 %
Conc.chall.: 3, 2, 1 %

20 Pirbright white guinea pigs (HOE: DHPK (SPF-LAC.)) were used in this skin sensitization study and 10 guinea pigs were used as irritation controls. The injection phase consisted of 3 series of 2 intradermal injections (0.05 ml) in the shoulders of the treatment and control group. After the first 2 intradermal injections, a dermal treatment with 10 % sodium laurylsulfate took place. 48 hours after the first two intradermal applications, the bandages were removed and the third intradermal injection followed (3 inj. of 3 % test substance in FCA diluted in oleum arachidis, 3 inj. DNCB and 3 inj. distilled water).

The animals were challenged by closed patch test, 14 days after the last exposure using 3 different concentrations (0.5 ml) per animal. After 24 hours the patches were removed.

Any sign of erythema and oedema was recorded 24 and 48 hours after the challenge.

No adverse skin reactions were observed.

Remark: This test is inadequate, because the concentrations used are too low.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 5-15
DWE: 140 mg/kg b.w.

Nitroblau, suspended in deionized water, was administered, by gavage, to 4 groups of 24 pregnant rats (BOR:WISW-SPF TNO). The test substance was daily administered at dosage levels of 15, 60 and 140 mg/kg b.w. The control group received the vehicle only. All dams were sacrificed at day 20 of gestation. The animals were observed daily for clinical signs. Individual body weights were recorded at day 0, 5, 10, 15 and 20. Food consumption was measured for the phases 0-5, 5-15, 15-20 and the entire period (0-20). Immediately following sacrifice, the uterus was removed, weighed and the number of (non)-viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs, of the dams, was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. The foetuses were examined

for skeletal defects and variations of the ossification process by Alizarin Red staining and for organic defects.

No animal died during the study. The urines of the treated animals were coloured violet, during the treatment. The high dose group animals showed in addition, a violet staining of the fur and tails. No irreversible structural changes were found.

The dose level without maternal and foetotoxicity was 140 mg/kg b.w.

Remark: no maternal toxicity was observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C labelled nitroblau was applied to the clipped skin of pigmented Long-Evans rats, either as a solution in DMSO (reference dose) or as a hair dye formulation*.

The test substance (1.5 %) was applied, to 3 female and 3 male rats, as a part of a hair dye formulation for a contact period of 30 minutes. A 10 % solution of the test substance in DMSO, applied to 3 males and 3 females for a contact period of 24 hours, served as a reference dose. The amount of test substance applied per animal was 15 mg.

The content of radioactivity was determined in rinsing water, treated skin areas, urines, faeces, organs and carcass.

The reference and formulation dose was left for 24 and 0.5 hours, respectively, and was then washed off with hair shampoo and warm water. Then the contact area was dried with cotton wools swabs.

95.85 - 96.52 % of the applied ^{14}C-amount was removed from the skin by rinsing 30 minutes after the beginning of the percutaneous treatment.

In the urines of rats 0.4 % (m) and 0.8 % (f) of the dose applied was excreted during 72 hours. In the faeces of rats 0.2 % (m) and 0.3 % (f) of the dose applied was excreted during 72 hours.

Very small ^{14}C-concentrations that were close to or below the detection limit, were found in the organs after 72 hours (0.00002 % for the large organs and 0.00003 % for the small organs, especially the thyroid). A relatively high ^{14}C-concentration was detected in the thyroid.

The treated area of skin, still contained a small ^{14}C-activity of 2.36 % (m) and 2.92 % (f) of the administered ^{14}C-activity for the formulation. The mean percutaneous absorption was about 1 % of the administered ^{14}C for the hair dyeing formulation.

*Composition of the formulation:

- ^{14}C labelled nitroblau 1.5 %
- 0-masse 46.3 %
- Texapon N25 2.9 %
- deionised water 49.3 %

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sB	*Salm.typh.*	TA97	frameshift mut	10-6000 µg/pl	-	-	r	AR
* sB	*Salm.typh.*	TA98	frameshift mut	10-6000 µg/pl	-	-	r	AR
* sB	*Salm.typh.*	TA100	base-pair subst	10-6000 µg/pl	-	-	r	AR
* sB	*Sacch cer*	D7	gene conv.	0.4, 2 and 10 mg/ml	-	-	r	AR
* sB	mouse lymp	L5178Y	TK+/- locus	10-2000 µg/ml	-	-	r	AR
* sB	hum. lymph.		chrom.aber	3, 15 and 75 µg/ml	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	mouse	NMRI	micronuclei	1000 mg/kg b.w.	-

Salmonella assay
3 strains of *Salmonella typhimurium* were exposed to sB, dissolved in DMSO and diluted with water, in the presence and absence of rat liver S9-mix. The dose levels tested were 10-6000 µg/plate. The negative control was DMSO and the positive control substances were sodiumacide, nitrofluorene, aminoanthracene and 4-nitro-o-phenylene-diamine.

There was no mutagenic effect found in the 3 strains, neither in the absence nor in the presence of S9-mix.

Remark: At least 4 strains are prescribed in the OECD guidelines.

Saccharomyces cerevisiae assay
Cell cultures of the yeast were exposed to sB, dissolved in DMSO, at concentrations of 0.4, 2 and 10 mg/ml, in the presence and absence of S9-mix. The negative control was DMSO and the positive control substances were ethylmethanesulphonate and cyclophosphamide.

There was no mutagenic effect found, neither in the absence nor in the presence of S9-mix.

Mouse lymphoma fluctuation assay
sB, dissolved in DMSO, was tested for genotoxicity in the mouse lymphoma fluctuation assay for mutations at the TK+/- locus, both in the absence and presence of S9-mix. Cells were treated with 10, 100, 200 and 500 µg/ml without S9-mix (24 hours exposure) and with 500,

750, 850, 1000, 1500 and 2000 µg/ml with S9-mix (4 hours exposure) (1500 and 2000 µg/ml were toxic concentrations). The negative control was DMSO and the positive control substances were ethylmethanesulphonate without S9-mix and dimethylbenzantracene with S9-mix.

Nitroblau induced no significant increases in mutation frequency at the TK+/- locus, neither in the absence nor in the presence of S9-mix.

Cytogenetics assay
Nitroblau, dissolved in DMSO, was tested in a cytogenetics assay using human lymphocytes, in the presence and absence of S9-mix. Cells were treated with 3, 15 and 75 µg/ml for 24 hours. 75 metaphases per culture were analysed for chromosome aberrations. The negative control was DMSO and the positive control was cyclophosphamide with S9-mix.

Nitroblau did not induce chromosomal aberrations, neither in the presence nor in the absence of S9-mix.

Micronucleus assay
Nitroblau, dissolved in 2 % carboxymethylcellulose, was administered orally in a single dose of 1000 mg/kg to 3 groups of NMRI mice (5/sex). The negative control group received 2 % carboxymethylcellulose and the positive control group received 40 mg cyclophosphamide/kg b.w. Bone marrow samples, of the 3 test groups, were taken 24, 48 and 72 hours after treatment, respectively. 1000 polychromatic erythrocytes were analysed in each group and the ratio of polychromatic to normochromatic erythrocytes was estimated.

Nitroblau did not induce micronuclei in bone marrow cells of the mouse.

Nitroblau had a cytotoxic effect 24 hours after administration, after 72 hours a toxic effect was not seen.

Remark: Although only one dose level was used, the study can be evaluated because there is clear indication that the substance reached the bone marrow, as shown by the toxic effect in the cells.

Sub	Species	Measure endpoint	Test conditions	Res.
* sA	Chin hamster	SCE	100, 300 and 900 mg/kg b.w.	-
* sA	rat	SCE	30, 100, 300, 600 and 900 mg/kg b.w.	-

Sister Chromatid Exchange Assays
2 hours before the animals were treated, BrdU (bromodeoxyuridine) was implanted subcutaneously. Nitroblau, dissolved in DMSO, was administered orally to Chinese hamsters (3-4 males/group) at concentrations of 100, 300 and 900 mg/kg b.w. and to Sprague Dawley rats (2-4 males/group) at concentrations of 30, 100, 300, 600 and 900 mg/kg b.w. The negative control was DMSO and the positive control substances were acetaminofluorene and aminoanthracene. Samples of the bone marrow were taken 24 hours after treatment and examined for SCE´s. Per animal 25 metaphase cells were evaluated.

Nitroblau did not induce any Sister Chromatid Exchanges in hamsters and rats.

11. Conclusions

A Quality Assurance Declaration was included by all tests, except for the acute oral toxicity test, one eye and one skin irritation study and the Salmonella assay.

General
Nitroblau is included in semi-permanent hair dyes and colour setting lotions at a maximum concentration of 1.5 %.

In permanent hair dyes the maximum concentration is 1.5 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 0.75 % only.

Acute toxicity
The test substance is moderately toxic via the oral route (LD_{50} mouse c. 1770 mg/kg b.w.; LD_{50} rat 1660 mg/kg b.w.) and slightly toxic via dermal exposure (LD_{50} rabbit >2000 mg/kg b.w.).

Irritation
No signs of irritation were observed in the eye and skin irritation tests in rabbits. A concentration of 2 % in the eye and 3 % in the skin irritation study with guinea pigs, showed no signs of irritation.

Sensitization
The concentrations in the sensitization test are too low. The test cannot be evaluated.

Semichronic toxicity
In a 90-day feeding study, Wistar rats were fed 30 mg nitroblau/kg b.w., by gavage once daily. All animals showed violet coloured urine. No adverse effects were seen at 30 mg/kg b.w., the only dose level tested.

Teratogenicity
In a teratogenicity study, Wistar rats were fed 0, 15, 60 and 140 mg nitroblau/kg b.w. No animal died during the study. The urine of all animals was coloured violet and in addition the fur and tails of the high dose animals were stained violet. No irreversible structural changes were found. The dose level without maternal and foetotoxicity was 140 mg/kg b.w.

Genotoxicity
Nitroblau was tested for its mutagenic potential in *in vitro Salmonella*, *Saccharomyces cerevisiae*, Mouse lymphoma, Cytogenetics and *in vivo* in the Sister Chromatid Exchange and micronucleus assays.

Nitroblau was negative in all tests, therefore nitroblau is considered to be not genotoxic.

Absorption
^{14}C labelled nitroblau was applied to the skin of rats in a hair dye formulation and as a 10 % solution in DMSO. Most of the substance was recovered by rinsing (95.9-96.5 %). The cutaneous absorption was 1 % of the administered ^{14}C for the hair dyeing formulation.

Conclusions
Nitroblau was found moderately toxic in the acute oral toxicity test and slightly toxic after dermal administration to rabbits.

Nitroblau showed no signs of irritation. The sensitization test was carried out inadequately. In the 90-day study with rats, 30 mg/kg b.w. was considered to be the NOAEL.

In the teratogenicity study, no irreversible structural changes were observed in the foetuses of the rat, after administration of 140 mg/kg b.w.

Nitroblau is not genotoxic. The cutaneous absorption was 1 % of the administered ^{14}C for the hair dyeing formulation.

For normal use of hair dye, the following calculation can be made:

750 mg nitroblau comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing 0.75 % nitroblau). With a maximal penetration of 1 %, this results in a dermal absorption of 7.5 mg per treatment, which is 0.125 mg/kg b.w. (assuming a body weight of 60 kg).

0.53 g nitroblau comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 1.5 % nitroblau). With a penetration of 1 %, this results in a dermal absorption of 5.25 mg per treatment, which is 0.0875 mg/kg b.w.

So a margin of safety of 240 can be calculated between the figure for human exposure to oxidative hair dye and the no adverse effect level found in rats in the 90-day study (limited study, only 1 dose tested). It should be noted that no effects were observed in the teratogenicity study at 140 mg/kg b.w. For the semi-permanent hair dye a safety margin of 343 can be calculated. It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week.

Classification: B.

Industry should provide data on skin sensitization potential from in-use data in the context of the volume used, together with any available information on the toxicological profile of the compound, e. g. from animal studies and/or, from experience in use in either the consumer or occupational context.

B 75: *METHYLGELB*

1. General

1.1 Primary name

Methylgelb

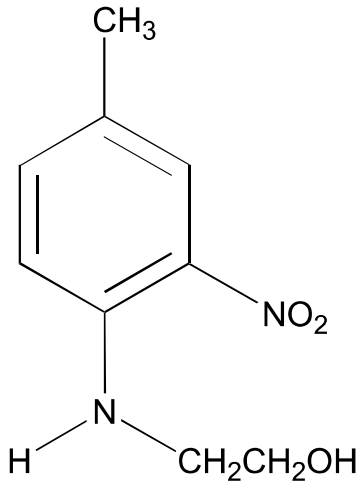
1.2 Chemical names

1-methyl-3-nitro-4-(2′-hydroxyethyl)-amino-benzene
4-(2′-hydroxyethyl)-amino-3-nitro-toluene
1-(2′-hydroxyethyl)-amino-4-methyl-nitrobenzene
4-methyl-2-nitro-(2′-hydroxyethyl)-aniline
2-((4-methyl-2-nitrophenyl)amino)-ethanol

1.4 CAS no.

100478-33-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{12}N_2O_3$
Mol weight:196

1.7 Purity, composition and substance codes

sA: Methylgelb (GHS 191184); purity ≥98 %.

Formulations in which substance is used.

Code	Formulation	Quantity
fA	methylgelb	1.0 %
fB	methylgelb + Welloxon (9 % H_2O_2)	0.5 %
fC	hairdye formulation 7606	0.3 %

Composition of formulations

fA:	^{14}C-sA	1 %
	mixture of salts	0.7 %
	ammonia, 25 %	0.36 %
	isopropanol	3.9 %
	WAS	2.0 %
	water, deionised	44.44 %
	formulation base	47.60 %
fB:	^{14}C-sA	0.5 %
	p-toluylendiamine, sulfate	1.75 %
	mixture of resorcinol and m-aminophenol	0.68 %
	mixture of salts	0.35 %
	ammonia, 25 %	2.83 %
	isopropanol	1.95 %
	WAS	1.0 %
	water, deionised	17.15 %
	formulation base	23.80 %
	Welloxon (cont. 9 % H_2O_2)	50 %
fC:	sA	0.3 %
	sodium laureth sulfate (40 % active)	23 %
	cocamide diethanolamine	5 %
	glycol distearate	0.5 %
	methyl, ethyl, propyl, butyl parabens	0.1 %
	1,2´-hydroxyethyloxy-3-nitro-4-aminobenzene	0.3 %
	N-methyl-N-hydroxyethylamino-3-nitro-methylamine	0.3 %
	HC-blue 1	0.3 %
	N-methyl-3-nitro-p-phenylenediamine	0.3 %
	water	to 100 %

1.8 Physical properties

Subst. code: sA
Appearance: orange red crystalls
Melting point: 74°C
Boiling point: >259°C

Density:1.319 Temp.:20°C
Vapour Press.:2.8E-04 Pa. temp.: 20°C
2.9E-03 Pa. temp.: 50°C
4.23E-03 Pa. temp.: 60°C
Surf.tens.: 0.0533 N/m. temp.: 20°C
Log P_{ow}: 2.1
The substance decomposes at 259 °C.

1.9 Solubility

Sol.water: 351 mg/l temp.: 20°C.
Sol.fats: 24800 mg/kg temp.: 37°C.

sA is soluble in dimethylsulphoxide, acetone and ethanol; and slightly soluble in water.

2. Function and uses

sA exists as a free base. It is included in hair tinting products and colouring setting lotions at a maximum concentration of 1 %. In oxidative hair dye formulations the maximum concentration included is 2 %. Since the oxidative hair dyes mixed with hydrogen peroxide before use, the concentration at application is 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Exp. hr.
sA	oral	rat (f)	1436 mg/kg b.w.	
sA	oral	rat (m)	1564 mg/kg b.w.	
sA	oral	mouse (f)	1750 mg/kg b.w.	
sA	oral	mouse (m)	1600 mg/kg b.w.	

A 10 % suspension of sA in gum Arabic was administered to rats and mice via stomach tube. Groups of 6 male and 6 female Wistar rats received a single dose of 900, 1700 or 2500 mg/kg. Groups of 10 male and 10 female CF1 mice received a single dose of 1000, 1500, 2000 or 2500 mg/kg. The animals were observed for 14 days (clinical signs and body weight), followed by a post mortem examination. sA caused a limitation of activity and orange colouration of the urine and extremeties. Both effects had disappeared 24 h after application.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	Exp. hr.
sA	derm	rat	>2000 mg/kg b.w.	24

The study was performed according to OECD guideline 402. 2000 mg/kg b.w. sA in water was administered on a patch to the clipped skin of 5 male and 5 female Sprague Dawley rats. The test area was covered with tape. After 24 h exposure the test substance was wiped off. Clinical observations were made for 14 days, followed by a post mortem examination in all animals. sA caused signs of general malaise (chromodacryorrhoea and ruffled fur) in 6 animals during the first 2 days. Body weight gain was decreased in female rats.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 3 m
DWE: 45 mg/kg b.w.
LED: 90 mg/kg b.w.

Method: sA, dissolved in water, was administered by oral gavage to four groups of 10 male and 10 female Sprague Dawley rats once daily for 3 months. Test doses were 0, 10, 45 and 90 mg/kg b.w., respectively.

Observations: Mortality, clinical condition and behaviour (daily), ophtalmoscopic changes (week 0 and 13), body weight (weekly), food and water consumption (weekly), haematology (all animals of the 0 and 90 mg/kg b.w. groups in week 0 and 13: MCH, PCV, MCHC, MCV, Hb, WBC, PT), clinical chemistry (0 and 90 mg/kg b.w. groups; week 0 and 13: GOT, GPT, AP, urea, glu, Na+, K+, total prot, alb, alb/glob ratio, creat, chol, TG), urinalysis (week 0 and 13: pH, volume, spec. gravity, prot, blood, glu, ketones, urobil, bil, reducing subst., sed, colour), organ weights (10 organs), macroscopic changes and histopathology (36 organs).

Results: No toxic signs or mortality occurred. Due to renal excretion of sA, bedding of all dosed groups were dose-relatedly orange-yellow-coloured. There was a slight reduction of both overall body weight gain and food consumption in males of the 10 and 90 mg/kg b.w. groups. Haematological chemistry data did not show dose-related changes. One female of the 90 mg/kg b.w. group showed hyaline casts during urine investigation at week 13. Terminal investigations: Absolute and relative liver weights of the 90 mg/kg b.w. group were slightly decreased when compared to the controls. Macroscopic observations of the kidneys showed a loamy colour, dilatation of renal pelvis and deposits in the renal pelvis in the 90 mg/kg b.w. group. The incidence of lobular structure of the liver was increased in the control and the lower dose groups, but occurred less frequent in the 90 mg/kg b.w. group. No sA related microscopic changes were observed in any organ or tissue.

4. Irritation & corrosivity

4.1 Irritation (skin)

(1.)
Route: skin
Species: guinea pig
Subst.: sA

Exposure: 5 d
Dose: 0.5-1 ml
Concentr.: 5 %

Method: 0.5 to 1 ml of a 5 % solution of sA in distilled water containing 0.5 % tylose was tested on 15 Pirbright white guinea pigs. The test substance was applied on patches to the clipped dorsal skin. This treatment was carried out once daily for 5 consecutive days. 5 h after each exposure, skin reactions were assessed according to Draize. Two days after the last application, a final observation of the skin was made.

Results: No changes in behaviour were found. Due to the colouring of the skin areas, erythema could not be recognized. However, based on the absence of oedemas or necrosis sA could be classified as not irritating for the skin of guinea pigs.

(2.)
Route: skin
Species: rabbit
Subst.: sA
Exposure: 4 h
Dose: 0.5 g
Concentr.: 100 %
Pr.Irr.Index: 0
Effect: not irrit.

Method: The study was performed according to OECD guideline 404. 0.5 g of sA was applied on a patch soaked with 1 ml aqua dest. to the clipped dorsal skin of 3 female New Zealand white rabbits. The area was occlusively covered with tape and a dressing. After 4 h the residual test substance was wiped off. At 1, 24, 48 and 72 hours after patch removal, dermal irritation was scored and other local and systemic signs were examined.

Results: No general toxic effects were noted. sA had no irritant or corrosive effect on the intact rabbit skin at any time.

4.2 Irritation (mucous membranes)

(1.)
Route: eye
Species: guinea pig
Subst.: sA
Exposure: 24 h
Dose: 0.1 ml
Concentr.: 1.5 %

Method: 0.1 ml of a 1.5 % solution of sA in aqua dest. was instilled into the right eye of five female Pirbright white guinea pigs. The left eye remained untreated and served as a control. Eye irritation was scored according to the scale of Draize, at 0.5, 1, 2, 3, 4, 6 and 7 h after application. Further readings, at 24 h and once each following day, were carried out after the instillation of one drop of a 0.1 % fluorescein-sodium solution.

Results: None of the animals showed any reaction to the treatment.

(2.)
Route: eye
Species: rabbit
Subst.: sA
Exposure: 24 h
Dose: 0.1 ml
Concentr.: 100 %
Pr.Irr.Index: 0.2
Effect: not irrit.

Method: The study was performed according to OECD guideline 405. 0.1 ml of undiluted sA, containing 25 to 40 mg SA, was applied to the right eye of three female New Zealand white rabbits. The left eye remained untreated and served as a control. Eye reactions were read at 1, 24, 48 and 72 h after application. Eye irritation and corrosion were scored.

Results: Minimal oedema of the conjunctivae was observed in one rabbit 1 h p.a. and minimal redness of the conjunctivae in another animal at 24 p.a. No further irritant effects could be noted at any reading time.

5. Sensitization

(1.)
Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 0.25 %
Conc.chall.: 0.25 %
Result: negative

Method: The study was performed according to OECD guideline 406. Ten female and ten male Pirbright guinea pigs were induced by three intradermal injections of 0.05 ml of a 25 % sA solution on both the right and left clipped shoulder region. The three injected solutions were 0.25 % sA in distilled water, 0.25 % sA in Freund´s complete adjuvant and Freund´s complete adjuvant plus distilled water (1:1), respectively. After one week 0.5 ml of a 0.25 % sA solution in distilled water was applied on a closed patch to the shoulder regions for 48 h. A second group of ten female and ten male guinea pigs served as a control.

Two weeks after the last induction the animals were challenged on the left flank with 0.5 ml of a 0.25 % sA solution in distilled water on a patch. The right shoulder served as a control. After 24 and 48 h skin reactions were read.

Results: Slight erythema was observed in two animals of the test group at 24 h post application and in one animal of the control group at 24 and 48 h post application. According to the method of Magnusson and Kligman, sA can be classified as not sensitizing to the skin of guinea pigs.

Remark: Both the induction and the challenge concentration (0.25 % sA) were based on a range finding test. At a concentration of 0.5 % sA slight erythema was observed, while at 0.25 % no primary skin irritation occurred.

(2.)
Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 5, 50 %
Conc.chall.: 50 %
Result: positive

Remark: only summarized data were available.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 6-15
DWE: 60 mg/kg b.w.

Method: Doses sA (dissolved in 0.5 % CMC in water) of 0, 10, 30 or 60 mg/kg b.w. were administered by oral gavage to four groups of 24 pregnant Sprague Dawley rats. On day 20 of gestation the animals were sacrificed.

Observations: Until day 20 the dams were clinically observed and body weights and food consumption were measured. On day 20 complete autopsy and macroscopic examination of the organs were carried out. Ovaries and uteri were examined: fetal sex ratio, fetal body weights, number and position of implantations (live fetuses, early and late intra-uterine deaths) and the number of corpora lutea were determined. Fetuses were examined for external, skeletal and visceral deviations.

Results: Maternal body weight gain and food consumption of the lowest dose group were slightly increased when compared to all other groups. Reproduction data showed no significant or dose related differences between the groups. Fetal examination: in the 60 mg/kg b.w. group significantly more foetuses showed a dilatation of the oesophagus. This has no functional relevance. No further treatment related effects on the fetuses were observed. Malformation frequencies were highest in the control group. Up to 60 mg/kg b.w. sA did cause no maternal toxicity, no embryotoxic effects and no structural irreversible effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

Method: ^{14}C labelled sA, included in two different hair dye formulations (fA and fB) or dissolved in DMSO/water (5/4) at a concentration of 3.33 %, was applied to the clipped dorsal skin of three male and three female Sprague Dawley rats. After 30 min. the substance was washed off with shampoo, water and absorbent cellulose tissues. Rinsing was continued until the rinsing water and tissues were free of colour. The skin was covered with gauze for 72 h,

after which the animals were killed. Radioactivity of rinsings, treated skin, urine, faeces, organs (13) and carcass was estimated by liquid scintillation counting.

Results: The majority of the applied ^{14}C (97.8 % to 99.7 %) was removed from the skin by rinsing after the cutaneous treatment. The mean ^{14}C content of the skin at the application site was 0.29 % (fA), 0.55 % (fB) and 0.18 % (sA solution) of the applied ^{14}C. The mean percutaneous absorptions were 0.21 % for fA and 0.24 % for fB. The absorption of sA in DMSO/water was significantly higher: 0.69 % of the applied ^{14}C. Excretion: After cutaneous application means of 0.21 % (fA), 0.23 % (fB) and 0.70 % (sA in DMSO/water) of the applied ^{14}C were recovered in urine and faeces within 72 h. Of the absorbed amount of sA, 80 % to 85 % was excreted in urine and 14 % to 19 % in faeces. 85 to 93 % of the totally absorbed amount was excreted in the first 24 h after application. Carcass: The remaining mean amounts of ^{14}C in the carcass 72 h after application were near the detection limit and varied from 0.0025 % to 0.0042 % of the administered dose. Organs: 72 h after application mean concentrations of ^{14}C were near or below the detection limits in all organs. The detection limits were about 0.00002 %/g for large organs, 0.0002 %/g for small organs. Relatively highest concentrations were noted in fat (fB), thyroid (fA), liver (fA, sA solution), skin (sA solution), spleen (fB) and kidney (sA solution). No accumulation of ^{14}C was observed.

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	*Salm.typh.*	TA98	frameshift mut	4-3000 µg/plate solvent DMSO	-	-	r	
* sA	*Salm.typh.*	TA100	basepair subst.	4-3000 µg/plate solvent DMSO; toxic at 2500;	-	-	r	
* sA	*Salm.typh.*	TA1535	basepair subst.	4-3000 µg/plate solvent DMSO	-	-	r	
* sA	*Salm.typh.*	TA1537	frameshift mut	4-3000 µg/plate solvent DMSO	-	-	r	
* sA	*Salm.typh.*	TA1538	frameshift mut	4-3000 µg/plate solvent DMSO	-	-	r	
* sA	mouse lymp cel L5178Y		mutation HGPRT-loc.	50-800 µg/ml DMSO; toxic at 800	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Indicator tests
-Bact., Non mammalian eukaryotic, *In vitro* mammalian.

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	HeLa S3 cells		UDS	0.0064 to 500 µg/ml	-	-		

-*In vivo* mammalian, Host mediated.

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	Chin. hamster		SCE in bone marrow	100, 333 and 1000 mg/kg b.w. in DMSO	-

9. Carcinogenicity

Skin painting carcinogenicity study

Method: A formulation containing 0.3 % sA was applied to the clipped interscapular skin of 60 male and 60 female Swiss mice: 0.05 ml per animal, three times weekly for 20 months. Mortalities and clinical signs were observed daily, body weights were determined weekly. After 9 months of treatment clinical tests, haematology, urinalysis and necropsy were carried out in 10 males and 10 females. At the end of the study all animals were killed. Organs (32), tumors and other lesions were examined histopathologically.

Results: In treated animals chronic inflammation of the skin occurred significantly more often when compared to the control group. The tumors seen were those commonly occurring in swiss mice. No significant increase in tumor incidence was noticed in the treated groups. It was concluded that in this skin painting procedure sA had no carcinogenic effect on mice.

Remark: Only a summary of the results was available.

11. Conclusions

General
Methylgelb is included in hair tinting products and colouring setting lotions at a maximum concentration of 1 %. In oxidative hair dyes the concentration included is up to 2 %. The final concentration at application when mixed with peroxide will be 1 %.

Acute toxicity
The substance is moderately toxic after oral administration, based on acute oral toxicity tests with LD_{50} values of 1436 (rat) and 1600 mg/kg b.w. (mouse). It was slightly toxic after dermal administration (LD_{50} > 2000 mg/kg b.w. in rat).

Irritation
100 % methylgelb was not irritating for the eyes and skin of rabbits. Eye and skin irritation were also determined in guinea pigs, according to the Draize scoring system. A 1.5 %

suspension of methylgelb in distilled water was not irritating for the eyes of guinea pigs, and a 5 % suspension was not irritating for skin.

Sensitization
Methylgelb administered both intradermally and epicutaneously at a concentration of 0.25 % in the maximization test of Magnusson and Kligman, was not sensitizing to the skin of guinea pigs.

A second maximization test with guinea pigs with concentrations of 5-50 % methylgelb was positive (based on summarized data only).

Semichronic toxicity
During 3 months methylgelb was administered by oral gavage to Sprague Dawley rats in doses of 0, 10, 45 or 90 mg/kg b.w. No toxic signs or mortality occurred. One female of the 90 mg/kg b.w. group showed hyaline casts in the urine at week 13. In the 90 mg/kg b.w. group absolute and relative liver weights were slightly decreased when compared to the control group. In the same group macroscopic observation of the renal pelvis revealed a loamy colour, dilatation and deposits. 45 mg/kg b.w. is considered to be a no-observed-adverse-effect-level.

Teratogenicity
A teratogenicity study in rats showed that methylgelb in concentrations up to 60 mg/kg b.w. does not cause maternal, embryotoxic or structural irreversible effects.

Genotoxicity
Methylgelb did not cause mutagenic effects in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538. It also did not induce mutations at the HGPRT locus of mouse lymphoma cells. An *in vivo* SCE test with Chinese hamster bone marrow cells and an *in vitro* UDS test in HeLa cells were both negative.

Absorption
In an *in vivo* skin absorption test in Sprague Dawley rats mean percutaneous absorptions were determined of 0.69 % for sA dissolved in DMSO/water, 0.21 % for fA and 0.24 % for fB (formulation mixed with 9 % hydrogen peroxide 1:1).

Conclusion
Based on the irritation tests in rabbits, methylgelb was considered to be not irritating for both eyes and skin. Tested according to the method of Magnusson Kligman, methylgelb can be classified as sensitizing to the skin of guinea pigs. The mean dermal absorptions were 0.69 % for a methylgelb suspension in DMSO/water, 0.21 % for the formulation without hydrogen peroxide (fA), and 0.24 % for the formulation with hydrogen peroxide (fB). In a teratogenicity test in rats, methylgelb did not cause maternal, embryotoxic or structural irreversible effects up to a concentration of 60 mg/kg b.w. Methylgelb was not mutagenic in an Ames test and in a mouse lymphoma assay. An *in vitro* chromosome aberration test was not performed. An unscheduled DNA synthesis assay and a SCE test were negative. In the 3 m feeding study effects were found at the 90 mg/kg b.w. level. The no-observed-adverse-effect-level was considered to be 45 mg/kg b.w.

The following human risk calculation for normal use can be made: The final on head concentration of methylgelb in a formulation will be 1 %. A maximum amount of 100 ml of the permanent hair dye formulation comes in contact with the human skin. This corresponds to 1000 mg methylgelb. As skin penetration for the formulation containing 50 % hydrogen peroxide (fB) was 0.24 %, dermal absorption will be 2.4 mg methylgelb per treatment. Assuming a body weight of 60 kg, the exposure of a human per kg body weight will be 40 µg/kg b.w. So a safety margin of 1125 can be calculated between the figure for human exposure to permanent hair dye and the no-effect-level of 45 mg/kg b.w. found in the 3 m rat study. In the same way a safety margin of 3215 can be calculated for hair tinting products and colouring setting lotions (based on maximum usage concentration of 1 % methylgelb and a maximum usage volume of 35 ml).

It should be noted that the no-effect-level found in rats in based on daily exposure for 90 days, whereas human exposure is unlikely to be more frequent than once a month for permanent hair dye and once a week for semipermanent hair dye.

Classification: B.

Industry should provide data on skin sensitization potential from in-use data in the context of the volume used.

B 77: *METHOXYBLAU*

1. General

1.1 Primary name

Methoxyblau

1.2 Chemical names

1-((2′-methoxyethyl)-amino)-2-nitro-4-(di-(2′-hydroxy-ethyl)-amino)-benzene

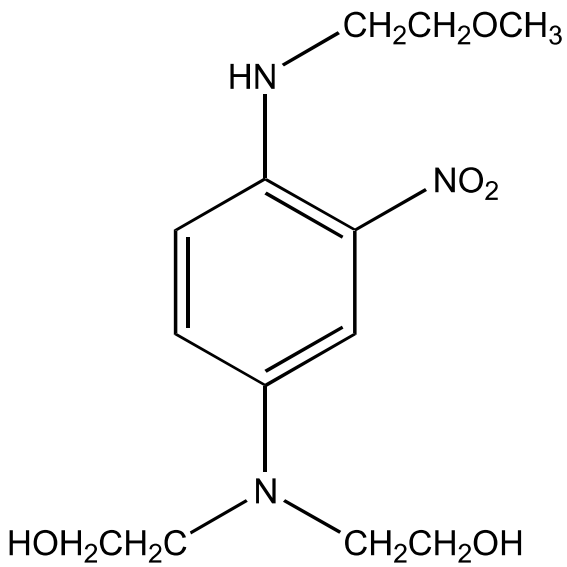
1.3 Trade names and abbreviations

COS 338

1.4 CAS no.

23920-15-2

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{13}H_{21}N_3O_5$
Mol weight: 299

1.7 Purity, composition and substance codes

sA: 1-((2′-methoxyethyl)-amino)-2-nitro-4-(di-(2′-hydroxy-ethyl)-amino)-benzene
(purity: 99 %)

1.8 Physical properties

Subst. code: sA

Appearance: violet christaline powder

Melting point: 64-65 °C

1.9 Solubility

The test substance exists as a free base or as a hydrochloride. Methoxyblau is freely soluble in methanol, ethanol and isopropanol and soluble in water.

2. Function and uses

Methoxyblau is included in hair tinting products and colouring setting lotions at a maximum concentration of 2 %.

In oxidative hair dye formulations the maximum concentration included is 3 %.

Since the oxidative hair dyes are mixed with hydrogen peroxide before use, the concentration at application is 1.5 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	mouse (f)	1275 mg/kg b.w.
sA	oral	rat (f)	1250 mg/kg b.w.
sA	oral	rat (m)	1250 mg/kg b.w.

The test compound (diluted in 5 % distilled water) was administered orally to female CF-1 mice (10/group) at single doses of 1000, 1250, 1500 or 1750 mg/kg b.w. and to Wistar rats (6/sex/group) at 1000, 1250 or 1500 mg/kg b.w.

During an observation period of 14 days, mortalities and clinical toxicological observations were recorded daily and body weights were noted weekly. The organs of all animals were also examined.

During the observation period a blue colouration of the extremities and urine was observed at all dose levels. Also a reduced activity and exitus were observed at all dose levels, till day 7.

The test substance is moderately toxic.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 90 d
DWE: 100 mg/kg b.w.

Methoxyblau, dissolved in water, was administered by gavage, once daily to 4 groups of Sprague Dawley rats (10/sex) (Crl: CD (SD)BR) for 90 days. The test substance was administered at dosage levels of 10, 50 or 100 mg/kg b.w. The control group received distilled water only. All animals were sacrificed at the end of the study.

All animals were observed twice daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophtalmoscopic examination was performed on day 0 and during week 13 on all control and high dose animals. Blood samples were taken from all animals of the control and high dose group, for haematological and clinical chemistry investigations, on day 0 and during week 13. Urine samples were collected on day 0 and during week 13. Organ weights (c. 10) were measured. Macroscopy and histopathology (c. 35 organs/tissues) was performed.

1 animal of the control group died during the study, due to an intubation error. The fur around the genital region and the tail was coloured lilac in the rats of the 50 and 100 mg/kg b.w. group. The urine of the 100 mg/kg b.w. group was coloured dark lilac. No toxic effects occurred.

The dose level without adverse effect was 100 mg/kg b.w.

Remark: This test is inadequate, because no effects were observed.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin
Species: guinea pig
Subst.: sA
Exposure: 4 hr
Dose: 0.5-1 ml
Concentr.: 5 %

0.5-1.0 ml of a 5 % aqueous test solution thickened with 0.5 % thylose, was applied open epicutaneously to the clipped back of 15 female Pirbright white guinea pigs, once daily for 4 days. The test substance was not rinsed. Observations for signs of dermal irritation were recorded at 5 hours and then daily for 5 days after patch removal.

Erythemas could not be recognized, because the skin areas were blue stained.

No other signs of irritation were observed.

4.2 Irritation (mucous membranes)

Route: eye
Species: guinea pig
Subst.: sA
Dose: 0.1 ml
Concentr.: 1.5 %

0.1 ml of the test substance (1.5 %) was instilled in the conjunctival sac of the right eye of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls.

The eyes were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after application. At 24 hours, additional examinations were carried out upon the instillation of 0.1 % fluorescein-solution.

In one animal redness of the conjunctivae was observed. After 6 hours this effect was disappeared.

5. Sensitization

Subst.: sA
Species: guinea pig
Method: MagnussonKligman
Conc.induc.: 3 %
Conc.chall.: 1, 2, 3 %

20 Pirbright white guinea pigs (Hoe: DHPK) (10/sex) were used in this skin sensitization study, 10 guinea pigs were used as negative controls and 10 (5/sex) as positive controls.

The induction phase consisted of 3 series of 2 intradermal injections (0.05 ml) in the shoulders of the treatment and the control group (pretreated with 10 % sodium laurylsulfate). The intradermal injections were divided as follows: 2 injections of methoxyblau in oleum arachidis, 2 injections of methoxyblau (3 %) in FCA and 2 injections of FCA/distilled water 1:1.

Day 1-7: examination on local tolerance. Day 8, dermal induction of 0.5 ml (3 %) test substance. The occlusive patch application lasted for 48 hours. Day 12-21: rest period.

The animals were challenged by closed patch test, 14 days after the last exposure using three different concentrations per animal, that is 3 %, 2 % and 1 %. The test sites were examined 24 and 48 hours after the challenge.

No adverse skin reactions were observed.

Remark: This test is inadequate, because the concentrations used are too low (did not induce irritation).

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 5-15
DWE: 90 mg/kg b.w.

Methoxyblau, dissolved in deionized water, was administered, by gavage, to 4 groups of 24 pregnant rats (BOR: WISW-SPF TNO). The test substance was daily administered at dosage levels of 10, 30 or 90 mg/kg b.w. The control group received the vehicle only. All dams were sacrificed on day 20 of gestation. The animals were observed daily for clinical signs. Individual body weights were recorded at day 0, 5, 10, 15 and 20. Food consumption was measured for the phases 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for organic imperfections.

No animal died during the study. The haircoat of all animals appeared smooth and brightly. During the treatment period, the urine of the rats of the 30 and 90 mg/kg b.w. group was coloured violet. No irreversible structural changes were found.

The dose level without maternal and foetotoxicity was 90 mg/kg b.w.

Remark: No maternal toxicity was observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C-labelled methoxyblau was applied to the clipped dorsal skin of Sprague Dawley rats (Him: OFA, SPF) for 30 minutes and then washed off. In the 3 studies 3 rats/sex were used. The test substance was integrated in two different hair dyeing formulations* or was used as a solution in DMSO/water 3:1. Hair dyeing formulation II was mixed with hydrogen peroxide before application. The amount of test substance applied per animal was 20.0 mg of formulation I (2 %) and II (2 %) and 20.6 mg of the 6.67 % solution of the test substance in DMSO/water.

The content of radioactivity was determined in rinsing water, treated skin areas, faeces, organs and carcass.

The formulation or the solution was left for 30 minutes and was then scraped off using a spatula, followed by a rinse-off using about 100 ml of shampoo-solution and water of about 37 °C. Rinsing was continued until the rinsing water and the absorbent tissue which used to dab the skin dry, were free of colour.

97.9-99.7 % of the applied ^{14}C-amount was removed from the skin by rinsing 30 minutes after the beginning of the percutaneous treatment.

The absorbed amount of ^{14}C-labelled compound was excreted via faeces (56-57 %) and via urine (40-41 %). 66 to 79 % of the absorbed amount was excreted in the first 24 hours.

Very small ^{14}C-concentrations, that were in most cases below the detection limit were found in the organs after 72 hours (0.00002 % for large organs and about 0.00003 % for small organs, especially the thyroid). Relatively high ^{14}C-concentrations were detected in thyroid and liver in studies A and B and in kidney and liver in study C.

The treated area of the skin still contained a small ^{14}C-activity of 0.28 % of the administered ^{14}C-activity for formulation I, 0.66 % for formulation II and hydrogen peroxide and 0.08 % for the solution of the test substance.

Significant sex differences occurred mainly in study B (formulation II and Welloxon), where the ^{14}C-content in the treated skin, in urine and faeces was higher with males than with females.

The *mean percutaneous absorption* was 0.049 % of the administered ^{14}C for hair dyeing formulation I, 0.055 % for formulation II and 0.0145 % for the solution.

* Composition of the formulations I and II:

	I (%)	**II (%)**
- ^{14}C-labelled methoxyblau	2.00	2.00
- p-toluylendiamine, sulfate	—-	1.75
- mixture of Resorcinol and m-Aminophenol	—-	0.68
- mixture of salts	0.70	0.35
- ammonia, 25 %	0.36	1.00
- isopropanol	3.90	1.95
- WAS	2.00	1.00
- deionized water	43.44	15.64
- formulation base	47.60	23.80
- ammonia, 25 %	—-	1.83
- Welloxon (containing 9 % H_2O_2)	—-	50.00

8. Mutagenicity

Sb.	**Species**	**Strain**	**Meas.endp.**	**Test conditions**	**res -act**	**res +act**	**sp +a**	**ind +a**
* sA	*Salm.typh.*	TA97	frameshift mut.	1-10000 µg/pl	-	-	r	
* sA	*Salm.typh.*	TA98	frameshift mut.	1-10000 µg/pl	(+)	(+)	r	
* sA	*Salm.typh.*	TA100	base-pair subst.	1-10000 µg/pl	-	-	r	
* sA	*Salm.typh.*	TA98-NR	frameshift mut.	1-10000 µg/pl	-	-	r	
sA	mouse lymph.	L5178Y	HGPRT	157-3000 µg/ml	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	mouse	NMRI	micronuclei	125, 415 and 1250 mg/kg b.w.	-

Salmonella assays

3 strains of *Salmonella typhimurium* were exposed to methoxyblau diluted in DMSO, in the presence and absence of rat liver S9-mix. The dose level tested was 1-10000 µg/pl. The negative control was bidest; the positive control substances were 4-nitro-o-phenylendiamin and 2-aminofluoren. Concentrations from 6000 µg/plate were toxic to the bacteria. Methoxyblau showed a weak positive result in strain TA98. The other results were negative.

Remark: At least 4 strains are prescribed in the OECD guidelines.

The second Ames test was carried out, due to the positive result obtained with strain TA98 in the first Ames test. In this test the strain TA98-NR, which is capable to detect false positive results of substances containing a nitro-group, was used. The strain was exposed to methoxyblau dissolved in DMSO, with and without S9-mix. DMSO alone was the negative control; the positive control substances were 2-nitrofluorene and 2-aminofluorene.

Methoxyblau was not genotoxic in strain TA98-NR, with and without S9-mix.

Mouse lymphoma fluctuation assay

Methoxyblau was tested for genotoxicity in the mouse lymphoma fluctuytion assay at the HGPRT-locus (6-thioguanine resistance), both in the absence and presence of a rat mitochondrial fraction. Cells were treated with sA, dissolved in DMSO, in duplicate at 188, 375, 750, 1500 or 3000 µg/ml in the absence of S9-mix and 157, 313, 625, 1250 or 2500 µg/ml in the presence of S9-mix. Concentrations from 1500 and 1250 µg/ml, respectively, were toxic to the cells. 4-nitroquinoline-1-oxide and benzo(a)pyrene were included as positive controls in the absence and presence of S9-mix, respectively.

Methoxyblau has no genotoxic activity at the HGPRT locus of L5178Y mouse lymphoma cells, either in the absence or presence of S9-mix.

Micronucleus assay

Methoxyblau, dissolved in DMSO, was administered orally to 5 groups of NMRI mice (6/sex) at single dose of 125, 415 and 1250 mg/kg b.w. The positive control group was treated with cyclophosphamide at 30 mg/kg b.w. The negative control group received the vehicle only. The bone marrow smears were prepared 24 hours after the administration and in the highest dose group also 48 and 72 hours after the administration. 1000 polychromatic erythrocytes were analysed in each group (5/sex) and the relationship of polychromatic to nonchromatic erythrocytes was calculated.

Methoxyblau did not induce higher frequencies of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse.

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	Chin. hamst.	ovary cell	SCE	0.001-1 mM	-	-	r	AR
* sA	rat	hepato cytes	UDS	0.01-1 mg/ml	-	-	r	AR
* sA	HeLa S3 cells		UDS	0.0064-500 µg/ml	-	-	r	AR
* sA	hamster	SHE	transfor-mation	10-500 µg/ml	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	rat	Wistar	UDS	80, 250 and 700 mg/kg b.w.	-

Sister Chromatide Exchange assay
Methoxyblau, dissolved in DMSO, was tested for Sister Chromatid Exchanges in Chinese hamster ovary cells (Kl-cells) in the absence and presence of S9-mix. The positive controls were 2-nitro-p-phenylene-diamine and 2-acetylamino-fluorene. 100 Metaphases per culture were taken into account.

Methoxyblau did not induce SCE´s in the test system with or without metabolic activation.

Unscheduled DNA synthesis
Methoxyblau, dissolved in DMSO, was assayed with and without S9-mix at dose values from 0.01-1 mg/ml, in a cell culture medium containing ^{3}H-thymidine at 1 µCi/ml. The positive control substance was 2-acetyl-amino-fluorene. Incorporation of ^{3}H-thymidine per mg DNA was calculated for each of the sixfold tested dose values and compared with the negative control.

Methoxyblau did not induce UDS in rat hepatocytes, neither in the absence nor in the presence of S9-mix.

Methoxyblau, dissolved in DMSO, was assayed with and without S9-mix at dose values from 0.0064-500 µg/ml, in a cell culture medium containing ^{3}H-thymidine at 5 µCi/ml. The positive control substances were benzo(a)pyrene with S9-mix and 4-nitroquinoline-1-oxide without S9-mix. Incorporation of ^{3}H-thymidine per µg DNA was calculated for each of triplicate tested dose values and compared with the negative control.

Methoxyblau was toxic to the cells at concentrations of 100 and 500 µg/ml without S9-mix and at 500 µg/ml with S9-mix. Methoxyblau did not induce UDS in HeLa cells, neither in the absence nor in the presence of S9-mix.

Cell transformation assay

Syrian hamster embryo (SHE) cells were treated with methoxyblau, dissolved in DMSO, and assessed for its potency to transform Syrian hamster embryo cells. SHE cells were treated with concentrations of methoxyblau of 100, 200, 350 and 500 µg/ml for 4 hours with S9-mix and at 10, 25, 50 and 100 µg/ml for 4 and 48 hours without S9-mix. Toxic properties were observed at 500 and 100 µg/ml, respectively. Untreated cells and the cells treated with DMSO served as the negative controls. Cells treated with N-methyl-N´-nitro-N-nitrosoguanidine in the absence of S9-mix and cells treated with benzo(a)pyrene in the presence of S9-mix served as positive controls. In each dose group 1000 colonies were scored for the occurrence of transformation. Methoxyblau did not induce transformation in the SHE-culture.

Unscheduled DNA synthesis

Methoxyblau, dissolved in DMSO, was administered to groups of Wistar rats (6/sex), by gavage, at concentrations of 80, 250 and 700 mg/kg b.w. The positive control group received 100 mg/kg b.w. methylmethane sulphonate. The negative control group received DMSO. 24 hours after administration the animals were sacrificed and the livers were removed. Liver preparations were incubated with ^{3}H-thymidine and then washed, fixed onto slides and stained. 100 cells per animal were microscopically examined and grains/nucleus counted.

Methoxyblau did not induce UDS *in vivo***.**

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the acute oral toxicity test, eye and skin irritation study, *Salmonella* assays and Sister Chromatid Exchange assay.

General

Methoxyblau is included in hair tinting products and colouring setting lotions at a maximum concentration of 2 %. In oxidative hair dye formulations the maximum concentration included is 3 %. Since the oxidative hair dyes are mixed with hydrogen peroxide before use the concentration at application is 1.5 % only.

Acute toxicity

The test substance is moderately toxic orally (LD_{50} mouse 1275 mg/kg b.w.; LD_{50} rat 1250 mg/kg b.w.).

Irritation

The eye and skin irritation tests were carried out with guinea pigs, instead of rabbits, the species normally used and for which the Draize scoring system is applicable. A concentration of 1.5 % in the eye and 5 % in the skin irritation study with guinea pigs showed no signs of irritation.

Sensitization
The concentrations in the sensitization test are too low. The test cannot be evaluated.

Semichronic toxicity
In a 90-day feeding study, Sprague Dawley rats were fed 0, 10, 50 or 100 mg methoxyblau/kg b.w., by gavage once daily. 1 animal of the control group died during the study. The animals of the 50 and 100 mg/kg b.w. group showed lilac coloured fur and urine. No toxic effects were observed.

The dose level without adverse effects was 100 mg/kg b.w.

Teratogenicity
In a teratogenicity study, Wistar rats were fed 0, 10, 30 and 90 mg methoxyblau/kg b.w.. No animal died during the study. The urine of the mid and high dose group was coloured violet. No irreversible structural changes were found.

The dose level without maternal and foetotoxicity was 90 mg/kg b.w.

Genotoxicity
Methoxyblau was tested for its mutagenic potential in *in vitro Salmonella*, Mouse lymphoma, Sister Chromatid Exchange, Unscheduled DNA synthesis and cell transformation assay and *in vivo* in the micronucleus and Unscheduled DNA synthesis assays.

Methoxyblau was negative in all tests, except for the *Salmonella* assay (strain TA98), in which a positive result was found. This observation, however, was reevaluated in a second *Salmonella* assay using a nitroreductase deficient strain (TA98-NR). The result was then negative.

On the basis of all available data, methoxyblau is considered to be not genotoxic.

Absorption
^{14}C-labelled methoxyblau was applied to the skin of rats in two different hair dye formulations (one of them contained hydrogen peroxide) or as a solution of the test substance in water/DMSO. Most of the substance was recovered by rinsing (97.9-99.7 %). The cutaneous absorption was 0.049 % of the administered ^{14}C for hair dyeing formulation I, 0.055 % for formulation II and 0.0145 % for the solution.

Conclusions
Methoxyblau was found moderately toxic in the acute oral toxicity test.

In the eye and skin irritation study no signs of irritation were observed.

The sensitization test was carried out inadequately.

In the 90-day study with rats, 100 mg/kg b.w. was considered to be the NOAEL. In the teratogenicity study, no irreversible structural changes were observed in the foetuses of the rat, after administration of 90 mg/kg b.w.

Methoxyblau has no genotoxic potential.

The cutaneous absorption was 0.049 % of the administered ^{14}C for hair dyeing formulation I, 0.055 % for formulation II and 0.0145 % for the solution.

For normal use of hair dye, the following calculation can be made:

1.5 g methoxyblau comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1.5 % methoxyblau). With a maximal penetration of 0.055 %, this results in a dermal absorption of 0.83 mg per treatment, which is 0.018 mg/kg b.w. (assuming a body weight of 60 kg). 0.7 g methoxyblau comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 2 % methoxyblau). With a penetration of 0.049 %, this results in a dermal absorption of 0.343 mg per treatment, which is 0.0057 mg/kg b.w.

So a margin of safety of 7273 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in rats in the 90-day study. For the semi-permanent hair dye a safety margin of 17500 can be calculated.

It should be noted that the NOAEL stems for a daily exposure for 90 days, whereas human exposure to permanent hair dye in unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week.

Classification: B.

Industry should provide data on skin sensitization potential from in-use data in the context of the volume used, together with any available information on the toxicological profile of the compound, e.g. from animal studies and/or, from experience in use in either the consumer on occupational context.

B 99: *CHLORORANGE*

1. General

1.1 Primary name

Chlororange.

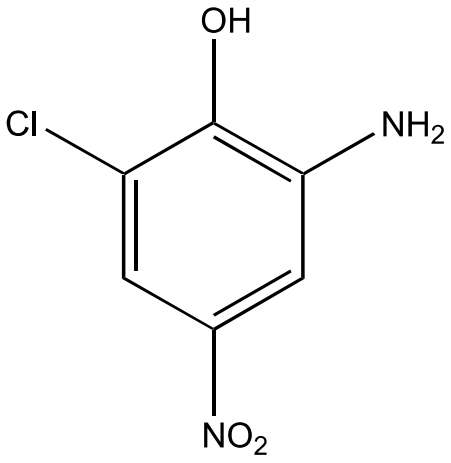
1.2 Chemical names

2-amino-6-chloro-4-nitrophenol
6-chloro-4-nitro-2-aminophenol
1-hydroxy-2-amino-4-nitro-6-chlorobenzene

1.4 CAS no.

6358-09-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_5N_2O_3Cl$
Mol weight: 206.60

1.7 Purity, composition and substance codes

sA: 6-chloro-4-nitro-2-aminophenol (purity > 98 %)

1.8 Physical properties

Subst. code: sA
Appearance: orange-yellow fine grained powder

1.9 Solubility

The substance exists as a monohydrate. It is good soluble in water at pH greater than 7.

2. Function and uses

6-chloro-4-nitro-2-aminophenol is used in oxidative hair dye formulations and colour setting lotions at a maximum concentration of 3 %.

Remark: This information is too brief.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
sA	oral	rat	>2000 mg/kg b.w.

The test compound (a 20 % dilution in deionised water) was given once by stomach tube to Wistar rats (5/sex) at a concentration of 2000 mg/kg b.w. The animals were observed for 14 days and at the end of the 14-day observation period all surviving animals were sacrificed and gross necropsies were performed.

During the observation period red-orange-stained urine was observed in all animals, up to 5 days. No mortalities were observed and no abnormalities were found in the animals necropsied on day 14. The test substance is slightly toxic.

3.7 Subchronic oral toxicity

Route: oral
Species: rat
Subst.: sA
Exposure: 90 d
Recov.p.: 4 w
DWE: 30 mg/kg b.w.
LED: 90 mg/kg b.w.

Chlororange was administered, by gavage, once daily to 4 groups SPF-Albino Wistar rats (15/sex) (Crl: Wi/Br) for 90 days. The control and high dose group included additionally 10 animals/sex, which were deprived from treatment after 13 weeks and remained for 4 subsequent weeks for recovery observations. The test substance was administered at dosage levels of 10, 30 or 90 mg/kg b.w. The control group received the vehicle (0.5 % methylcellulose) only. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination

and a hearing test were performed. Blood samples were withdrawn from 10 males and 10 females of each test group for haematological and clinical chemistry investigations, at week 6, 13 and 17. Urine samples were collected from 5 males and 5 females at each test group, at week 6, 13 and 17. Macroscopy and histopathology (c. 40 organs/tissues) was performed.

No animal died during the study. Urine of the 30 and 90 mg/kg b.w. groups was coloured orange during the study. In the 90 mg/kg b.w. group diarrhoea was observed. Body weight gain in the males of the 90 mg/kg b.w. group was significantly reduced, during the second half of the study, whereas food consumption was normal in all animals. In the females of the 90 mg/kg b.w. group lung and thymus weights were found to be increased in the high dose group.

The dose level without effect was 30 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin
Species: rabbit
Subst.: sA
Exposure: 4 hr
Dose: 0.5 ml
Concentr.: 2 %

0.5 ml of the test substance was applied occlusively on the right, clipped back of 6 New Zealand White rabbits. After 4 hours the patches were removed.

Observations for signs of dermal irritation were recorded at 30 and 60 minutes and 24, 48 and 72 hours after removal of the patches.

In all animals very slight erythema and in 2 animals very slight oedema was observed 30-60 minutes after patch removal. After 24 hours no irritation was observed. The Draize score was 0.3 (not irritating).

4.2 Irritation (mucous membranes)

Route: eye
Species: rabbit
Subst.: sA
Dose: 0.1 ml
Concentr.: 2 %

0.1 ml of the test substance was instilled into the conjunctival sac of the left eye of 6 New Zealand White rabbits. In 3 animals the test substance was rinsed after 4 seconds. The untreated right eyes served as controls.

The eyes were examined 1, 24, 48 and 72 hours after application. At 24 and 72 hours, additional examinations were carried out upon the instillation of one drop of 1 % fluorescein-solution.

In 1 animal redness was observed, 1 hour after application. No other irritating effects were observed. The Draize score was 0.08 (not irritating).

5. Sensitization

Subst.: sA
Species: guinea pig
Method: Magnusson Kligman
Conc.induc.: 10 100 %
Conc.chall.: 2 0.1 %
Result: negative

20 Pirbright guinea pigs were used in this skin sensitization study and 20 guinea pigs were used as irritation controls.

The induction phase consisted of 3 series of 2 intradermal injections in the shoulders of the treatment and the control group. The intradermal injections were divided as follows: 2 injections of 0.05 ml test substance (10 %), 2 injections of 0.05 ml of the test substance (10 %) in Freund´s Adjuvant Complete (FCA) and 2 injections of 0.05 ml FCA. The control group received 2 injections of 0.05 ml FCA, 2 injections of 0.05 ml FCA and propylene glycol (vehicle) and 2 injections of 0.05 ml propylene glycol.

Day 1-7: examination on local tolerance. Day 8, dermal induction of 0.5 g (100 %) test substance. The occlusive patch application lasted for 48 hours on the surface corresponding to the injections. Day 12-21: rest period.

On day 22, the challenge phase started using “Hill-Top” Chambers, namely 0.5 ml 2 % test substance and 0.5 ml 0.1 % test substance was occlusively applied for 24 hours on the left flank; the right flank received the vehicle. The control animals were treated the same way, using the vehicle only.

Any sign of erythema and oedema was recorded 24 and 48 hours after the challenge.

No skin reactions were observed in the test and control group on the areas treated with a 0.1 % dilution. The areas, which were treated with the maximum administration concentration (2 %) could not be evaluated due to the severe self-colouration of the sample. The control areas of both groups were without any signs. The test substance did not produce dermal sensitization.

6. Teratogenicity

Route: oral
Species: rat
Subst.: sA
Admin.Days: 5-15
DWE:: 90 mg/kg b.w.

Chlororange was administered, by gavage, to 4 groups of 20 pregnant SPF Albino Wistar rats (Crl: Wi/Br). The test substance was daily administered at dosage levels of 10, 30 or 90 mg/kg b.w. The control group received the vehicle (0.5 % Na-carboxymethylcellulose) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at day 0, 5, 10, 15 and 20. Food consumption was measured daily. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

No animal died during the study. Females of all dose groups had orange coloured urine troughout the application period. This discolouration is considered to be caused by the test substance. Mean maternal body weight gain and mean food consumption was significantly reduced during the treatment period in the females of the 90 mg/kg b.w. group. 5 foetuses of the 30 and 1 foetus of the 90 mg/kg b.w. group had oedema. This effect was considered to be coincidental. No irreversible structural changes were found.

The dose level without maternal toxicity was 30 mg/kg b.w. and the dose level without foetotoxicity was 90 mg/kg b.w.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin absorption *in vivo*, distribution and elimination

^{14}C-labelled chlororange (purity >98 %) was applied to the clipped dorsal skin of Sprague Dawley rats (HIM: OFA, SPF) for 30 minutes and then washed off. In the 5 studies, 3 rats/sex were used. The test substance was integrated in 2 different hair dyeing formulations* or was used as a solution in water/DMSO 1:1. A 9.99 % solution of the test substance was used in study C, a 3.0 % solution in studies D and E. Hair dyeing formulation II was mixed with Welloxon (containing 9 % hydrogen peroxide) before application. Oral application of the test substance was used as a reference and an additional experiment was performed to determine the blood level after oral application.

The mean mass of the test substance applied per animal was 30 mg (study A: cutaneous application with formulation I); 29.3 mg (study B: cutaneous application with formulation II); 30.3 mg (study C: cutaneous application with test substance solution); 30.9 mg (study D: oral application with test substance solution); 30.6 mg (study E: oral application with test substance solution). The formulation or the solution was left for 30 min and was then scraped off using a spatula, followed by a rinse-off using first about 100 ml of a 3 % solution of a proprietary shampoo and then water of about 37 °C. Rinsing was continued until the rinsing water and the absorbent cellulose tissue which was used to dab the skin dry were free of colour. The rinsings were collected. Than the treated areas were covered with 4 layers of gauze fixed by adhesive tapes. Additional covering by fixation of an air permeable, plastic, truncated cone to prevent licking of the treated area.

95.4 to 98.7 % of the applied ^{14}C was removed from the skin by rinsing 30 min. after the beginning of the cutaneous application.

The absorbed amount of ^{14}C-labelled test substance was excreted mainly via urine (89-92 %) and via faeces (8-11 %). The mean excretion within the first 24 hours was fast (89-93 %).

After oral administration 70 % was excreted via urine and 30 % via faeces. The mean excretion within the first 24 hours was fast (92.7 %).

The remaining mean amounts of ^{14}C in the carcass 3 days after cutaneous application (studies A-C) were below or near the detection limit, namely 0.0015-0.0059 % of the administered dose. The remaining mean amount of ^{14}C in the carcass 3 days after oral application was 0.219 % of the administered ^{14}C-amount. After cutaneous application all mean concentrations of the ^{14}C in the organs, after 72 hr, were near or below the detection limits (0.0004 % for thyroids and 0.00002 % for large organs). Relatively high concentrations were noted in thyroids and low concentrations were detected in brain, muscle and testes. After oral administration high ^{14}C concentrations were detected in kidneys, thyroids and liver. Low concentrations in testes, brain and muscle. The blood level after oral administration was highest at 35 min post application. A half-life of about 1 hr is estimated. The test substance is rapidly absorbed from the gastro-intestinal tract and also rapidly eliminated from the blood. The *mean percutaneous absorption* was 0.248 % of the administered ^{14}C for hair dyeing formulation I, 0.189 % for formulation II and 1.213 % for the solution.

* Composition of the formulations I and II:

	I	II	
	(%)	cream alone (%)	mixed with Welloxon (%)
- ^{14}C-labelled chlororange	3.00	6.00	3.00
- p-toluylendiamine-sulfate	—-	3.50	1.75
- mixture of resorcinol and m-aminophenol	—-	1.36	0.68
- mixture of salts	0.70	0.70	0.35
- ammonia, 25 %	0.36	2.00	1.00
- isopropanol	3.90	3.90	1.95
- WAS	2.00	2.00	1.00
- deionised water	42.44	29.30	14.65
- formulation base	47.60	47.60	23.80
- ammonia, 25 %	—-	3.65	1.83
- Welloxon (containing 9 % hydrogen peroxide)	—-	—-	50.00

8. Mutagenicity

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	*Salm.typh.*	TA97	frameshift mut.	1-6000 µg/pl	-	-	r	PC
* sA	*Salm.typh.*	TA98	frameshift mut.	1-6000 µg/pl	(+)	-	r	PC
* sA	*Salm.typh.*	TA100	base-pair subst.	1-6000 µg/pl	-	-	r	PC
* sA	*Salm.typh.*	TA98-NR	frameshift mut.	10-6000 µg/pl	-		r	PC
* sA	mouse lymph.	L5178Y	HGPRT	0.5-5000 µg/ml	-	-	r	AR
* sA	Chin. hamster	CHO	chrom.aber	0-500 µg/ml	e	-	r	AR
* sA	human lymph		chrom.aber	39.1-1250 µg/ml	-	e	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (AR = Aroclor, PH = Phenobarbital, MC = Methylcholantrene)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	mouse	NMRI	micronuclei	15, 50 and 150 mg/kg b.w.	-

Salmonella assays
3 strains of *Salmonella typhimurium* were exposed to chlororange diluted in dimethyl-sulphoxide (DMSO), in the presence and absence of rat liver S9 mix. The dose level tested was 1-6000 µg/plate. The negative control was DMSO; the positive control substances were 2-amino fluorene with metabolizing enzymes and sodium azide, 2-nitro fluorene and 4-nitro-o-phenylenediamin without S9 mix.

Chlororange showed, without metabolic activation, a weak positive result in strain TA98. The other results were negative. Concentrations of 3000 and 6000 µg/plate were toxic to the bacteria.

Remark: At least 4 strains are prescribed in the OECD guidelines.

The second Ames test was carried out, due to the positive result obtained with strain TA98 in the first Ames test. In this test the nitroreductase-deficient strain TA98-NR, was used. The strain was exposed to chlororange dissolved in DMSO, without S9 mix.

Chlororange was not mutagenic in strain TA98-NR, without S9 mix.

Mouse lymphoma fluctuation assay
Chlororange was tested for genotoxicity in the mouse lymphoma fluctuation assay at the HGPRT-locus (6-thioguanine resistance), both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in DMSO, in duplicate at 1.58, 5, 15.8, 50, 158, 500, 1580 and 5000 µg/ml in the absence of S9. 5000 µg/ml proved to be insoluble in the test culture medium. Therefore, the plus S9 treatments used a dose range of 0.5, 1.58, 5, 15.8, 50, 158, 500 and 1580 µg/ml sA.

4-nitroquinoline-N-oxide and benzo(a)pyrene were included as positive controls in the absence and presence of liver S9 mix, respectively.

Chlororange has no genotoxic activity at the HGPRT locus of L5178Y mouse lymphoma cells, neither in the absence nor presence of S9.

Cytogenetics assay
Chlororange was tested in a cytogenetics assay using duplicate cultures of Chinese hamster ovary (CHO) cells, both in the absence and presence of metabolic activation. Cells were treated with sA, dissolved in DMSO, at 5, 16.5, 50, 165, 500, 1650 and 5000 µg/ml. Chlororange was toxic at concentrations of 1650 and 5000 µg/ml. The final doses selected for analysis were 0, 50, 165 and 500 µg/ml. 100 metaphases from each culture were analysed for chromosome aberrations. Methyl methanesulphonate and cyclophosphamide were the positive controls in the absence and presence of liver S9, respectively.

In the absence of S9, chlororange was able to induce small, significant increases in numerical chromosomal aberrations, at 165 and 500 µg/ml (dose-related). However, the total number of structural and numerical aberrations did not exceed the defined control range (historical solvent control data). In the presence of S9 there was no indication of an increased number of aberrations.

Chlororange, dissolved in DMSO, was tested in a cytogenetics assay using human lymphocyte cultures from a male and a female donor, in the presence and absence of metabolic activation.

The test compound dose levels for analysis were selected by determining mitotic indices from a range of doses from 39.1-1250 µg/ml. The final concentrations were 312.5, 625 and 1250 µg/ml. 100 metaphases per culture were analysed for chromosome aberrations.

Methyl methanesulphonate and cyclophosphamide were the positive controls in the absence and presence of S9, respectively.

Chlororange, in the presence of S9, caused at 1250 µg/ml a slight increase in aberrations in the male culture. Aberrations were not induced in the absence of S9.

Micronucleus assay
Chlororange was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of NMRI mice. The test substance, dissolved in DMSO, was administered, by gavage, to the animals (6/sex) at concentrations of 15, 50 and 150 mg/kg b.w. Cyclophosphamide was the positive control. Samples were taken 24 hours after administration

for all dose levels. For the high dose and control groups additional samples were taken 48 and 72 hours after administration. In each group 1000 polychromatic erythrocytes of 5 males and 5 females were analysed for micronuclei.

Chlororange did not induce micronuclei.

Indicator tests (Bact., Non mammalian eukaryotic, *In vivo* mammalian)

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
* sA	Chin. hamster	ov.cel Kl	SCE	0.01-1 mM	-	-	r	AR

Abbreviations:
meas.endp. = measured endpoint
sp = species used for activation (r = rat)
res = result of test (+ = pos., - = neg., e = equivocal)
ind = inducer (Aroclor)

Indicator tests (*In Vivo* mammalian, Host mediated)

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
* sA	rat	Wistar	DNA repair (UDS)	15, 50 and 150 mg/kg b.w.	-

Sister Chromatid Exchange Assay
Chlororange, dissolved in DMSO, was tested for Sister Chromatid Exchanges (SCE´s) in Chinese hamster ovary cells (Kl-cells) in the absence and presence of metabolic activation. The positive controls were 2-nitro-p-phenylendiamin and 2-acetylaminofluoren. 100 metaphases per culture were taken into account.

Chlororange did not induce SCE´s in the test system with or without metabolic activation.

Unscheduled DNA synthesis
Chlororange was administered, by gavage, to 3 groups (6/sex) of Wistar rats (Crl:(WI)BR, SPF) in concentrations of 15, 50 and 150 mg/kg b.w. (volume 2 ml/kg b.w.; solvent DMSO). The positive control was methylmethane sulphonate. After 24 hours, liver preparations were made in addition of 185 kBq 3H-thymidine/million cells. 100 cells/animal were examined for unscheduled DNA synthsis.

Chlororange did not induce UDS.

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the two *Salmonella* assays and the Sister Chromatid Exchange assay.

General

6-chloro-4-nitro-2-aminophenol is used in oxidative hair dye formulations and colour setting lotions at a maximum concentration of 3 %.

Acute toxicity

The test substance is slightly toxic, based on the result of the acute oral toxicity test (LD_{50} >2000 mg/kg b.w.).

Irritation

A concentration of 2 % in the eye and 2 % in the skin irritation study showed no signs of irritation.

Sensitization

No signs of sensitization in guinea pigs were observed in the Magnusson Kligman test.

Semichronic toxicity

In a 90-day feeding study, Wistar rats were fed 0, 10, 30 or 90 mg chlororange/kg b.w., by gavage once daily. No animal died during the study. The animals of the 30 and 90 mg/kg b.w. groups showed orange coloured urine throughout the study. In the 90 mg/kg b.w. group, the following effects were observed: diarrhoea, reduced body weight gain in the males and increased kidney weights in the females. After the recovery period the following organ weights were increased: liver, kidney, lung and thymus.

The dose level without adverse effects was 30 mg/kg b.w.

Teratogenicity

In a teratogenicity study, Wistar rats were fed 0, 10, 30 or 90 mg chlororange/kg b.w. No animal died during the study. No irreversible structural changes were found.

The dose level without maternal toxicity was 30 mg/kg b.w. and the dose level without foetotoxicity was 90 mg/kg b.w.

Genotoxicity

Chlororange was tested for its mutagenic potential in *in vitro Salmonella*, Mouse Lymphoma, cytogenetic and Sister Chromatid Exchange assays and *in vivo* in the micronucleus assay. An unscheduled DNA synthesis test was also performed. Chlororange was negative in all tests, except for the *Salmonella* assay (strain TA98, without metabolic activation), in which a weak positive result was found. This observation, however, was reevaluated in a second *Salmonella* assay using a nitroreductase deficient strain (TA-98-NR) without metabolic activation. The result was then negative.

On the basis of the available data, there is equivocal evidence that the substance has genotoxic potential in mammalian cells *in vitro*. There is no evidence for genotoxic potential *in vivo*.

Absorption
^{14}C-labelled chlororange was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in water/DMSO.

Most of the substance was recovered by rinsing (95.4-98.7 %). The cutaneous absorption was 0.248 % for the formulation without hydrogen peroxide, 0.189 % for the formulation with hydrogen peroxide and 1.213 % for the solution.

Conclusions
Chlororange was found slightly toxic in the acute oral toxicity test.

A 2 % concentration of the test compound showed no eye and skin irritation.

Chlororange showed no signs of sensitization.

In the 90-day study with rats, 30 mg/kg b.w. was considered to be the NOAEL. In the teratogenicity study, no irreversible structural changes were observed in the foetuses of the rat, after administration of 90 mg/kg b.w., no maternal toxicity was observed at 30 mg/kg b.w.

Chlororange has no genotoxic potential.

The cutaneous absorption was 0.248 % for the formulation without hydrogen peroxide, 0.189 % for the formulation with hydrogen peroxide and 1.213 % for the solution.

For normal use in hair dye, the following calculation can be made: 3 g chlororange comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 3 % chlororange). With a maximal penetration of 0.189 %, this results in a dermal absorption of 5.67 mg per treatment, which is 0.095 mg/kg b.w. (assuming a body weight of 60 kg).

1.05 g chlororange comes in contact with the human skin in semi-permanent hair dye condition (based on a usage volume 35 ml containing maximal 3 % chlororange). With a penetration of 0.248 %, this results in a dermal absorption of 2.60 mg per treatment, which is 0.043 mg/kg b.w.

So a margin of safety of 317 can be calculated between the figure for human exposure to oxidative hair dye and the no adverse effect level found in rats in the 90-day study. For the semi-permanent hair dye a safety margin of 691 can be calculated.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month and human exposure to semi-permanent hair dye is unlikely to be more than once a week.

Classification: A, for a use concentration of *2 %*.

P 4: *3-(P-CHLOROPHENOXY)-PROPANE-1,2-DIOL*

1. General

1.1 Primary name

3-(p-chlorophenoxy)-propane-1,2-diol

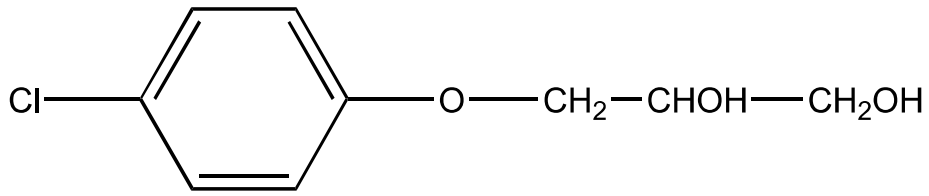
1.2 Chemical names

3-(p-chlorophenoxy)-propane-1,2-diol
Chlorphenesin
p-chlorophenyl-glycerol ether

1.4 CAS no.

104-29-0

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{11}ClO_3$

Mol weight: 202.64

1.9 Solubility

Slightly soluble in water (0.6 %), moderately soluble in glycerol (9.5 %) and alcohol (15 %).

2. Function and uses

Used in cosmetics up to 0.3 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} values (in mg/kg) are: oral in rats > 1400, in mice 1060, in guinea pigs 820, i. p. in rats 520, in mice 675 and 911; in guinea pigs 425, s. c. in mice 930.

3.4 Repeated dose oral toxicity

Full details are available of a 28-day oral toxicity study in rats given doses of 10, 100 and 1000 mg/kg compound by gavage as an aqueous suspension. Detailed autopsies were performed at the end of the exposure period and in addition serum immunoglobulin levels and B; T Lymphocyte ratios in blood and spleen were determined. Compound related mortality was seen at the top dose, 1/5 male animals dying. Other effects noted at this level were reduced weight gain, abnormal posture and gait, reduced haemoglobin levels, reduced spleen and thymus weight and evidence of nephrotoxicity. The only significant effects seen at 100 mg/kg were a slight reduction in haemoglobin levels. No pathology was seen in the spleen, lymph nodes, thymus or bone marrow at any dose level. The no effect level was 10 mg/kg with only marginal effects at 100 mg/kg.

Dogs given 75 or 150 mg/kg/day (route not specified) 5 days a week for 18 weeks, did not show any significant changes in behaviour or growth, in haematology or clinical chemistry, and in urine composition (summary report only).

3.5 Repeated dose dermal toxicity

In a repeated intramuscular injection test in mice, with 0.5 ml of a 0.6 % aqueous solution daily for 40 days there were no observable effects on growth or on the state of the organs.

3.7 Subchronic oral toxicity

In an oral 13-week study in rats given doses of 50, 100 or 200 mg/kg b.w./day by gavage, no effect on growth rate or food intake was observed. Examination of vaginal smears provided no evidence of interference with oestrus. No gross changes were observed at autopsy (a detailed report is not available).

4. Irritation & corrosivity

4.1 Irritation (skin)

A skin irritation test in rabbits was negative (no details).

In repeated insult patch tests with 18 humans, application of 0.05 ml of 0.2 % in hand cream, skin lotion and skin soothing milk on 5 successive days was negative, or produced slight erythema in some cases.

4.2 Irritation (mucous membranes)

An eye irritation test in rabbits with 1 % in glycerine did not provoke corneal irritation.

7. Toxicokinetics (incl. Percutaneous Absorption)

An oral dose of the labelled compound given to rats was rapidly absorbed and reached a peak concentration in the blood in 30 minutes. The half life in serum was 140 min. More than 1/2 of an oral dose was excreted in the urine in 4 hr partly as the unchanged compound. Four metabolites have been identified: 3-p chlorophenoxyacetic acid, a p-chlorophenoxyacetic conjugate of chlorophenol, and a conjugate of chlorophenesin.

An *in vivo* study to measure the percutaneous absorption of chlorphenesin has been carried out using a 0.05 % formulation of radiolabelled compound in cold cream and applied under occlusive dressing for up to 96 hours. By that time approximately 50 % of the dosed radioactivity had been excreted in the urine. These data indicate that chlorphenesin is well absorbed through the skin.

8. Mutagenicity

No evidence of mutagenic potential was obtained in a well-conducted Ames test with up to 0.5 mg/plate. Mutagenicity was examined also by the CHO/HGPRT locus bioassay. Treatment of the cells *in vitro* with up to 1.5 mg/ml did not demonstrate mutagenic potential (Colipa subm. III). A chromosomal aberration test with human lymphocytes exposed *in vitro* to up to 0.325 mg/ml was negative.

10. Special investigations

Chlorphenesin may affect the immune system: both stimulating and inhibiting properties have been reported. Lymphocyte function *in vitro* was found to be suppressed by 20-50 µg/ml culture medium. However although a reduction in thymus weight was seen at 1000 mg/kg in the 28-day study in the rat, no pathology was noted, nor were there any effects on T:B lymphocyte ratios. No effects were seen at 100 mg/kg or below.

11. Conclusions

Chlorphenesin has low acute toxicity, no significant irritant properties but no animal data are available on skin sensitization; however experience in use has not suggested significant sensitization properties. It has a relatively low toxicity on repeated oral exposure the no effect level being 10 mg/kg but with only a marginal effect at 100 mg/kg. No data are available on teratogenicity. The compound is well absorbed through the skin (about 50 % under occlusive dressing over 96 hours) and hence information is needed from a teratogenicity study.

12. Safety evaluation

Assuming extensive use of all cosmetic products total exposure would be to 27.6 g product (5.54 g ingested from oral hygiene at 22.06 g skin contact). Assuming that all products contain chlorphenesin at the maximum permitted level (0.3 %), total exposure is to 16.62 mg by ingestion and 66.18 mg by skin contact. Assuming 50 % absorption through the skin this gives a total absorbed dose of 49.80 mg. This is equivalent to 0.83 mg/kg.

Safety Margin over marginal effect level $= \frac{100}{0.83} = 120.$

Since this based on extreme estimates of exposure this is considered acceptable.

A teratogenicity study is needed.

Classification: B.

P 76: *1,5-PENTANEDIAL*

1. General

1.1 Primary name

1,5-pentanedial

1.2 Chemical names

1,5-pentanedial
Glutaraldehyde

1.4 CAS no.

111-30-80

1.5 Structural formula

$CHO\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CHO$

1.6 Empirical formula

Emp. formula: $C_5H_8O_2$
Mol weight: 100.13

1.8 Physical properties

(Chemical property: the substance reacts with amino groups and forms cross-links.)

1.9 Solubility

Glutaraldehyde is readily soluble in water and most organic solvents.

2. Function and uses

It is used as a preservative in rinse-off and non rinse-off cosmetics at up to 0.1 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Several studies have been carried out to investigate acute toxicity in rats, the LD_{50} being reported to be in the range 60-820 mg/kg b.w. Doses near the LD_{50} caused depression of the CNS, prostration, convulsions and respiratory difficulty; effects on the gastrointestinal tract

and lungs were noted at autopsy. LD_{50} values in the mouse were in the range 100-352 mg/kg b.w. and in the guinea-pig 50 mg/kg.

3.2 Acute dermal toxicity

Dermal LD_{50} values in the rabbit were 640-2000 mg/kg b.w. In a separate study the LD_{50} value was shown to increase with decreasing concentration, values of 735, 900 and above 2000 mg/kg b.w. being obtained with 50, 40 and 25 % glutaraldehyde respectively.

3.3 Acute inhalation toxicity

Inhalation studies in the rat resulted in an LC_{50} of 12.6 mg/l for 8 hour exposure and 24-40 ppm for 4 hour exposure. Rats and mice survived exposure for 4 hours to the vapour given off from a 2 % aqueous glutaraldehyde solution.

3.4 Repeated dose oral toxicity

Oral administration of 0.1, 0.5, 1.0 and 1.6 g/kg b.w./day to rats with the diet for 7 days produced growth depression with decreased weights of liver and kidneys being reported at 1.6 g/kg. No changes were seen with 1.0 g/kg. Two-week oral treatment of rats with 100 or 1000 ppm in drinking water (13 and 103 mg/kg b.w./day respectively) revealed hyperplasia of mucous glands in the stomach with 103 mg/kg, while 13 mg/kg was a NOAEL.

3.6 Repeated dose inhalation toxicity

Inhalation exposure of rats to 0.2, 1.0 and 3.0 ppm in the atmosphere for 9 days, caused reduced intake of food and water, weight loss and mortality at 3.0 ppm and haematological and clinical chemistry effects at 1.0 and 3.0 ppm. A few signs of toxicity were seen with 0.2 ppm.

3.7 Subchronic oral toxicity

A 12-week study in rats with up to 100 mg/kg b.w. in the drinking water showed growth retardation at the top-dose. There were no other relevant changes and the NOAEL was 25 mg/kg b.w. for males and 37.5 mg/kg b.w. for females. No clinical effects or tissue abnormalities were reported in the central and peripheral nerve fibres when groups of 3 rats were given glutaraldehyde at up to 0.5 % in the drinking water (about 500 mg/kg b.w./day) for 11 weeks.

3.9 Subchronic inhalation toxicity

A 90-day inhalation study in rats at 0.02, 0.05 and 0.2 ppm, 5 hrs/day, 4 days/week, revealed growth depression, and clinical signs of mucosal irritation (perinatal wetness and discharge) at 0.2 and 0.05 ppm. Microscopic examination showed lesions of the heart in 3/20 males of the high-dose group (which the authors did not consider to be treatment-related). The NOAEL was probably 0.02 ppm, but individual data were not provided.

4. Irritation & corrosivity

4.1 Irritation (skin)

Studies using a 4 hour occluded dressing in rabbits and 25 % aqueous glutaraldehyde solution resulted in severe inflammation and tissue destruction; a 1 % solution was the threshold for erythema production. Other investigators have however reported that aqueous concentrations up to 7 % glutaraldehyde produced no signs of irritation in rabbits using a 24 hour occluded dressing. In human volunteer studies in contact dermatitis patients and using a 24 hour covered patch, a solution of 1 % glutaraldehyde in petroleum produced irritant reactions in the majority (9/13) of patients. In another study a 5 % solution was shown to cause severe skin reactions after 2 occluded applications. However application of a 10 % aqueous solution of glutaraldehyde to the soles of the feet three times a week for 2 weeks produced no signs of irritation.

4.2 Irritation (mucous membranes)

Studies on rabbits have resulted in severe irritation being observed using aqueous concentrations of 2 % and above, with corneal opacities at 5 %. Slight irritation was observed at 0.2-1 % with no irritation at 0.1 %. In humans accidental instillation of $2^1/_2$ aqueous glutaraldehyde resulted in severe irritation and burning pain. Eye irritation has been reported in workers exposed to atmospheric levels of the order of 0.05-0.5 ppm glutaraldehyde.

8. Sensitization

A number of studies in volunteers have indicated that glutaraldehyde has sensitizing potential in humans. In an attempt to induce sensitization in 30 healthy volunteers, 5 % glutaraldehyde in petrolatum was applied ten times under occlusive dressing (for 48-72 hours) over $3^1/_2$ weeks. Challenge 2 weeks later using 0.5 % glutaraldehyde solution resulted in sensitization reactions in 7/30 subjects. In a separate study using a similar protocol but with an induction concentration of 0.1 %, no signs of sensitization were seen in the 102 subjects tested. In another study 13 'open' applications of 5 % glutaraldehyde to 20 subjects followed by 'open' challenge with a similar solution produced no evidence of sensitization. However when 7 applications (occluded) of 1 % glutaraldehyde and 3 applications of 2 % glutaraldehyde were used, evidence of mild reaction was seen on challenge with a 2 % solution in 6/20 individuals.

There are numerous reports of allergic contact dermatitis in subjects using aqueous solutions of glutaraldehyde as a disinfectant in hospitals. It is used as a 2 % solution as a cold sterilant for many types of instruments and contact dermatitis has most frequently been reported in nurses, clinical assistants and cleaning workers in hospitals. There are few reports of such reactions in non-occupational areas. It has been claimed that glutar-aldehyde is a relatively weak allergen since individuals sensitized to glutaraldehyde could wear cotton shirts that had been laundered using a liquid fabric softener containing 550 ppm glutaraldehyde for prolonged periods (up to 2 weeks) without any reaction. In another review it was stated that glutaraldehyde occasionally produces allergic skin sensitization. Although there is evidence that glutaraldehyde and formaldehyde do not in general cross react, some patients show positive reactions to both substances, presumably due to exposure to both. There are

insufficient data to come to any definite conclusions regarding the relative potencies of these two compounds as regards the induction of skin sensitization, although there is no evidence to indicate that glutaraldehyde is significantly worse than formaldehyde as regards non-occupational exposure.

There is evidence that occupational exposure to glutaraldehyde by inhalation has resulted in respiratory sensitization in some individuals. In view of the seriousness of such reactions the use of glutaraldehyde in cosmetic products which may lead to significant exposure by inhalation, e.g. as aerosols, should be avoided.

6. Teratogenicity

In a developmental toxicity study in mice, the animals were treated by gavage with a 2 % commercial product in amounts providing 16, 20, 24, 40, 50 and 100 mg a.i./kg b.w./day on days 6-15 of pregnancy. Increased incidences of resorptions and of malformed fetuses were seen with 40 mg/kg and more. With 24 mg/kg and less no increased incidence of malformations was seen, but signs of toxicity occurred in the maternal animals at all dose levels. In a second teratogenicity study in mice (dosed orally with 3.3, 10, or 30 mg/kg b.w./day on days 7-12 of pregnancy) the percentage of resorbed and dead fetuses were relatively high in all dose groups although the significance of these findings was not clear. Rats dosed orally with 25 or 50 mg/kg b.w./day, on days 6-15 of pregnancy, did not show increases in resorptions, deaths or malformations, although the top-dose induced decreased body weight gain.

No effects on either the maternal animals or the developing fetuses were however noted in another study in mice given oral doses of up to 24 mg glutaraldehyde/kg b.w./day on day 6-15 of pregnancy. At 40 mg/kg and above maternal survival and weight gain were decreased, and there was a slight increase in the incidence of fetal malformations at 50 mg/kg b.w./day; this was more marked at 100 mg/kg.

Glutaraldehyde was given to rats at levels of 50, 250 and 750 ppm in the drinking water on days 6-16 of gestation. This was equivalent to dose levels of about 5, 26 and 68 mg/kg body weight. Decreased water consumption was seen at 250 and 750 ppm. No effect was reported on body weight at any dose level, nor were any other signs of toxicity reported. Examination of the developing fetuses on day 20 revealed no adverse effect at any dose level up to 68 mg/kg body weight/day.

The study in rabbits involved gavage administrations using dose levels of 5, 15 and 45 mg/kg daily on days 7-19 of gestation. Marked signs of toxicity were seen at the top dose with 5/15 animals dying, and severe body weight loss in the remainder. Severe foetotoxic effects were seen with viable foetuses in only one animal. No effects were seen in the maternal animals given 15 mg/kg of below, nor were any significant effects seen on examination of the developing fetuses on day 29. The No Effect Level in this study was 15 mg/kg.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal application of 0.075 %, 0.75 %, or 7.5 % [1,5]-^{14}C glutaraldehyde, *in vivo* under occlusion for 24 hours, resulted in between 10 and 53 % dermal absorption in the rabbit and less than 9 % in the rat. The highest absorption occurred with the higher concentration due to

the skin damage and the value obtained with the 0.075 % solution would be expected to be most relevant to cosmetic use. The percentages absorption mentioned, do not include the considerable amounts of radioactivity which remained in the stratum corneum under the area of application. This retention may explain the long $t_{1/2}$ of 112 hrs in rats and of 77 hrs in rabbits observed upon dermal dosing, whereas after intravenous dosing the $t_{1/2}$ was only 9-11 hrs in rats and 12-18 hrs in rabbits. Dermal dosing resulted in reasonably uniform tissue distribution. Up to 80 % was excreted as CO_2 and up to 20 % in the urine.

8. Mutagenicity

Several assays have been carried out to investigate the ability of glutaraldehyde to induce gene mutation in *S. typhimurium*. In most cases the concentrations used were low due to the toxicity of the compound. Negative results were obtained. A positive result was however obtained using *Salmonella typhimurium* TA102 and also in E. coli WP2 uvr (pK101) strain, but not in E. coli WP2 (pK101). No indications of mutagenicity were observed in a HGPRT test with CHO cells treated *in vitro* with up to 0.1 mg/ml.

The ability of glutaraldehyde to induce chromosome aberrations has been investigated in a limited study using CHO cells. Three concentrations in the range 0.03-1.0 µg/ml in the absence of S9 and 1-10 µg/ml in the presence of S9 were used. The highest concentration used was estimated, from preliminary toxicity studies, to result in ca. 10 % - 30 % reduction in mitotic index. The next higher concentration resulted in a 50-100 % reduction. Cells were treated for 4 hours, and were harvested about 24 hours later, only a single harvest time being used. There was no evidence for any increase in chromosome aberrations at any concentration used, but the results were not confirmed in a separate experiment as currently recommended.

In a sister chromatid exchange (SCE) test cells were exposed to various concentrations up to 0.1 mg/ml; a significant increase in exchanges was seen only with 0.05 mg/mg (not with 0.1 mg/ml). The positive result was considered a chance event. A study to investigate the ability of glutaraldehyde to induce unscheduled DNA synthesis (UDS) in rat hepatocytes gave negative results but in the positive control substances were sometimes negative also. Glutaraldehyde did not induce sex-linked recessive lethal mutations in the fruit fly drosophila melanogaster.

Negative results have been obtained in an *in vivo* assay to measure UDS in hepatocytes in the mouse. The compound was given as a single oral dose of 30, 150 or 600 mg/kg and hepatocytes were harvested after 2 and 12 hours in each case.

Negative results were also obtained in a dominant lethal assay in mice given 30 and 60 mg/kg glutaraldehyde.

9. Carcinogenicity

No data are at present available from long-term bioassays. Long-term inhalation and skin painting studies are however apparently in progress as part of the NTP programme in the USA.

11. Conclusions

The available data indicate that glutaraldehyde is highly toxic by inhalation and moderately toxic when administered orally. Solutions of 1 % and above (using an occlusive dressing) produce skin irritation in humans. Studies in animals indicated that aqueous solutions of 2 % and above produce severe irritation, with no effects at 0.1 %. Experience in use of glutaraldehyde as a disinfectant in hospitals indicates that it has potential to induce skin sensitization. Occupational exposure has also occasionally induced respiratory sensitization.

The NOAEL in a 13 week toxicity study in rats with exposure through the drinking water, was 25 mg/kg. Growth retardation and signs of gastric irritation were seen at 100 mg/kg. In a 90 day inhalation study in rats the NOAEL was 0.02 ppm, with signs of mucosal irritation of the upper respiratory tract and reduced growth rate being noted at 0.05 ppm and above.

The mutagenic potential of glutaraldehyde has been extensively investigated. Negative results were obtained in most assays for gene mutation in bacteria. Negative data were also obtained in assays for gene mutation and chromosome damage in mammalian cells, in the sex linked recessive lethal assay in drosophila, and in *in vivo* liver UDS and dominal lethal assays.

The teratogenic potential of glutaraldehyde has been investigated in several species following oral administration. No adverse effects on the developing fetus were seen in rats and rabbits unless dose levels were used that were severely toxic to the maternal animals; the No Effect Level was 68 mg/kg and 15 mg/kg respectively. A similar value (24 mg/kg) was reported in mice, and no adverse effects were seen on the developing offspring at doses up to 24 mg/kg in a second study in mice. Another study gave less clear results.

Dermal absorption following application of a ca. 0.1 % solution under occlusive dressing in the rabbit was ca. 10 %. Lower values were obtained in the rat.

12. Safety evaluation

The NOAEL in the 90 day repeated dose oral toxicity study in the rat was 24 mg/kg/day; in the oral teratogenicity study in the rabbit (the most sensitive species) the NOAEL was 15 mg/kg/day. No teratogenic effects were seen unless severe effects were produced in the maternal animal. The critical NOAEL was taken as 15 mg/kg/day.

Exposure in use
Glutaraldehyde is used in both rinse-off and non-rinse off cosmetic products up to a concentration of 0.1 %.

Assuming an individual use all types of non-rinse off cosmetics (face cream, general purpose cream, body lotion, roll-on antiperspirant and hair styling products) and that they all contain glutaraldehyde, and furthermore they all are used extensively (rather than average use) the total exposure from such use will be to 20.3 grams a day [the value for 'normal' use would be 10.8 grams/day].

Making similar assumptions for rinse-off cosmetics (make-up remover, shower gel, shampoo, hair conditioner) the total exposure for an individual who uses all such products extensively is 1.7 grams assuming 10 % retention after rinse-off.

Thus an 'extreme' consumer using all types of products extensively, and assuming these contain glutaraldehyde at the maximum level would be exposed to 22 grams product per day.

This is equivalent to 22 mg glutaraldehyde per day.

Assuming 10 % dermal absorption this is equivalent to 2.2 mg absorbed per day, or 0.036 mg/kg.

SAFETY MARGIN

The safety margin for an extreme consumer is $\frac{15}{0.036} = 417$

This is acceptable.

Classification: A, but not to be used in aerosol sprays.

P 84: *SODIUM HYDROXYMETHYLAMINO ACETATE*

1. General

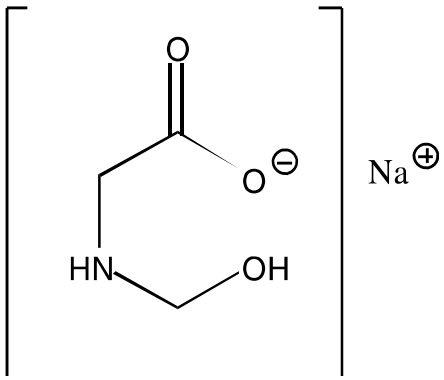
1.1 Primary name

Sodium hydroxymethylamino acetate

1.2 Chemical names

Sodium hydroxymethylamino acetate
(Sodium hydroxymethyl glycinate; Suttocide A)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_3H_6NO_3Na$
Mol weight: 127.1

1.9 Solubility

The compound is strongly alkaline, highly soluble in water, soluble in methanol, propylene glycol and glycerin, but insoluble in most organic solvents.

2. Function and uses

A preservative for use in cosmetics at concentrations of 0.05 % to 0.5 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}-values in rats were estimated to be 1.067 g/kg b.w., and 1.410 g/kg b.w. in two separate studies.

3.2 Acute dermal toxicity

The dermal LD_{50} in rabbits was > 2 g/kg b.w. The undiluted material applied dermally under occlusion caused a severe reaction to the skin probably as a result of the alkaline properties.

3.4 Repeated dose oral toxicity

A 28 day repeated dose study has been carried out in the rat with the compound administered orally by gavage at dose levels of 40, 160 and 640 mg/kg. There was a decrease in body weight gain in males at 640 mg/kg and in the serum total protein value which was outside the historical range of control values. There were some alterations in haematological parameters in this group which although within the range of historical controls were considered treatment related. Gross findings at necroscopy were reddening of the gastric mucosa in some animals at 640 mg/kg. Histological examination revealed 2 males and 5 females with focal subacute gastritis and 3 females with focal ulcerations at 640 mg/kg. There was also a death in this dose group, probably due to technical error but the possibility that it was compound related could not be ruled out. All other findings were considered coincidental or of no biological significance. The No Effect Level was 160 mg/kg.

3.7 Subchronic oral toxicity

In a subchronic oral toxicity study, 4 groups of 10 rats/sex received by gavage 0 (control), 10, 40 or 160 mg/kg b.w./day as a 2 % aqueous solution for 90 days. There were no clinical signs of toxicity or changes in body weight gain, food intake, haematology, clinical chemistry or urine examinations. Gross or microscopic examinations did not reveal any treatment-related effect. The No Effect Level in this study was thus the highest dose used, namely 160 mg/kg (as had been observed in the 28 day study which had been carried out after the 90 day study, primarily to identify toxic effects and target organs).

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin irritation tests in rabbits, showed a 5 % aqueous solution to be moderately irritating, while a 0.5 % solution produced only slight, transient irritation. In a repeated dermal application test, guinea pigs received 0.5 ml aqueous dilutions of 50, 7.5, 0.75 and 0.38 % under occlusion on days 1, 3 and 6 of one week period. No signs of oedema or irritation were observed.

4.2 Irritation (mucous membranes)

Eye irritation tests in rabbits conducted with 100 mg undiluted powder showed moderate irritation when the eye remained unwashed, and mild irritation when the eye was washed after treatment. A 5 % aqueous solution was mildly irritating if not washed out, and not irritating if washing was applied. Relatively mild, transient effects were also seen with a 50 % aqueous solution.

5. Sensitization

Sensitization was examined in guinea pigs, by the Landsteiner test, the maximization test and the Buehler test. In the Landsteiner test, 0.1 ml 0.1 % solution in saline was injected intradermally ten times, once every other day. After a two weeks rest period, the intradermal challenge injection of 0.05 ml 0.1 % solution did not reveal any sensitizing properties. In the maximization test, the induction treatment consisted of 6 intradermal injections of 0.1 ml 5 % solution, followed, 8 days later, by topical application of 0.3 g moistened powder. On day 22, a topical challenge treatment with a 50 % aqueous dilution produced a positive reaction in 7 out of 10 animals. When the challenge was repeated 7 days later, with 5 % and 0.5 %, 4/10 and 2/10 animals respectively reacted positively. These results indicate that the substance has some sensitizing properties. In the Buehler test, 0.5 ml 0.5 % aqueous solution was applied topically 10 times during 3 weeks. After 2 weeks rest, animals were challenged with a 0.5 % solution; there was no evidence of sensitization in any animal.

The ability of a 0.5 % solution of the substance to induce skin sensitization in human volunteers (102) has been investigated. The induction regime consisted of 9 patch applications (24 hour occlusion) over 3 weeks. There was no evidence of skin sensitization in any subject.

6. Teratogenicity

The ability of the compound to produce adverse effects on the developing foetus has been investigated in the rat. In a sighting study deaths were seen at 750 mg/kg, and thus dose levels of 150, 300 and 450 mg/kg were used in the main study. This study used 27 mated females per group given these dose levels on days 6 to 15. There was no significant difference in the percentage gravidity between the groups (92.6 % to 96.3 %). There were 2 maternal deaths at 150 mg/kg both attributable to technical error on dosing. There was post-dose salivation in some animals at all dose levels and there was also decreased activity at 450 mg/kg. There was 1 fetal death at 300 mg/kg which was considered coincidental. There were no significant differences in the total number of implantation sites, corpora lutea, viable and non viable foetuses, foetal sex distribution and body weight, early or late resorptions, number and percent of pre- and post-implantation loss. No soft tissue malformations were observed and there was no significant difference in the skeletal variations. Skeletal malformations were seen in 8 fetuses, 7 from one litter at 150 mg/kg; these were not considered treatment related. The study thus provided no evidence that the substance had any teratogenic potential. The No Effect Level in this study was 300 mg/kg with minor effects seen in the maternal animals at 450 mg/ kg.

8. Mutagenicity

A number of mutagenicity studies have been carried out on this substance. An Ames test using up to 0.5 mg/plate in 5 strains of *S. typhimurium*, with and without metabolic activation did not indicate mutagenic properties. A somewhat limited study to investigate unscheduled DNA synthesis (UDS) in rat hepatocytes using an autoradiographic method and concentrations up to 20µg/ml gave negative results. Higher concentrations were not used because of cytotoxicity. The positive control gave the expected result. The data obtained were not however confirmed in an independent experiment. Positive results were obtained in a metaphase analysis study for clastogenicity in CHO cells at 30 and 60 µg/ml in the absence of S9 and at 90 µg/ml in the

presence of S9. A clear increase in chromosome aberrations (including exchanges) was seen. Effects were confirmed in an independent experiment. Negative results were obtained in an *in vivo* bone marrow micronucleus test in mice. Animals were treated once orally with 375, 625 or 875 mg compound/kg b.w. Five mice of each sex per group were examined at 30, 48 and 72 hrs after treatment. No increased incidence of micronucleated cells was observed. No data are however available in any other tissue *in vivo*.

11. Conclusions

In summary sodium hydroxymethylamine acetate has low acute toxicity by the oral and the dermal route, and has marked irritant properties due to its alkaline nature. The in use concentration however produced no significant irritant effects. There was evidence of sensitization potential. Challenge with a 50 % solution induced sensitization in 70 % of animals in a maximization test; the corresponding value with a 0.5 % solution was 20 %. No data are available on sensitization in humans. The No Effect Level in repeated dose studies (28 and 90 days) using the oral route was 160 mg/kg; at 640 mg/kg signs of marked toxicity to the gastrointestinal tract were noted. Negative results were obtained in a Salmonella assay for gene mutation and in a limited assay for unscheduled DNA synthesis in hepatocytes *in vitro*. Positive results were however obtained in a metaphase analysis for chromosome damage in CHO cells. Negative results were obtained in an *in vivo* bone marrow assay for clastogenicity using oral doses up to 875 mg/kg. No *in vivo* mutagenicity data are available in other tissue. In a teratogenicity study in the rat using the oral route the compound gave no evidence of any adverse effects on the developing fetus at dose up to 450 mg/kg; slight effects on the maternal animals were seen at this dose level.

Classification: B

12. Safety evaluation

The NOAEL for sodium hydroxymethylamino acetate in a 90 day oral toxicity study was 160 mg/kg.

Exposure in Use

Industry have requested that this preservative be allowed for use in all types of product up to a maximum concentration of 0.5 %.

Total exposure, assuming an induvidual extensively uses all types of product and that all these products contain this preservative at the maximum permitted level will be 27.6 grams (5.54 g oral hygiene products, 21.06 g skin contact).

Total exposure to the preservative would be 138 mg/day.

No data are available on skin absorption as therefore 100 % absorption is assumed as a worst-case. Thus 138 mg are absorbed per day. This is equivalent to 2.3 mg/kg.

The safety margin = $\frac{160}{2.3}$ = 70.

The exposure data are based on extreme use and this is considered acceptable. However data from an *in vivo* assay to measure UDS in the liver are needed to provide adequate reassurance that activity seen *in vitro* is not expressed *in vivo*.

P 90: *7-ETHYLBICYCLOOXAZOLIDINE*

1. General

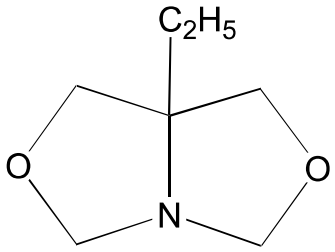
1.1 Primary name

7-Ethylbicyclooxazolidine

1.3 Trade names and abbreviations

Oxaban-E,
Bioban/Amine CS-1246,
Zoldine ZE,
Oxazolidine E,
P1601

1.5 Structural formula



1.7 Purity, composition and substance codes

The material is specified by Colipa as being >97.5 % pure, however the substances tested did not reach this specification, with purity being as low as 96 %. The major impurities were 4, 4-dimethyl oxazolidine and 4-ethyloxazolidine (2 %) and water (2 %).

1.8 Physical properties

Chemical property:

Oxaban E is a formaldehyde releasing preservative. Analysis of hand cream containing 0.15 and 0.3 % compound revealed levels of free formaldehyde of 329 and 812 ppm respectively. The corresponding values for a shampoo containing similar amounts of Oxaban E was 331 and 308 ppm respectively.

1.9 Solubility

The compound is soluble in water and most organic solvents, exceptions are cyclohexane and 1, 4-dioxane.

2. Function and uses

The proposed use in cosmetics is in none-rinse-off products, excluding oral hygiene and mucous membrance products, at concentrations up to 0.3 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

The compound has low acute toxicity. Oral LD_{50} values in rats are 3.7 g/kg b.w. (females) and 5.3 g/kg b.w. (males). The dermal LD_{50} value in rabbits was 1.95 g/kg b.w. (combined value for abraded and unabraded skin). An inhalation (aerosol) LC_{50} of 3.1 mg/litre air was obtained from a 4h, whole body exposure of male and female rats to an aerosol mist of mass median aerodynamic particle size in the range 3.9-4.7 µm. Based on the acute toxicity data, dermal and oral absorption appears to be of a similar order, though no studies of absorption/permeation were performed.

3.4 Repeated dose oral toxicity

In a 28 day oral study, groups of 5 rats/sex received 100, 300 or 1000 mg/kg b.w./d given in deionised water. Both 1000 and 300 mg/kg b.w./d produced local effects on the stomach, indicative of an irritant effect. Significant changes in many haematological and clinical chemistry parameters were seen in both sexes receiving 1000 mg/kg b.w./d: evidence of anaemia, increased WBC count with neutrophil and lymphocyte numbers increased, thrombocyte numbers were increased, serum potassium and phosphate levels were increased, with glucose levels reduced. Increases in relative organ weights were seen for liver, adrenals, testes and kidneys; absolute values for adrenals were increased in both sexes, despite the reduced body weight seen at 1000 mg/kg b.w./d. Microscopic examination was limited with no changes reported in spleen, liver, kidney, adrenals, heart and testes. Total protein levels were reduced in males. In the 300 mg/kg b.w./d groups there was evidence of anaemia in males and of increased thrombocyte counts in both sexes. Serum phosphate levels were increased in both sexes whilst glucose levels were reduced in both sexes (not statistically significant in females). Adrenal weight was significantly increased in females. At 100 mg/kg b.w./d non significant decreases in serum glucose and WBC numbers were seen in both sexes together with evidence of increased adrenal weights. Though there was some evidence of compound related effects at the lowest dose these were not statistically significant and 100 mg/kg b.w./d may be taken as the no effect level.

3.5 Repeated dose dermal toxicity

A 21 day dermal toxicity study in rats (n = 6/sex/group) at dose levels of 30, 100 or 300 mg/kg b.w./d applied daily for 5d/week in a deionised water solution (0.75, 1.25 or 3.75 % w/v) produced dose-related irritation. Eschar formation was observed in the majority of top dose animals and in 3 males and 2 females from the mid-dose group. Dose related increases were seen in relative adrenal weights in females and GPT in both sexes, though these did not achieve

statistical significance. A dose related increase in relative kidney weights was seen in females, reaching statistical significance at the top dose.

4. Irritation & corrosivity

4.1 Irritation (skin)

A test of primary skin irritancy in rabbits using 0.5 g of undiluted material of unknown purity produced severe irritation following 24 h occluded exposure. A 4 h occluded exposure produced signs of mild irritation, but no tissue destruction. Repeated application (21 days) of a 0.7 % solution (6.7 mg/ml) produced erythema in rats. Repeated application of a 0.3 % solution (12 applications over 3 weeks) produced mild irritation in one of 100 human volunteers.

4.2 Irritation (mucous membranes)

A preparation of unknown specification (probably > 96 %) was a severe irritant to the rabbit eye, with washed eyes showing more damage than unwashed. The effects persisted for 7 days. No dilutions were tested.

5. Sensitization

A skin sensitization test performed by the Buhler method was defined as inconclusive (no details given. A second test using the Landsteiner and Jacobs procedure (a non-adjuvant technique) showed a 0.5 % solution to be non-sensitising. More recently full details of a test in guinea pigs using the Magnusson-Kligman Maximisation procedure have been provided. The induction concentrations used were 5 % for the intra-dermal injection and 25 % for the topical application, with challenge concentrations of 0.5 and 1.0 % topically (a concentration of 5 % produced transient irritant effects). There was no evidence of sensitization in any animal. A study in 100 human volunteers found no sensitization potential following repeated application of a 0.3 % solution.

6. Teratogenicity

In a teratogenicity study groups of 25 mated female rats received 50, 250 or 650 mg/kg b.w./d on days 6-15 of gestation, by gavage in deionised water. There were clear effects at 650 mg/kg b.w./d: decreased maternal body weight and increased incidences of malformation (eg cleft palate and umbilical hernia of intestines). The fetal effects were concentrated in 4 litters including the dam with lowest body weight gain. No increases in malformations or variations were recorded at the mid and low doses. The NOAEL in this study was 250 mg/kg b.w./day.

8. Mutagenicity

Regarding mutagenicity studies, results from a well performed Ames test showed no evidence of mutagenicity at concentrations between 6 and 600 µg/plate $\pm$ rat liver S9; cytotoxicity was evident at 300 µg/plate and above.

An assay for chromosomal aberrations in CHO cells showed no evidence of clastogenicity with rat liver S9, but without S9 mix a slight increase in the numbers of cells with aberrations and in the numbers of aberrations per cell were reported. No details of individual cultures or types of aberrations are given, making it difficult to assess the significance of the results. The concentrations used were 0.5 to 4 µl/ml, with only slight cytotoxicity (about a 15% decrease in mitotic index) seen at the top dose. Furthermore only one harvest time was investigated, and the results were not confirmed in an independent experiment. Results of a second study in CHO cells are available. Again only low concentrations could be investigated due to the marked cytotoxicity of the compound. Concentrations of 0.0125 µg/ml - 0.1 µg/ml were used in the presence of S9 and 0.0125 - 0.05 µg/ml in the absence of S9. Higher concentrations resulted in complete inhibition of mitosis. There was no evidence of any significant increase in chromosome aberrations. Taken together these results indicate that Oxaban E does not have significant clastogenic potential in CHO cells.

An assay for unscheduled DNA synthesis in rat primary hepatocytes showed increased activity at all concentrations (0.25 - 4 µl/ml) but this was not dose-related or significant and results were negative.

11. Conclusions

Oxaban E has low acute toxicity by the oral and dermal routes and appears to have appreciable absorption through the skin. The compound itself produced marked skin and eye irritancy, but no significant skin irritation was seen at 0.3 % in human volunteer studies. There was no evidence of skin sensitization in a number of animal models including the Magnusson Kligman maximization test, nor in 100 human volunteers when subject to repeated patch testing at 0.3 %. In a 28 dietary study in the rat marked haematological effect were seen (anaemia, increased thrombocytes) together with gastric lesions, presumably due to the irritant effects of the compound, at 1000 mg/kg. The NOAEL was 100 mg/kg. In a 21 day dermal study in the rat, no effects were seen at 100 mg/kg apart from local skin irritancy. The NOAEL was again 100 mg/kg. There was no evidence of mutagenicity in assays for gene mutation in Salmonella, clastogenicity in CHO cells and unscheduled DNA synthesis in hepatocytes. In an oral teratogenicity study evidence of adverse effects on the developing fetus were seen only at doses producing effects on the maternal animals. The NOAEL was 250 mg/kg in these studies.

Classification: A

12. Safety evaluation

Calculation of Safety Margins
The NOAEL for Oxeban E in a 28 day repeated dose toxicity study in the rat was 100 mg/kg.

Exposure in use
Oxeban E is used only in non-rinse off cosmetic products at up to 0.3 %.

Assuming an individual use all types of non-rinse off cosmetics (face creams, general purpose creams, body lotions, roll-on anti-perspirants and hair styling products) and that they all contain Oxeban E, and furthermore they are all used extensively (rather than average use) the

total exposure from such use will be 20.3 grams product/day. Assuming that all products contain the maximum amount of preservative, namely 0.3 % this is equivalent to 60.9 mg Oxeban E.

Since no data are available on skin absorption, for the purposes of this safety evaluation 100 % absorption is assumed.

Thus absorbed dose per day is 60.9 mg, which is equivalent to about 1 mg/kg/day.

The safety margin thus $= \frac{100}{1} = 100.$

This is based on very conservative assumptions on use and is acceptable.

P 91: *3-IODO-2-PROPYNYL BUTYL CARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butyl carbamate

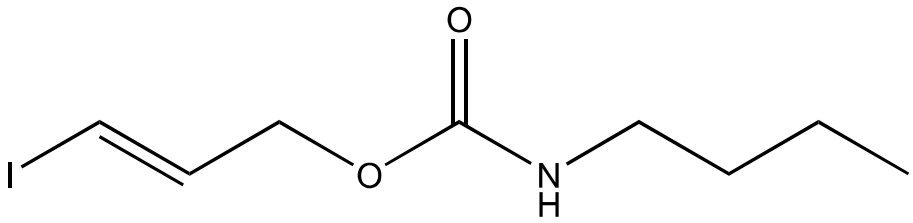
1.2 Chemical names

3-iodo-2-propynyl butyl carbamate
iodo propynyl butyl carbamate

1.4 CAS no.

55406-53-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}NO_2I$
Mol weight: 281

1.9 Solubility

It has low solubility in water (156 ppm at 20°C) and is soluble in organic solvents.

2. Function and uses

It is proposed for use as a preservative in all types of cosmetic products at up to 0.1 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal

signs of toxicity were seen at 500 mg/kg or below. In a percutaneous toxicity study in rabbits a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing resulted in no deaths. The only signs of toxicity seen were slight irritant effects at the site of application.

3.7 Subchronic oral toxicity

In a sub-chronic study rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg. Histological examination showed hepatocyte enlargement at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The no-effect level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritancy study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe oedema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours.

4.2 Irritation (mucous membranes)

Severe effects were noted in an eye irritation study in rabbits. The substance (0.1g) produced moderate to severe hyperaemia, chemosis and discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. If the compound was washed out of the eye 20-30 seconds post instillation only transient irritant effects were seen.

The eye irritancy of a 0.5 % solution of IPBC in corn oil has been tested in rabbits and also the effects of 0.5 % IPBC in a baby shampoo. Groups of 6 animals were used in each case. No signs of any irritant effects were seen with the corn oil formulation. In the case of the baby shampoo, signs of slight irritant effects were seen for about 24-48 hours, but similar effects were seen in the 'control' baby shampoo that did not contain IPBC. Thus 0.5 % IPBC in corn oil or in a baby shampoo formulation produced no eye irritation.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximisation test. Induction concentrations were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson & Kligman maximisation tests have been carried out on formulations containing 0.5 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical)

were used. In the second case the concentrations were 0.1 % and 0.5 % respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation. There was no evidence of sensitization in either test. These studies suggest that the compound does not have any significant potential for skin sensitization.

6. Teratogenicity

Teratogenicity studies have been carried out in both the rat and the mouse. In the study in rats compound was given on day 6-15 or gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The no-effect level was 50 mg/kg. A similar dosing regime was used in the study in mice. No compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The no-effect level was 50 mg/kg.

6.2 Two-generation reproduction toxicity

A two generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week pre-mating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound related effects were seen at any dose level on clinical chemistry or at necropsy. Reduced weight gain was seen in the males at 300 ppm and above in the initial generation during the pre-mating period and at 750 ppm in the females. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index at 750 ppm; postnatal growth of the offspring however was not affected. No effects were seen on the development of the offspring. The no-effect level in this study was 120 ppm test compound in the diet. (This dietary level is roughly equivalent to a dose of the order of 10 mg/kg body weight). No marked effects were seen on fertility or general reproductive performance at any dose level.

7. Toxicokinetics (incl. Percutaneous Absorption)

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C radio-labelled material. Following iv administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the liver and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicates that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

8 Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies. Negative results were obtained in the Salmonella assay versus strains TA1535, 1537, 1538, 98

and 100 but this study was limited by investigating only 3 concentrations (6.2-55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537, 98 and 100 and concentrations of 1-1000µg/plate against TA 1535. In all cases the top concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 %-25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

The compound is a carbamate and studies have been carried out to investigate whether significant blood cholinesterase inhibition occurs in the rat following intravenous administration. The compound was given in PEG/400: water vehicle at 2-16 mg/kg and blood samples taken and analysed for erythrocyte cholinesterase activity at 15, 30, 60 minutes and 2 and 5 hours post dose. No effects on blood cholinesterase levels were observed.

11. Conclusions

The substance has moderate acute toxicity by the oral route and low toxicity following dermal exposure. It is a mild to moderate skin irritant, but it is a severe (corrosive) eye irritant; however concentrations of 0.5 % do not produce any eye irritation. Negative results were obtained in 3 Magnusson & Kligman maximization tests for skin sensitization. In a sub-chronic (90 day) oral study in the rat the No Effect Level was 50 mg/kg.

Mutagenic potential has been investigated in Salmonella assays for gene mutation and in a study to investigate Unscheduled DNA Synthesis (UDS) in hepatocytes. Negative results were consistently obtained. There was no evidence for any teratogenic potential in studies in 2 species (rat and mice) nor for any significant effects on reproductive performance in a two generation fertility study in rats. The compound is well absorbed orally but is rapidly metabolised and excreted.

No further toxicity data are required. Calculations of safety margins are based on the maximum permitted concentration being 0.1 % in all cosmetic products.

Classification: A (for use at 0.1 %)

12. Safety evaluation

The NOAEL for 3-iodo-2-propenyl butyl carbamate in a 90 day repeated dose oral toxicity study in the rat was 50 mg/kg.

Exposure in Use

Maximum concentration 0.1 % in all types of product.

Total exposure, assuming extreme worst-case scenario (all products contain this preservative at the maximum permitted amount and all are used extensively) is to 27.6 g/day (from 27.6 grams product comprising 5.54 g oral hygiene and 21.06 g skin contact).

No data are available on skin absorption, although the results obtained in acute toxicity studies suggest a lack of significant absorption. However assuming 100 % absorption, the worst-case scenario would indicate absorption of 27.6 mg preservative per day.

This is equivalent to 0.46 mg/kg.

The Safety Margin = $\frac{50}{0.46}$ = 108.

This is acceptable.

S 8: *2-ETHYLHEXYL-P-DIMETHYLAMINOBENZOATE*

1. General

1.1 Primary name

2-ethylhexyl-p-dimethylaminobenzoate

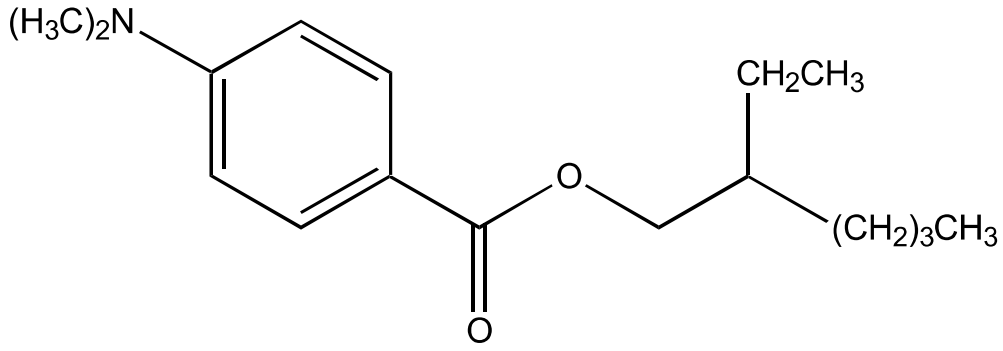
1.2 Chemical names

2-ethylhexyl-p-dimethylaminobenzoate

1.3 Trade names and abbreviations

Padimate O
Escalol 507

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{17}H_{27}O_2N$
Mol weight: 277.4

1.7 Purity, composition and substance codes

The substance is stated by the manufacturer to contain not less than 98.5 % of active ingredient. Maximum absorption 310 nm. Not known to polymerise.

1.8 Physical properties

Appearance: Yellow fluid.

1.9 Solubility

Soluble in isopropyl alcohol, mineral oil, and ethanol. Insoluble in water.

2. Function and uses

Use level: up to 8 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat: Values for the oral LD_{50} varied from 3 to 15 g/kg b.w.

3.8 Subchronic dermal toxicity

Rabbit. A 13-week dermal toxicity study was carried out in groups of 20 animals at dose levels of 140 and 280 mg/kg b.w. No significant abnormality was detected.

Rat. A 28 day oral study was carried out according to GLP. Groups of 10 male and 10 female rats were used, and the doses of a.i., given daily by gavage, 7 days a week, were 0, 100, 300 and 1000 mg/kg b.w./day (groups 1, 2, 3 and 4, respectively). Groups 1 and 4 also contained 5 additional animals of each sex, which were treated identically with the other animals in their groups, except that they were not sacrificed for some 4 weeks after the cessation of dosing, to study recovery.

There was one death, in a female animal of group 1, following venepuncture. Clinical observations showed little change, except for salivation among group 4 animals after dosing. Body weight was depressed in group 4 males in weeks 3 & 4, and this did not recover during the drug free follow up period (DFFU). Mean body weight gain was also depressed in these animals throughout the dosing period. There was no significant effect on food consumption during the treatment period, but there was a temporary fall in DFFU animals, particularly in males, during the recovery period.

All animals were subject to necropsy, and microscopic examinations of a considerable number of organs was carried out in animals of groups 1 & 4, as well as histological examination of all macroscopic lesions. Blood was sampled before sacrifice. Ophthalmoscopy was carried out before the experiment and in week 4, with the use of a mydriatic.

The chief abnormal findings were:

(a) Blood. Haemoglobin, haematocrit, and mean corpuscular volume were decreased in males of group 4; in females, the same findings were made, except that MCHC was also reduced in these animals. All these findings were normal in DFFU animals. Alanine aminotransferase was increased in males of group 4, and this finding persisted in DFFU animals. In males of group 4 there was a fall in globulin, and in both males and females a rise in the A/G ratio; total bilirubin was raised in male animals of group 4, and triglycerides and gamma glutamyl transferase in females of group 4; but these changes were not found in DFFU animals.

(b) Macroscopic pathology. Females of group 4 showed roughness of the splenic capsule in 3/10 animals. The testis was soft in 4/10 animals of group 4, and small in 8/10 animals of the

same group. There were no abnormalities in prostate, epididymes or seminal vesicles. In DFFU animals the testes were small in 3/5 animals of group 4.

(c) Organ weights. (i) Mean absolute weights: spleen increased in group 4 females; liver increased in females of group 3, and in males and females of group 4; testis and pituitary reduced in males of group 4. In the DFFU animals the mean weight of testis was reduced in group 4 animals. (ii) Relative weights, organ to body weight: spleen and liver increased in males and females of group 4; pituitary increased in group 4 females. (iii) Relative weights, organ to brain weight: spleen and kidney increased in females of group 4; liver increased in males of groups 2 & 4 and females of groups 3 & 4; testis & pituitary decreased in males of group 4. In recovery animals, the kidney weight was reduced in males of group 4, but there were no changes in the testes or pituitary weights.

(d) Histopathological findings. In male animals of group 4, there was moderate or moderately severe testicular atrophy in all animals, and epididymal oedema in 7/10. There was slight splenic pigmentation in males in group 4, more marked in females of group 3, and severe in females of group 4. The histological appearance of the pituitary gland was not remarkable. In recovery animals, the histopathology of the testis was normal, but hypospermia was noted in treated animals.

This seems to have been a well conducted study, with a clear effect. The investigators put the NOAEL at 100 mg/kg b.w./day, in view of the pigmentation of the spleen. This may be thought unduly conservative, in which case a NOAEL of 300 mg/kg b.w./day might be considered.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Solutions of 5 % a.i. were applied to both intact and abraded skin for 24 hours under occlusion. The test was negative.

Man. Occlusive patch tests with 5 % a.i. in yellow soft paraffin were applied for 48 hours. There was no adverse reaction.

4.2 Irritation (mucous membranes)

Rabbit. A Draize test using concentrations of 2 % and 5 % in mineral oil showed slight transient irritation.

5. Sensitization

Guinea pig. Ten male animals had an initial intracutaneous injection of 0.05 ml of a 0.1 % solution of a.i. in saline, followed by 9 injections of 0.1 ml 3 days a week. After a 12 week rest period, a challenge dose of 0.05 ml was given. There was no evidence of sensitization.

Man. (a) Fifteen applications of a 4 % solution of a.i. in soft paraffin were made under occlusion over 3 weeks. A challenge application was made after a 2 week rest. There was no adverse reaction.

(b) A mixture of 7 % a.i. with 3 % oxybenzone was used in 150 subjects in a repeated insult patch procedure. No abnormality was found.

(c) Ninety subjects were similarly tested using 8 % a.i. with 3 % benzophenone. The test was negative, although there were occasional slight irritant responses during induction.

(d) A panel of 156 subjects was similarly tested with 7 % a.i. in soft paraffin. The test was negative.

6. Teratogenicity

Rat. Dermal applications of 2 ml/kg b.w. of a preparation (concentration of a.i. not specified) were made daily from days 6 to 16 of pregnancy. In the test group 7/56 foetuses had bilateral wavy ribs and 2/56 had unilateral wavy ribs. There were no such findings in the control group. This effect is not regarded by the authors as indicating teratogenic activity, as they consider it a common finding in rats of this strain, but the reason for its appearance in foetuses of the test group only is unexplained.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man. An 8 % ethanolic solution of ^{14}C labelled a.i. was applied over 100 cm^2 of forearm skin in 4 male and 4 female subjects. After the ethanol had dried, the areas were covered with a gauze pad for 24 hrs. Blood was sampled at times 0, 2, 4, 8, 24, 48 and 72 hours. Three consecutive 24 hour collections of urine were made. No radioactivity was found in the blood; the urine contained (mean percentages of total radioactivity) 2.45 % (males) and 1.18 % (females). The main amount of radioactivity was found in the washings of the skin in the areas of application. The mean recovery of radioactivity was 95.7 %. Assuming a body area of 1.8 m^2, a concentration of a. i. of 8 %, and an application of 0.5 mg of formulation per cm^2, the total amount absorbed may be calculated as 13 mg, or about 0.2 mg/kg b.w. Taking the NOAEL found in the subchronic oral study as 100 mg/kg b.w./day, the safety factor may be calculated as about 500.

8. Mutagenicity

A standard Ames test was negative. A second similar test is also reported negative, but figures are given for plates with activation only.

A chromosomal aberration test was carried out in human lymphocytes. The study was carried out in accordance with GLP standards. Suitable positive, negative and solvent controls were used. The assays were carried out with and without metabolic activation using S9 mix. Following a dose ranging study, levels of a. i. of 315 to 5010 µg/ml of a. i. were used for testing. The solvent was ethanol. Experiments, with or without activation, were carried out in duplicate, with 2 experiments using harvesting at 24 hrs, and 1 at 48 hrs. The positive controls gave marked effects. The test compound showed no evidence of capacity to produce chromosomal aberrations.

A micronucleus test was carried out in the mouse, using a dose which caused disorders of gait and hypotonicity. The a.i. was given intraperitoneally in a dose of 5000 mg/kg b.w. to 3 groups of 10 animals. Positive and negative control groups were included. Sacrifice was at 30, 48 and 72 hours. The test was negative.

10. Special investigations

Phototoxicity

Guinea pig. The ears of 10 animals were depilated and a formulation containing 7 % a.i. and 3 % oxybenzone was applied several times to one ear with vigorous rubbing. The untreated ear

served as a control; 2 of the animals had 8-methoxypsoralen applied as a positive control. Thereafter the animals were exposed to UV radiation (wavelength not stated) for 2 hrs. The test was negative; the positive controls showed marked effects.

In another test, a similar preparation was applied to the nuchal area with occlusion for 2 hrs. This was followed by irradiation with 3 J/cm^2 at 320-400 nm. Suitable positive and negative controls were used. The test was negative.

Man. In a poorly reported test, a mixture of 7 % a.i. and 3 % oxybenzone was tested in 26 human subjects. No adverse effects were seen.

In another similar test, a 5 % ethanolic solution was used. At 30 J, the control area showed more damage than the test area.

Ten fair-skinned subjects were treated with a mixture of 7 % a.i. and 2 % oxybenzone under occlusion for 24 hrs. A control was similarly applied. After removal of the patches, a further application was made to the skin and irradiation was carried out using 1 m.e.d. of UVB followed by 12 minutes of UVA. The test was negative.

11. Conclusions

Evaluation
Acute toxicity is low. A 13 week dermal test in the rabbit showed no effect up to 280 mg/kg b.w./day. A well conducted 28 day rat oral study showed a no adverse effect level of 100 mg/kg b.w./day. Tests for irritation of the skin and mucous membranes were carried out at less than the proposed use level. A test for sensitization in guinea pigs was carried out with a low concentration, which did not cause any irritation. Tests in man, in the range of use level, did not show any sensitization. In one of the tests for phototoxicity in the guinea pig, the dose of radiation and its wavelength are not given and the application tested contained oxybenzone as well as the a. i. In the second test, the dose of radiation (3 J/cm^2) was small, and the wavelength used was 320-400 nm, which is inappropriate for a UVB blocker. In the tests in man, no figure is given for the amount of UVA irradiation. On the whole, the tests presented for phototoxicity and photosensitivity are poor, but seem to be negative. An Ames test and a test for chromosomal aberration *in vivo* were negative. Tests for photomutagenic activity have not been carried out. The experimental procedure used in the test for teratogenic activity was unsatisfactory, and the results are anomalous. Tests for percutaneous absorption suggest that about 0.2 mg/kg b.w. may be absorbed; taking this figure in conjunction with the no effect level found in the oral rat study, a safety factor about 500 may be calculated.

It is believed that numerous further investigations have been carried out with this compound (e.g., mouse embryo fibroblast repair test); these should be submitted.

Classification: C.

S 28: *2-ETHYLHEXYL-4-METHOXYCINNAMATE*

1. General

1.1 Primary name

2-ethylhexyl-4-methoxycinnamate

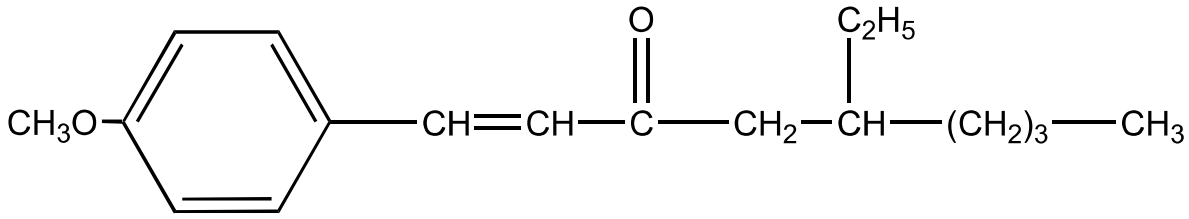
1.2 Chemical names

2-ethylhexyl-4-methoxycinnamate

1.3 Trade names and abbreviations

Parsol MCX

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{18}H_{26}O_2$
Mol weight: 290

1.8 Physical properties

Appearance: Colourless pale yellow slightly oily liquid.

1.9 Solubility

Miscible with alcohols, propylene glycol, etc.
Immiscible with water.

2. Function and uses

Use level up to 10 %.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}: Mouse, greater than 8 g/kg b.w. Rat, greater than 20 ml/kg b.w.

3.4 Repeated dose oral toxicity

Rat. Three week oral study. Groups of 5 male and 5 female animals were given 0, 0.3, 0.9 and 2.7 mg/kg b.w./day by gavage for 3 weeks. All animals of the top dose groups exhibited loss of body weight and a reduced relative and absolute weight of the thymus. Male rats showed a decrease in absolute weight of the left kidney and female rats showed a decrease in the absolute weight of the heart. At the two lower doses, the only significant alteration observed was an increased absolute weight of the pituitary gland in male rats receiving the lowest dose. As the number of animals was small, the investigators considered this not to be biologically significant. The NOAEL was put at 0.9 ml/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Four groups of 12 male and 12 female SPF rats received the compound in the diet at levels of 0, 200, 450 and 1000 mg/kg b.w./day. During the experiment the usual clinical observations were carried out, as well as extensive haematological and biochemical studies. Full gross necropsy was carried out on all survivors. Histological investigations were carried out in half the animals of the control and top dose groups. The organs studied included the heart, lungs, liver, stomach, kidneys, spleen, thyroid and retina. In the remaining animals histological examination of the liver only was carried out. Six control animals and 6 top dose animals were allowed to recover over 5 weeks, and then examined.

The results of the experiment showed no dose related mortality. The kidney weights of top dose animals were increased, but were normal in the recovery animals; the increase was attributed to a physiological response to an increased excretion load. There was a diminution of glycogen in the liver, and a slight increase in iron in the Kupffer cells in the high dose animals. Two of these also showed minimal centrilobular necrosis of the liver with some infiltration; similar less marked findings were made in 2 of the control animals as well. These findings were attributed to infection. High dose females had increased GLDH which reversed during the recovery period. The NOAEL was put at 450 mg/kg b.w./day.

3.8 Subchronic dermal toxicity

Rat. Thirteen week dermal study. Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55.5, 277 and 555 mg/kg b.w./day applied to shaved skin 5 days a week for 13 weeks. (The top dose is believed to be about 135 times the amount which would be used daily by the average consumer). Various laboratory and clinical tests were carried out during the experiment.

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination. The NOAEL is 555 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Guinea pig. The a.i. was applied undiluted twice daily to 20 animals for 16 days. There were no signs of irritation.

Man. Occlusive applications of undiluted a.i. were made to 60 subjects, of whom 20 had sensitive skin. The applications were made for 24 hours. Observations at removal of the patches, and 24 and 48 hours later, showed no evidence of a reaction.

In 51 male and female subjects, similar patch tests were carried out. The dilution of the a.i. (if any) was not stated. There was no irritation.

A formulation (concentration not stated) tested on the skin of 50 subjects caused no adverse effect.

In 53 subjects, a Draize repeated insult patch test at a concentration of 2 % caused no irritation.

In 54 subjects, a Draize repeated insult patch test of a 7.5 % dilution of a.i. in petrolatum caused no irritation.

4.2 Irritation (mucous membranes)

Rabbit. Groups of 4 animals had 0.1 ml of a test preparation instilled into the conjunctival sac (concentration not stated). No further treatment in one group; in the other, the instillation was followed by washing out. There were no signs of irritation.

A Draize test carried out with undiluted a.i. was found to be practically non-irritant.

5. Sensitization

Guinea pig. Twenty animals received applications of undiluted a.i. twice daily for 16 days. After a 3 day interval without treatment, a daily challenge application was made for 3 days. There was no evidence of sensitization.

Two groups of 4 animals were used. Animals of one group were exposed to 0.05 ml injections of undiluted a.i. daily for 5 days. In the other group, 0.025 ml of a 50 % acetone solution of a.i. was applied to 2 cm^2 areas of shaved skin on either side. There was no evidence of sensitization.

Man. A Draize repeated insult patch test was carried out at a concentration of 2 % in 53 subjects. There was no sensitization.

In 54 subjects, a formulation of 7.5 % a.i. in petrolatum was applied for 48 hours under occlusion for 11 applications. After a 14 day rest, a challenge application of a single dose was made. There was no adverse reaction.

In an extensive series of patch tests carried out in man, the a.i. was found to be very rarely responsible for allergic contact effects.

A 10 % solution of a.i. in dimethylphthalate was used. A total of 58 subjects was recruited, 12 males and 46 females, aged 18-63. Of these, 6 subjects failed to complete the test for reasons unconnected with the experimental procedure.

Induction applications were made on the skin of the back, for 24 hours with occlusion, 3 times a week for 9 applications. Following a rest period of 2 weeks, a further patch was now applied to a new site on the back for 24 hours with occlusion. The area was inspected at 0, 24 and 48 hours after removal of the patch. No adverse reaction was noted at any stage of the experiment.

6. Teratogenicity

Rabbit. Groups of 20 female animals were mated and given a.i. in doses of 0, 80, 200 and 500 mg/kg b.w./day by gavage during the period of organogenesis. Except for a slight reduction of maternal and foetal weight in the top dose animals, no abnormality was found.

Rat. Following a pilot study, groups of 36 rats were mated and treated with 0, 250, 500 and 1000 mg/kg b.w./day of a.i. (probably by gavage) during days 6-14 of pregnancy. Owing to an error, the preparation of the control foetuses led to their destruction, so this part of the test was repeated under identical conditions. Subgroups of each dose group were allowed to litter normally and rear the offspring. The percentage of resorptions in the high dose group was elevated by comparison with the other groups. The investigator records, however, that this relatively high rate is the usual one with this strain of rat in this laboratory, and he attributes the difference to an unusually low level of resorption in the other groups. No other abnormality was found.

7. Toxicokinetics (incl. Percutaneous Absorption)

Tests for percutaneous absorption

(a) *In vitro* tests.

Rat. Naked rat skin. This was studied in a chamber experiment. The investigators used a 1 % solution of a.i. in Carbitol and the amounts applied were 120, 360 and 1200 µg/cm^2. Most of the material was found in the stripped skin; there was less in the stratum corneum, and least in the chamber. The approximate amounts found in the chamber were: after 6 hrs, 1.13 %; after 16 hrs, 11.4 %; and at 24 hrs 17.9 %. The figures for the horny layer and the strippings combined were, respectively, 31.4 %, 44.4 % and 45.7 % (percentages of applied doses). The amount of a.i. applied did not seem to affect the results.

In another set of experiments, various amounts of ”Parsol 1789“ (4-tert-butyl-4’-methoxydibenzoylmethane) were added to the a.i. in the formulation. There seemed to be no effect on the absorption of the a.i.

Pig. A similar experiment using mini-pig skin was carried out in which ”Parsol 1789“ was used as well as the a.i. Using 3 sorts of formulation, about 3 % of a.i. was found in the chamber in 6 hrs. Using the concentrations proposed for a particular commercial use (i.e., 2 % of ”Parsol 1789“ and 7.5 % of a.i.) about 2.2 % of the amount of a.i. applied was found in the chamber. It is calculated by the authors that the total absorption for a 75 kg consumer would be about

70 mg, or 0.9 mg/kg b.w. This figure may be too high; a different calculation gives a value of 0.2 mg/kg b.w.

Man. A test on human abdominal skin in a chamber was carried out. With 7.5 % a.i., about 0.03 % is found in the chamber in 2 hours, 0.26 % in 6 hours, and 2.0 % in 18 hours. Various combinations of a.i. and "Parsol 1789" were investigated. A calculation shows that these results might indicate an absorption of about 0.2 mg/kg b.w. in use.

(b) *In vivo* tests.
Man. Eight healthy volunteers had small amounts of radioactive a.i. applied to the interscapular region. One group of 4 had the material applied under a watch glass; the other 4 had it applied on gauze, with occlusion in one case. Tests for absorption of a.i. were negative except for about 0.2 % in urine. The concentrations used were not stated.

In a preliminary experiment, a capsule containing 100 mg of a.i. was taken orally. As a lipophilic substance, the a.i. is very likely to be metabolised; it is known in any case to be hydrolysed by plasma esterases, although slowly. The cumulative excretion of 4-methoxycinnamate in the urine over 24 hours was studied by GC/MS of the methyl ester derivative (This method would also detect 4-hydroxycinnamic acid). Over 24 hours, an amount of cinnamate was found in the urine equivalent to about one-fifth of the amount that would have been accepted if all the dose of a.i. had been absorbed. Nearly all of the metabolite was found in the first 6 hours.

In the main part of the experiment, an o/w cream containing 10 % a.i. was used. Applications of 2 grams of this material (= 200 mg a.i.) were made to the interscapular area of each of 5 male subjects, aged 29 to 46. The area of skin covered was 750 cm^2. After application, the area was covered with 3 layers of gauze, left in place for 12 hours. Blood was taken at times 0, 0.5, 1, 2, 3, 5, 7, and 24 hours. Urine was collected at 0, 2, 3, 4, 5, 6, 7, 12, 24, 48, 72 and 96 hours.

The control plasma samples showed a level equivalent to about 10 ng/ml before any application had been made. There was no evidence of any rise in plasma levels during the experiment. The urine showed a "physiological" level of 100 to 300 ng/ml. No significant increase in this amount was found in any sample. The experiment seems to have been carefully conducted. The authors conclude that very little, if any, of the compound was absorbed after application to the skin, compared with the reasonably well marked absorption after ingestion.

8. Mutagenicity

Salmonella mutagenesis assays were performed on the usual strains. There was a positive result with TA 1538 without metabolic activation. This was thought to have been a batch effect. From another laboratory, a very weak positive was found with TA 1538 without activation, at 10 µl/plate; it was not found in 2 replicates, nor in a second Ames test.

A test for mutagenesis and crossing over in *S. cerevisiae* was negative.

A test using Chinese hamster V 79 cells showed a very slight increase in mutant colonies with dose.

A test in human lymphocytes *in vitro* was negative.

A test for cell transformation in Balb/c 3T3 cells was negative.

A test for unscheduled DNA synthesis was negative.

Feeding tests in *Drosophila*:

There was an increase in the frequency of sex-linked recessive lethals; this was attributed with fair certainty to a batch effect.

There was no evidence of mutagenicity in feeding tests (adults and larvae).

Somatic mutation and combination tests using wing structure were negative.

Mouse. A standard micronucleus test was carried out. No effect was found up to 5000 mg/kg b.w.

Test for photomutagenic activity

These were carried out in cells of *S. cerevisiae*, which had previously been shown not to be affected by a.i. (*supra*). Doses of a.i., dissolved in DMSO, ranged from 0.06 to 625 µg/ml, and radiation up to 500000 J m^{-1} UVA and up to 12000 UVB (50 and 1.2 J cm^{-1}). Chlorpromazine was used as the positive control. Suitable negative controls were also employed. The experiment appears to have been well carried out. The results show that UVA and (more markedly) UVB are mutagenic; and that the a.i. protects against this effect in a dose dependent manner.

10. Special investigations

Test for capacity to produce phototoxicity

Man. In 10 subjects, patches were applied for 24 hours and the areas then exposed to a suberythematous dose of UV irradiation. There was no evidence of phototoxicity.

Test for capacity to produce photosensitization

Test which "showed that the product did not provoke photosensitization." No details supplied.

Test for inhibition of UV-induced tumors

Hairless mouse. The animals were exposed to repeated doses of UV simulating the solar energy spectrum. After a rest period, 3 applications a week were made to an area of skin of 12-o-tetradecanoyl phorbol-13-acetate (at first at 10 µg/ml, but later at 2 µg/ml, as the higher concentration was found to be irritant). Suitable controls were used. The test group was completely protected by 50 % a.i., and 7.5 % gave an effect equivalent to reducing the insolation four-fold. It had been suggested that the a.i. could itself have been a promoter, but there was no evidence of this.

11. Conclusions

The compound appears to have low acute toxicity. A subchronic oral toxicity study showed a NOAEL of 450 mg/kg b.w./day. A subchronic dermal study showed NOAEL of 550 mg/kg b.w./day. The a.i. does not irritate the mucous membranes in conventional animal tests. The

data presented suggest that the compound is not a skin irritant or sensitiser in animals; however, tests for sensitization were carried out at levels below the proposed maximum use level. Clinical investigation shows that this compound is very rarely responsible for allergic contact dermatitis in man. There is no carcinogenicity study, but an extensive range of mutagenicity studies has been carried out. A test for photomutagenicity in *S. cerevisiae* was negative, although the dose of UVB used was rather low; photomutagenicity tests in mammalian cells *in vitro* have not been carried out. Animal studies for teratogenic activity were negative. Percutaneous absorption was studied in naked rat, minipig, and human skin *in vitro*; and experiments in man were carried out *in vivo*. The *in vitro* experiments show that there is a decreasing amount of absorption as one goes from rat skin to human skin; the last suggests that about 0.9 mg/kg b.w. might be absorbed. Experiments with radioactive a.i. indicate that only about 0.2 % of the applied amount appears in the urine. In a detailed study that compared oral and percutaneous absorption, using GC/MS, although about one-fifth of 100 mg of ingested a.i. was found in the urine, none at all was found when 200 mg were applied to the skin in a concentration of 10 %.

A safety factor may be calculated by taking the oral subchronic NOAEL as 450 mg/kg b.w., and the maximum absorption as 0.9 mg/kg b.w. in use. These are conservative values, and indicate a safety factor of about 500. Against this, it should be noted that the dermal and oral subchronic NOAELs are similar, but there was some irritation at the site of application of the dermal material, and this may have facilitated absorption. Teratogenic activity in the rat and rabbit show a NOAEL of 500 mg/kg b.w./day.

Classification: B.

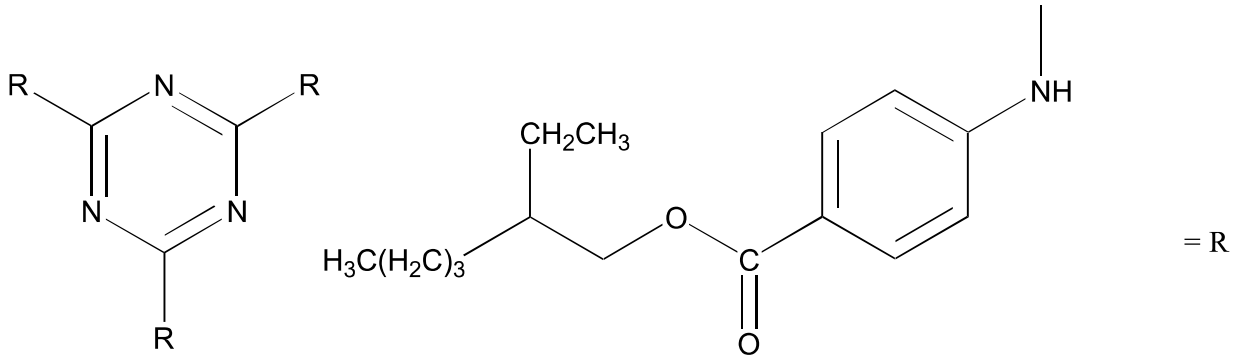
S 69: *2,4,6-TRIANILINO-(P-CARBO-2'-ETHYLHEXYL-1'-OXI) 1,3,5-TRIAZINE*

1. General

1.1 Primary name

2,4,6-trianilino-(p-carbo-2'-ethylhexyl-1'-oxi)1,3,5-triazine.

1.5 Structural formula



1.6 Empirical formula

Emp. formula:$C_{48}H_{66}N_6O_6$.
Mol weight: 823.1.

1.7 Purity, composition and substance codes

Stated by manufacturer to be more than 98 % pure.

1.9 Solubility

Insoluble in water; soluble in isopropyl myristate, olive oil, ethanol.

2. Function and uses

Also used as a stabiliser in light sensitive plastics, dyes, etc. Proposed use level in sunscreen preparations: up to 5 %. Absorption maximum 312 nm.

TOXICOLOGICAL CHARATERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat and mouse. The acute oral toxicity was in general greater than 10 g/kg b.w. Dermal application up to 2 g/kg b.w. did not cause any abnormality.

3.7 Subchronic oral toxicity

Rat. Oral. In a 13 week study, groups of 10 male and 10 female animals were given 0, 1000, 4000 and 16000 ppm in the diet. There was a dose related increase in the weights of the liver in female animals only. However, there was no evidence of liver damage on histological examination, and clinical chemistry tests were normal; because of these findings, and the fact that they occurred in female animals only, the liver changes are considered not to be significant, and the no effect level is put at 16000 ppm, or about 1150 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Two groups of 6 animals were used, one group with scarified skin and one group without. A 10 % dilution of a.i. in olive oil was applied for 24 hours with occlusion. There was definite erythema in 4/6 animals with scarification, and slight erythema in 2/6 animals with intact skin. No abnormality was found after 7 days.

In another experiment, groups of 3 male and 3 female NZW animals were used. A 50 % suspension of a.i. in physiological saline was applied to intact and scarified skin with occlusion for 24 hours. Vehicle controls were used. No abnormality was found.

In another experiment, groups of 6 males and 6 females were used; 3 of each sex had scarification of the area of application. Concentrations of up to 2 % of the a.i. were applied for 24 hours with occlusion; the material was formulated in various o/w creams, in emulsions, and in a formulation used commercially. The last had no adverse effects, but the concentrations of a.i. were only 0.9 % and 1.8 %. The emulsions and o/w preparations showed slight erythema and oedema in the first few days, but the maximum Draize score at any time was 2.

In another experiment, a 50 % suspension in water was applied under semi-occlusive conditions for 24 hours to 3 animals. There was no evidence of irritation.

Guinea pig. A commercial preparation containing 2 % of a.i. was applied daily for 5 days. No abnormality was found.

Man. Fifty subjects were tested, 28 males and 32 females. Concentrations of 5 % and 10 %, formulated as emulsions and as oily solutions, were applied for 24 hours with occlusion. There was one reaction to the 5 % solution in oil. Otherwise no abnormality was found.

4.2 Irritation (mucous membranes)

Rabbit. Four standard Draize tests are reported. Evaluation is uncertain in two of the experiments, because of doubts about the concentrations used. Slight changes were found with a 10 % solution in olive oil, with and without rinsing. Findings were normal after 48 hours. A suspension of (probably) 50 % in saline caused no abnormality. A suspension of (probably) 41 % caused slight changes only. In another study, up to 50 % in olive oil was used; no abnormalities were found. Overall, the substance appears to be only slightly irritating to mucous membranes, if at all.

Chick chorio-allantoic membrane. The probable concentrations of a.i. used were 0.64 % and 6.4 %. No abnormalities were found at either concentration.

5. Sensitization

Guinea pig. A commercial preparation containing 2 % of a.i. was used. It was applied daily to the skin, 5 days a week for 3 weeks. After a 2 weeks rest, the same preparation was applied 3 times to a fresh site. No reaction was seen.

A Magnusson-Kligman maximisation test was carried out in 40 animals, 20 test and 20 controls. The induction concentration of a.i. was 5 % in olive oil intradermally, and 60 % dermally, with occlusion for 48 hours. The challenge was made with 40 % solution in olive oil. There were no significant differences between control and test groups.

Man. Sixty subjects were tested by applications of a commercial preparation containing 2 % a.i., applied for 24 hours with occlusion. No reaction was seen. Of the 60 original subjects, 10 had the test material applied to the same sites 5 times at intervals of 48 hours with occlusion. The application was repeated after 10 days rest and again after a further 10 days. No reaction was seen.

Test for capacity to produce photosensitization

Guinea pig. Two groups of 9 animals were used. The concentration of a.i. was probably 0.5 %, and 0.5 ml was applied to the skin of the neck for 15 minutes. The positive control was 2 % 3,3',-4,5-tetrachlorosalicylanilide (TCSA). Applications were followed by 15 minutes from UV irradiation from a quartz lamp 75 cm from the site. The lamp was type Q-600, made by Quarzlampen-Gesellschaft m.b.H., Hanau. The procedure was repeated 5 times. After a 10 day rest, 2 challenge applications were made, the test being the same as the induction application, but the positive control being 0.1 % TCSA, followed by irradiation as before. Later, the test and positive control solutions were applied as follows: „1 % of emulsion Ka99 and 0.1 % TCSA in 8 % soap solution... Soap solution without the test agent was applied as a control to the opposite flank of the test animals..." (This seems to indicate that the concentration of a.i. on this occasion was 0.02 %). Again, irradiation was applied. There was no evidence of photosensitization. The positive control, however, gave either very weak reactions or none.

Test for production of allergy.

Man. A 1 % solution of a.i. in olive oil was applied to the skin of a panel of 8 subjects known to be allergic to para-aminobenzoic acid derivatives. No reaction was produced. No details given.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man. A 0.5 % solution of a.i. in ethanol/hexane was applied to the forearms of 8 subjects. (Note, however, that the maximum permitted use level is 5 %). After 30 minutes the area was repeatedly stripped, 20 times in all. The concentration of a.i. in each stripping was estimated by HPLC. The authors state that in 20 strippings, 87 % of the applied material was recovered. The area treated was between 1 and 2 cm^2. If one calculates from these figures the percutaneous

absorption using the method proposed by Rougier et al, and extrapolating to the full body surface area, this gives an absorption of between 18 and 37 mg/kg b.w.

A photoacoustic method was used to measure penetration into the skin. As this method is not as yet a validated one for this purpose, it was not possible to evaluate it.

8. Mutagenicity

A standard Ames test was carried out. The maximum concentration used was 5000 µg/plate (limit of solubility, 500 µg/plate). There was no evidence of mutagenicity.

Mouse. A micronucleus test was carried out at a dose of 2100 mg/kg b.w. There was no evidence of micronucleus production.

10. Special investigations

Test for tolerance on repeated use.
Man. In 45 subjects, of whom 14 had sensitive skin and allergic conditions, a commercial formulation containing 2 % a.i. was applied daily. During 3 weeks of exposure, no adverse reaction was seen.

Test for capacity to produce phototoxic and photoallergic effects.
Guinea pig. Dunkin Hartley albino animals were used. Tests for phototoxicity and photoallergenicity were carried out in the same animals, according to the method of Guillot et al (1985) J. Toxicol. 4, 117.

Two groups of animals were used. Group 1 consisted of 3 male and 2 female animals which were treated with the a.i. but not irradiated. Group 2 consisted of 10 male and 10 female animals which were both treated and irradiated.

(a) Phototoxicity. The a. i. was applied to the clipped skin of the interscapular area over about 4 cm^2, with occlusion for 90 minutes. The animals of group 2 were then irradiated with 2 lamps: one with a spectrum of 400 to 310 nm („UVA lamp“) and one with a spectrum of 350 to 285 nm („UVB-lamp“). The dose of UVB radiation was chosen to be a minimal erythema dose. Both lamps were used for 5 minutes (energy produced 0.43 J/cm^2) at a distance of 10 cm from the skin. This was followed by irradiation with the UVA lamp for 90 minutes at a distance of 5 cm from the skin; energy released 12 J/cm^2; total energy thus 12.5 J/cm^2, of which 1 % was UVB. Readings were carried out 6 and 24 hours after irradiation.

(b) Photoallergy. On day 4, following wax depilation, the applications were repeated in the same way to both experimental groups, but as well 4 intradermal injections of 1.0 ml of FCA, diluted 50/50 with saline, were made at the sides of the application site. The applications were then repeated on days 7 and 9. Animals of group 2 were irradiated with the UVA and UVB lamps as before, for 15 minutes at 5 cm, and with the UVA lamp for 40 minutes at 5 cm, after the removal of each patch. Total energy 7.1 J/cm^2 and UVB 6 %.

After a 14 day rest period, a new area of skin was treated with a.i. as before. Animals of group 2 were then irradiated with the UVA lamps for 90 minutes at 5 cm (energy released 12 J/cm^2).

Reading was at 6, 24 and 48 hours after the irradiation. No abnormality was found in any of the experiments.

No contemporaneous positive controls were used, but the investigators present tables of experiments carried out under identical conditions in their laboratory, in which the effects of various phototoxic and photoallergenic compounds are recorded and shown to be positive.

Test for developmental effects.
Chick embryo. Two series of experiments were carried out, injections being made on day 1 and day 5 respectively, the doses being lower for the latter. The LD_{50} was 45 mg (day 1) and 25 mg (day 5). Mortality was dose related. There was a significant increase in the length of the metatarsus, and some bichemical changes, in chicks of the group given 10 mg on the fifth day.

Miscellaneous tests.
Rat. Doses up to 500 mg/kg b.w. by mouth had no effect on blood pressure, or on carrageenan-induced oedema of the paw.

11. Conclusions

The concentrations of a.i. used in some of the tests for irritation of mucous membranes and for sensitization were low in relation to the proposed use level. On the whole, however, these tests were acceptable, and negative. Acute oral and dermal toxicity were low. A subchronic oral study in the rat gave a NOAEL of about 1150 mg/kg b.w./day. An Ames test and a micronucleus test in the mouse were negative. There were no tests for chromosomal aberration *in vitro*, and no tests for photomutagenicity. Tests for phototoxicity and photoallergenicity were negative. The test for photosensitization was unsatisfactory, in view of the poor positive controls. The tests for percutaneous penetration were difficult to interpret; the penetration may be substantial.

Classification: C.

REVIEW OF THE USE OF BORIC ACID IN ORAL HYGIENE PRODUCTS

Recent data on the effects of boric acid on the reproductive system (testicular toxicity and developmental toxicity) have prompted a request to review these data, and to consider the adequacy of the safety margins arising from the oral hygiene use. Since boric acid in talc is not absorbed through normal skin, such use does not give rise to concern.

EFFECTS OF BORON (AS BORIC ACID OR BORAX) ON THE REPRODUCTIVE SYSTEM

Effects on male fertility

There are a number of reports in the literature of adverse effects of boron on male fertility.

Limited details are available on studies in Eastern Europe (Russia) showing reduced testicular weight and sperm count in rats exposed to drinking water containing 6 ppm boron for 6 months, the NOAEL being 0.3 ppm in the drinking water. Interest in such studies was prompted by claims of a high incidence of male infertility in certain parts of the USSR where boron levels in drinking water were 0.4-1.2 mg/l but few details are available. However, attempts to confirm the Russian studies in rats failed, with no effects being seen in the gonads of animals given 0.3, 1 and 6 ppm boron (as borax) in drinking water for up to 90 days.

Short-term (14 day) exposure of rats to high levels of boron (1 gram/kg as boric acid) resulted in marked testicular toxicity (atrophy, severe degenerative changes).

An earlier extensive series of studies on the effect of boron (given as either boric acid or borax) to rats and dogs, involving both 90 day and 2 year repeated dose, and reproductive studies, has clearly indicated effect on male fertility. In the 90 day study in rats boron levels in the diet of 1750 and 5210 ppm (as boric acid or borate) produced signs of general toxicity (reduced weight gain, skin lesions) as well as degenerative changes in the testis. The NOAEL was 525 ppm. In two year studies the NOAEL was 325 ppm in the diet with testicular damage at 1170 ppm. In a reproductive toxicity study no effects were seen at 117 and 350 ppm on fertility, lactation, litter size and development. Similar effects were seen in dogs. In a 2 year study no effects were seen on the testis at dietary levels of 58-350 ppm but marked toxicity was seen at 1750 ppm. In neither the rat nor the god studies were dose levels given on a mg/kg body weight basis. The NOAEL for the rat (350 ppm) is roughly equivalent to a dose level of the order of 50 mg/kg/day.

The same group of workers have published more recently a sub-chronic study in rats specifically to investigate testicular damage. Animals were fed 500, 1000 and 2000 ppm boron (as borax) in the diet for 30 and 60 days. No significant adverse effects were seen at 500 ppm. At 1000 ppm and above dose related effects on the testis were observed (reduced weight, degenerative changes). Infertility was shown to be persistent for at least 8 months indicating prolonged germ cell depletion. The no effect level was 500 ppm in the diet. No data were provided in the daily dose in mg/kg body weight but this is estimated to be of the order of 75 mg/kg/day.

Recently full details have been published of a reproductive toxicity study in mice using a continuous breeding protocol. Male and female mice were exposed to boric acid in the diet for 27 weeks at levels of 1000, 4000 and 9000 ppm, stated to be equivalent to 152, 636 and 1262

mg/kg boric acid body weight/day. Marked effects on fertility were seen at 4000 ppm (reduced to 5 % control value in later stages of study and 9000 ppm (sterility at all time points). No significant effects on fertility were seen at the lowest dose. The only effect reported in this group was a slight reduction in sperm motility, but this did not affect fertility, a significant reduction in sperm motility. A crossover mating trial of the controls and 4000 ppm group confirmed that reduced fertility was solely due to affects in the males. The NOAEL in this study was 152 mg boric acid/kg affects in the males. 27 mg boron/kg body weight/day.

Studies to investigate the mechanism of action of boron as a testicular toxin have been reported using short-term exposure (up to 4 weeks) to high levels of boron (9000 ppm). Under these conditions the first effect seen was a reduction in basal serum testosterone levels from day 4 with an inhibition of spermiation from day 7. Widespread exfoliation of apparently viable germ cells and pachytene cell death appeared during the second week. Extrem epithelial disorganisation and germ cell loss was noted after 28 days. There was no evidence of any accumulation of boron in the testis.

Thus in summary ingestion of boron, either as boric acid or borax, has produced severe testicular damage in rodents and dogs, the effects resulting in prolonged impairment of fertility. Effects have consistently been shown at levels in the diet of 500 ppm and above (around 75 mg/kg b.w. per day) with a no-effect level of about 25-50 mg/kg b.w./day.

DEVELOPMENTAL (TERATOGENICITY) STUDIES

The teratogenic potential of boric acid has recently been investigated in rats and mice.

Pregnant rats were given boric acid in the diet at 1000, 2000 and 4000 ppm throughout gestation and also at 8000 ppm on day 6-15 of gestation. These levels were estimated to be equivalent to 78, 163, 330 and 539 mg/kg boric acid b.w. per day. Animals were sacrificed and the uteri and contents examined on day 20. A significant reduction in maternal weight gain was seen at 330 mg/kg and above, with histological evidence of nephrotoxicity and hepatoxicity in maternal animals at 163 mg/kg/day. Regarding effects on the developing offspring, the percentage of resorption and fetal deaths was increased at 539 mg/kg. No significant effects were seen on litter size or viability at the other dose levels. An increase in gross malformations was seen at 330 mg/kg and above, including abnormalities of the eye, CNS and cardiovascular system. An increase in skeletal malformations was seen at 163 mg/kg. Total body weight was significantly reduced at all dose levels and in addition there was an increase in the number of litters with one or more affected implants (non live implants plus fetal abnormalities) at the lowest dose level. The NOAEL in this study for effects on maternal animals was 78 mg boric acid/kg b.w. per day but a NOAEL was not identified for effects on the offspring since some adverse effects were seen at the lowest dose level investigated namely 78 mg boric acid/kg/day or 14 mg boron/kg/day.

Regarding the studies in mice animals were dosed with 1000, 2000 or 4000 ppm boric acid in the diet on day 0-17 of gestation. These doses were equivalent to 248, 452 and 1003 mg/kg boric acid/kg b.w./day. The animals were then killed and their uteri and contents examined. Maternal weight gain was reduced at the top dose and there was some histopathological evidence of nephrotoxicity and hepatoxicity at all dose levels in the maternal animals. A marked decrease in fetal weight was seen at the top dose with a significant effect at 452 mg/kg. Skeletal malformations were marked by increased at the top dose also. No significant effects were seen on the developing fetuses at 248 mg/kg.

Thus in this study the NOAEL for effects on the developing fetuses was 248 mg boric acid/kg b.w. per day or 43 mg boron/kg/day, a dose level that was associated with some evidence of maternal toxicity, boric acid or approximately 0.5 mg boric acid per b.w. per day for a 60 kg individual.

Calculation of Safety Margins.

For testicular effects NOAEL = 24 mg boron/kg
= 14 mg boric acid/kg

$$\text{Safety Margin} = \frac{143}{0.5} = 286$$

This is acceptable.

For developmental effects: No NOAEL identified.

Lowest effect dose = 78 mg boric acid/kg (fetotoxicity) if a NOAEL of 7.8 mg/kg is assumed (1/10 LOAEL) then

$$\text{Safety Margin} = \frac{7.8}{0.5} = 16$$

This is inadequate.

ESTIMATION OF EXPOSURE AND SAFETY MARGINS

Exposure from oral hygiene use
Boric acid is permitted for use in oral hygiene products at concentrations up to 0.5 %.

Based on data recently provided by Colipa on usage, the following exposures for an average and an extensive user are anticipated.

Product	Typical quality per application	Frequency per day	Exposure per day	
			normal	extensive
toothpaste	1.4g	1-2	1.4g	2.8g
mouthwash	10g	1-5	10g	50g

Assuming 1 gram of mouthwash (10 %) and 0.24 g (17 %) of a toothpaste, is swallowed total ingestion of an extensive user is to 5.48 grams of product. Assuming that all products used contain the maximum amount of boric acid (0.5 %) this is equivalent to 27.4 mg.

If the maximum permitted concentration were reduced to 0.1 %, the Safety Margin would be 90.

It is suggested that this would be provisionally acceptable, but that data from a developmental (teratogenicity) study be requested in the rat to define more clearly a NOAEL for effects on the developing offspring.

Classification: B.

REPORT ON STRONTIUM HYDROXIDE

Application I submitted by the industry requests permission to use strontium hydroxide $Sr(OH)_2$ as an alkaline pH regulator in depilatory products.

The terms of the request are the following:

inclusion of Sr $(OH)_2$ in Annex III, part 1, subject to the following restrictions:

– field of application: pH regulator in depilatory products
– maximum authorised amount in cosmetic products: 3.5 % calculated as strontium, up to a maximum pH of 12.7.

Conditions of use and printed warnings: Keep out of reach of children.

Avoid contact with the eyes.

BACKGROUND

The pK values of reducing mercaptans which are used to break down the disulphide bonds in hair keratin generally indicate that their ionization constant is rather low.

For this reason alkalis must be used to allow the formation of more soluble thioglycolates (which are therefore more effective over a shorter period).

Calcium thioglycolate is one of the most commonly used reducing agents but is not very soluble in water: 7 g requires 100 ml of water.

A depilatory should be just effective enough to break down the structure of the hair without harming the corneal layer. This depends on two key factors:

– length of contact
– the pH value used.

Experience has shown that a pH of 12.5 is the optimum pH at which the keratin in the hair is broken down by thioglycolate within a few minutes of contact, avoiding damage to the corneal layer.

This short contact time, combined with the buffering power of the skin and careful removal of the product as soon as depilation has taken place, makes it possible for the physiological pH to be recovered without any difficulty.

Tests carried out by Zviack and Rouet using the same quantity of thioglycolic acid but modifying the alkalinity of the experiment, while still obtaining the same pH, showed that the speed at which depilation was achieved differed in each case and that in some cases skin irritation occurred.

The most significant data are summarised in the following table:

Alkali	pK	Speed of depilation (min)	Irritation
Calcium	12.6	7	0
Strontium	13.2	5	0
Sodium	14.8	4	+

This experiment confirms the data obtained in companies which manufacture depilatories, which consider that sodium hydroxide is not very suitable (very soluble, producing an excessively high pH and a risk of skin irritation which is difficult to control) and prefer strontium hydroxide, often in combination with calcium hydroxide, because the first substance ensures an adequate rate of depilation and the second´s relatively low solubility maintains a reserve of alkali which is only used up during the final moments of contact. In both cases, the pH is much more tightly controlled and the contact time is kept within the advisable limits.

TOXOCOLOGICAL PROFILE

There are practically no specific data on $Sr(OH)_2$. However, there is extensive literature on Sr´s various salts and compounds.

In general, Sr behaves in a similar way to Ca and can be substituted for it in various biological systems without altering its activity.

Sr´s metabolic activity is similar to that of calcium and the toxicity of its salts is due to the anion.

Acute Toxicity
Weak and fairly similar to Ca and Mg.

LD_{50}.	Oral	mice:	3100 mg/kg weight.	Chloride.
		rats:	2250 mg/kg weight.	Chloride.
		rats:	10600mg/kg weight.	Fluoride.
		rats:	2750 mg/kg weight.	Nitrate.
LD_{50}.	Intravenous:	mice:	148 mg/kg weight.	Chloride.
		rabbits:	1060 mg/kg weight.	Chloride.
LD_{50}.	Intraperitoneal:	rats:	1000 mg/kg weight.	Bromide.
		mice:	908 mg/kg weight.	Chloride.
		mice:	4400 mg/kg weight.	Fluoride.
		rats:	800 mg/kg weight.	Iodide.
		rats:	540 mg/kg weight.	Nitrate.
		rats:	900 mg/kg weight.	Lactate.

Sub-acute/sub-chronic toxicity
Oral: Adult rats: No adverse effects were caused by ingestion of 50 mg/kg weight of Sr nitrate for 8 weeks.

Using newborn rats which were force-fed an aqueous solution of Sr chloride for 13 days it was established that the NEL (no-effect level) was 100 mg/kg weight.

Rats treated with Sr chloride for three months (0.34 % in drinking water) had an increased level of Sr in their bones but no adverse effects were recorded.

Chronic toxicity
Oral: Mice which were given Sr lactate in their drinking water for 402 days showed nothing more than reduced weight gain.

Inhalation: Rodents were exposed to chronic inhalation of Sr nitrate, revealing the NEL to be 3.2 mg/m^3.

Intestional absorption
There is practically no absorption of Sr´s insoluble salts in the intestine.

Sr´s soluble salts are absorbed in the intestine at a rate of between 5 % and 25 % of the ingested dose.

90 % of the Sr absorbed gastrointestinally is eliminated in the urine.

Percutaneous absorption
There is virtually no percutaneous penetration of Sr salts. An experiment carried out on rabbit skin (occlusive application over a two-hour period of 5.6 g of a product containing 11.5 % Sr peroxide) showed that there was no change in blood levels two hours after application.

Reproduction, foetal toxicity, teratogenicity
Experiments on rats show that ingestion of 2000 mg/kg weight of Sr carbonate throughout pregnancy did not produce malformations.

Experiments on rats injected subcutaneously with varying doses of Sr nitrate (25-200 mg/kg weight) between days 9 and 19 of pregnancy produced no teratogenic effects. Quantities of Sr present in the foetuses were similar in all groups and the saturation dose seemed to be 50 mg/kg weight.

Foetal development of the rats was unaffected by ingestion of Sr chloride in drinking water during pregnancy (the dose varied between 2 and 80 mg/l).

Skin irritation
Sr nitrate can produce skin irritation in rats, guinea pigs and rabbits.

Sr peroxide caused a slight skin irritation in rabbits when a 6 % solution was applied by occlusion over a 24-hour period.

Skin sensitization
The reliability of the test carried out using Sr peroxide in guinea pigs which concluded that there might be a slight skin-sensitising potential is in doubt, since the conditions under which it was applied were clearly irritating.

CONCLUSION

Toxicological data on compounds containing Sr show that it is very unlikely that Sr hydroxide can cause problems at a pH less than 13.

It is proposed that the request made in Application I be accepted.

(It should be established whether depilatory products contain a warning ensuring that they are used only on undamaged skin which has not been recently subjected to sunburn).

In view of the substance´s alkalinity, contact time on the skin should be as short as possible.

Classification: A.

REPORT ON STRONTIUM PEROXIDE

Submission No 1 for strontium peroxide requests permission for its use at a strength of 6 %, exclusively as a hairdressing product, by hairdressing professionals and with all trace of it to be subsequently rinsed away.

The data supplied, referring to the method of application, and other data supplied by people within the profession, indicate the use of a mixture of powders containing strontium peroxide (SrO_2), probably together with other peroxides and masking and thickening agents. The product is diluted and mixed with the required quantity of H_2O_2 (30 volumes) until a smooth, creamy consistency is obtained. This is then immediately applied with a brush over the full length of the hair.

It is highly alkaline (pH > 10) and the release of the reactive oxygen brings about bleaching of the darker shades of hair after approximately 30 minutes' contact. Both the hair and the scalp are then thoroughly washed with shampoo and rinsed with water.

The dossier submitted includes an acute toxicity study of topical application on rats (limit test), enabling the lethal dose to be established at over 2000 mg per kilo of body weight. Given the way the product is used, this figure may be considered acceptable.

The primary skin irritation test, carried out over 24 hours on albino rabbits using the occlusive patch test with SrO_2 at 6 % (diluted in water) resulted in a level of erythema, eschar and oedema equivalent to a primary skin irritation index of 0.7 on the Draize scale. The product should therefore be considered slightly irritating to the skin of a rabbit.

An identical study, carried out using H_2O_2 in place of water, places SrO_2 in the same category, but the index is slightly higher.

Bearing in mind that SrO_2 is not likely to remain in contact with the scalp for more than some 30 minutes, that the scalp is not covered in any way, and that several weeks will elapse between treatments, the risk of irritation may be considered very slight.

The sensitization study was carried out on 20 albino guinea pigs. After checking for the absence of individual reactions by means of a 48-hour topical and occlusive application of the product containing SrO_2, the sensitization protocol was applied to each guinea pig. This involved intradermic injection of Freund's adjuvant to the rib area of each animal followed (over a period of 15 days) by seven topical applications of the product containing SrO_2. There was then a rest period of 12 days before the product containing SrO_2 was applied to the abdominal region under an occlusive patch for a period of 48 hours to provoke the reaction. After removal of the occlusive patch, the application zone was examined after 6, 24 and 48 hours. These inspections identified visible macroscopic skin reactions in both the initiating and the induction zones. It was decided that the animals should undergo histopathological examination (to assess the appearance of experimentally-induced eczema).

The inspection six hours after removing the occlusive patch revealed the need for histopathological examination of 3 of the 20 guinea pigs in the test. The result of this test

showed that two animals had a "clear orthogenic reaction" and only one an "actual allergic reaction".

If this is taken to mean that the three animals were sensitised by SrO_2, this then means a class II sensitisation level (i.e. a maximum of 25 % of the animals).

If we consider that the orthogenic reaction does not necessarily mask an allergic reaction, the sensitization level would be type I (no more than 10 % of the animals sensitised). These two hypotheses would rank the sensitising properties of SrO_2 as WEAK or VERY WEAK, respectively.

In contrast to the tests referred to earlier, this sensitization test was carried out using not SrO_2 but a formula (a mauve-coloured powder) of which all we are told is that it contained 11.5 % strontium peroxide.

It is difficult to regard sensitization tests carried out using a finished product, the composition of which is not fully known, as definitive, since the unknown ingredients may affect the response.

The conditions under which the sensitization test is performed require the use (in the form of an occlusive patch for at least 48 hours) of the maximum quantity before the skin becomes irritated. The slightly irritating properties of SrO_2 in a 24-hour occlusive patch were demonstrated during the skin irritation test. Under such circumstances, it is difficult to reach valid conclusions, given that the application dose might in some animals present an irritation potential which could invalidate the interpretation of the sensitization potential. For these reasons, it is not desirable to request a new sensitization test using SrO_2 (instead of the finished product).

Finally, the submission document contains a study of *in vivo* penetration, carried out using rabbits and with the same formula as that used for the sensitization study. The results are to some degree contradictory and so it is difficult to interpret them properly.

The aim of the trial was to see whether application of the product to the skin, under virtually identical conditions to its normal use by hairdressing professionals, would result in an increase in SrO_2 levels in blood and accordingly presumes that SrO_2 can be absorbed through the skin.

The product contains 11.5 % of SrO_2 and 5.6 g (diluted in 12 ml of 30 volumes H_20_2) were used. The six test rabbits were shaved the day before the trial and their blood analysed to establish the strontium content before the trial. The trial was carried out by applying the product, in the diluted form described above, to 100 cm^2 of their skin and using a semi-occlusive patch to maintain contact for two hours. At the end of this time, blood samples were taken (a double quantity so that the analyses could be repeated if necessary).

The detection limit for Sr is of the order of 25 ppb.

Blood levels before the test varied between 0.15 and 0.30 ppm, providing an average value of 0.22 ppm.

After the test, they varied between 0.15 and 0.22 ppm, yielding an average value of 0.21 ppm.

Only one rabbit showed anomalous behaviour, with a significant increase in blood Sr after application of the product: rising from 0.15 ppm before the application to 0.40 ppm after application. For this animal, the analysis was repeated with the second sample and surprising results were obtained: 0.20 ppm before and less than 0.05 ppm after the test. There is no explanation for these anomalous results.

If however the data from this animal are disregarded, it is fair to say that the absence of any increase in Sr in the blood suggests that none of the Sr present in the SrO_2 of the product tested was absorbed through the skin.

No trials other than those cited have been submitted indicating the toxicological profile of SrO_2, and for this reason it could be useful to examine some aspects of research on other strontium salts.

Acute toxicity for hexahydrated strontium chloride corresponds to an LD_{50} of 12.4 g per kilo body weight (oral pathway in the rat).

The effect of $SrC_{12}6H_2O$ in newly-born rats : Rats were selected with a litter of 8 young. From day 2 to day 15, during lactation, each litter received a solution of a determined dose of $SrC_{l2}6H_2O$ once a day via intubation.

The elements used were Sr, Mo, Li and B. In each experiment and at each of the three doses tested, 2 or 3 litters of 8 new-born rats were used. In all cases, half of each litter were used as controls and received distilled water.

The dose of 100 mg per kilo body weight did not have any adverse effects, there being no deaths, rachitis or dentine lesions; weight increase was optimal and no histopathological lesions were detected.

Short-term toxicity of hexahydrated strontium chloride in the rat: After administration the authors carried out an exhaustive control of the weight of the animals organs, as well as a histopathological evaluation. Only for the thymus was there an abnormal increase in weight in male rats subjected to a daily dose of 1200 and 4800 ppm over a 90-day period.

Doses of 0.75 and 300 ppm did not lead to any increase in thyroid weight in the male rat. Female rats were not affected at any of the doses used.

Histopathological studies were also carried out on this organ in the form of 5-micron histological cuts, with haemalum and eosin staining.

The study does not mention the criteria adopted for establishing the four effect levels, which for the male rats subjected to a dose of 4800 ppm were as follows:

without increase in thyroid activity	2 animals.
very mild increase	3 animals.
mild increase	3 animals.
moderate increase	4 animals.

Mutagenicity using 127 metallic compounds. This study was published in 1980. In an initial screening, they studied growth inhibition of bacillus subtilis strains (one without a deficiency, or rec+, the other with a recombination repair deficiency, or rec-). In each case, a precise

concentration of a metal compound was used, with impregnation of filter paper disks located in the bacteria plate culture and measurement in mm of the longitude of the inhibition provoked by each strain.

When inhibition is greater in the rec- strain than in the rec+ strain, it is clear that the chemical compound in question damages cellular DNA.

In this study, a variant was used which involved keeping the plates of the different strains with filter disks pre-impregnated with the metal compounds over a 24-hour period at 4°C, before proceeding to normal incubation at 37°C during the entire night. The authors report that this protocol increases test sensitivity 20 to 50-fold for many drugs.

For $SrC_{12}6H_2O$ the result was negative.

However, positive results were obtained for 44 compounds, including various compounds of arsenic, silver, barium, bismuth, celsium, chrome, platinum and rhodium. In all cases, strains of *Escherichia coli* and *salmonella* were used.

Metal-induced DNA synthesis infidelity: the study estimated the fidelity of DNA replication *in vitro* and showed that many metal ions can alter it.

The model utilised was a synthetic polynucleotide formed by deoxytimidine and deoxyadenosine monophosphates: Poly d (A-T).

This polynucleotide can be synthesised with an error of less than 2.10^6 using DNA polimerase I of *Escherichia coli*. In the protocol used the correct copy contains only dAMP and dTMP. Incorporation of dCTP and dUTP signal errors in replication.

40 metallic compounds were tested in the experiment. The authors report that Sr did not affect DNA synthesis fidelity. However, alterations did take place in the case of silver, beryllium, cadmium, cobalt, chrome, manganese, nickel and lead.

Effect of metallic ions on RNA transcription. For this experiment, they used a RNA polimerase of Escherichia coli, the initial model being poly d (A-T) in the presence of various metal ions, with a view to determining transcription fidelity: one incorrect nucleotide (cytidinmonophosphate - CMP) for 200 correct nucleotides, in the presence of Mg2+.

Various metal ions tested, known to be non-mutagenic or non-carcinogenic, and including Sr+, did not lead to erroneous incorporation of CMP during transcription of poly d (A-T).

Moreover, various studies provide a wide range of reliable data indicating that strontium is not teratogenic, that it is not toxic for the embryo and has no effect on the reproductive process.

The toxicity of strontium depends to a large extent on the naturalness of the anion.

Finally, we should remember that use of strontium peroxide could theoretically involve alkaline aggression. The following points deserve mention:

– Aqueous alkaline solutions may attack the cutaneous tissue.

– The corneal layer can be a very effective defence because it has various barriers capable of minimising the adverse effects.

– The surface lipids make it difficult for the aqueous alkaline solution to wet the corneal layer.

– The barrier function of the corneal layer itself, in particular:

  a) the peculiar „membrane“ of the numerous superimposed corneocytes, which consists of a dense proteic sheath with abundant glutamyl-lisin links that are very resistent to alkaline aggression; this protein sheath is found together with a very hydrophobe lipid sheath formed by ceramides.

  b) the proven barrier capacity posed by the corneal layer in the form of various acidophilic molecules which are synthesised and accumulated inside the corneocytes, especially lactic acid, pyroglutamic acid and urocanic acid.

In the more seborrhoic body zones, such as the scalp, where there may be up to 900 glands per cm^2, the surface lipids act as an effective barrier to alkaline aggression.

Healthy skin is a good barrier, and only diseases which alter the functionality of the grannular layer (negatively affecting the keratohyaline and the Odland corpuscles) severely impair its resistance to its chemical aggression (examples: icthyosis and psoriasis).

The abundance of sebaceous glands in the scalp allows rapid recovery of the level of surface lipids, given that in 30 minutes they normally secrete up to 100 microgrammes of fat per cm^2 from the reservoir attached to the glands.

Comparison may be made with the skin of the legs, were surface lipids are much more scarce and sebaceous secretion is a lot less.

Given that alkaline aggression depends on the combination of two factors - the degree of alkalinity entering into contact with the skin and the contact time - the response of cutaneous tissue should take all the circumstances into account.

Ten minutes of contact with an alkaline product on the skin of the legs (a time considered optimum for depilation) may be as harmless as 30 minutes contact of the same product on the scalp!

Taking all these data into account, it can be said that strontium peroxide may safely be used in the conditions requested, given that neither percutaneous absorption, acute toxicity, nor sensitization potential (probably very weak, which is considered to be insignificant) can be considered as giving grounds for concern.

The mild irritation potential detected suggests, however, that one should avoid contact with the mucous memebrane and with skin suffering from a disorder impairing the barrier function of the corneal layer.

Conclusions: Classification A.

Caution: Potentially irritating to the eyes and damaged skin.
Concentration: 4.5 % Sr in preparations listed for use.

OPINIONS ADOPTED DURING THE 54TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 10 December 1993

A 11: *RESORCINOL*

1. General

1.1 Primary name

Resorcinol

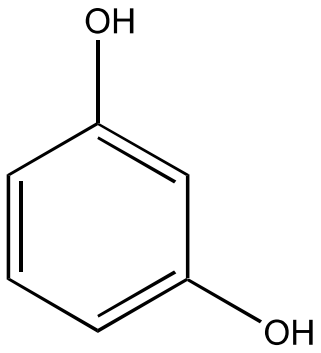
1.2 Chemical names

1,3-dihydroxybenzene
1,3-benzenediol
m-benzediol
m-dihydroxybenzene
m-dioxybenzene
3-hydroxycyclohexadien-1-one
m-hydroxyquinone
3-hydroxyphenol

1.4 CAS no.

108-46-3

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_6O_2$
Mol weight: 110.11

2. Function and uses

Resorcinol is an oxidative hair dye; max. use: 2.5%; 1.25% in combination with H_2O_2; 0.5% in hair lotions and shampoos.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: rat, oral: 370 mg/kg
rat, oral: 980 mg/kg
rabbit, dermal: 3,360 mg/kg

3.5 Repeated dose oral toxicity

In 17-day oral studies no gross or microscopic lesions attributable to resorcinol (>99 % pure) administration were observed in groups of five F344/N rats (0, 27.5, 55, 110, 225 or 450 mg/kg) and five B6C3F1 mice (0, 37.5, 75, 150, 300, 600 mg/kg) of each sex. All female and four male mice (600 mg/kg) and one male mice (300 mg/kg) died during the study. Absolute and relative thymus weights were significantly decreased in the high-dose female group of rats. Clinical findings were observed in male (225 and 450 mg/kg) and female (55, 110, 225, 450 mg/kg) rats; and in male (150, 300, 600 mg/kg) and female (300 and 600 mg/kg) mice.

No biological significant change in organ weights was observed in rats and mice. The NOAELs were: 27.5 mg/kg for F344/N rats and 75 mg/kg for B6C3F1 mice (NTP, 1992).

3.7 Subchronic oral toxicity

30 males and 30 females of SPF Wistar rats (Mura Han 67 PF) received by oral intubation 20 mg/kg/day (amount administered 10 ml/kg water solution per 5 days/week) of resorcinol for 12 weeks. No clinical, histological anomalies or cumulative toxicity were found. The dose of 20 mg/kg/day represents the NOAEL.

In a 13-week oral study with resorcinol (>99 % pure) no-chemical related gross or microscopic lesions were observed in both sexes of F344/N rats (0, 32, 65, 130, 260 or 520 mg/kg) and B6C3F1 mice (0, 28, 56, 112, 225 or 420 mg/kg). Groups of 10 rats of each sex were considered. All females and eight male rats treated with 520 mg/kg and eight mice of each sex treated with 420 mg/kg of resorcinol died of chemical-related toxicity during the study. Absolute and relative liver weights were significantly increased in male (130 and 260 mg/kg) and female (65, 130, and 260 mg/kg) rats. Absolute and relative adrenal gland weights were significantly increased in all surviving dosed groups of rats. Significant decreases were noted in absolute and relative adrenal gland weights for male (28, 56, 112, and 225 mg/kg) mice.

3.8 Subchronic dermal toxicity

Resorcinol in arachis oil (1.5 g of resorcinol in 100 ml, i.e. 154 mg/kg/day) was injected s.c. (2 ml) to four albino rats twice a day for 47 days. The results showed goitrogenic action in 1 of 2 animals after 47 days.

Resorcinol applied (1% and 3% in unguentum) on unshaved ears and flank of 10 male guinea-pigs (10 animals dose) for 14 days showed acanthosis (max. 8 d), hypergranulosis and orthohyperkeratosis, oedema on ears and flank, and papillomatosis only on ears (max. 8 d).

Female rats (11 animals treated) which received Resorcinol diacetate (0.4 mmol/100 g, i.e. 800 mg/kg, in water solution with 3 % glycerin) by s.c. injection 2 times/day x 9-12 days (5 rats) or 22-25 day (6 rats) showed goitrogenic action after 12 days.

4. Irritation & corrosivity

4.1 Irritation (skin)

In three albino rabbits the dose of 2.5 % w/v (0.5 % in aqueous gum tragacanth containing 0.05 % sodium sulphite) applied for 24 h on shaved skin resulted not irritating (reading at 72 h).

4.2 Irritation (mucous membranes)

The compound instilled into one eye of three albino rabbits (1 h, 2.5 % w/v in water containing 0.05 % sodium sulphate) showed mild conjunctival redness in all animals.

5. Sensitization

20 albino Guinea pigs, 3 % in water with 2 % Natrosol 250, 2 % Tween 80 and 0.05 % sodium sulphite: no inflammation and no allergic reactions were observed (3 week treatment and 2 wk. challenge reaction).

Human-skin sensitization: Resorcinol (1% in paraffin solution) showed positive reaction in 2.1 % (1.9 % males and 2.2 % females) of 877 persons suffering from primary contact dermatitis. Resorcinol (0.2 % in salicyl-alcohol) gave positive response in a patch test in eczematous subject (erythema) and acute dermatitis with salicyl-resorcinol solution in alcohol applied dermally. Resorcinol (5 % in water) showed allergic reactions in 7.9 % of 340 patients (eczematous) tested. Resorcinol gave positive reaction for cross-sensitivity/patch test with: resorcinol monoacetate; hydroquinone; pyrocatecol; phenol; pyrogallol; hydroxy-quinone; phoroloroglucinol; hexylresorcinol; orcinol; cathecol; 4-phenyl cathecol; pyrogallic acid; 3,5-dihydroxybenzene; resorcinol mono methyl ether; resorcinol dimethyl ether; and floroglucinol. Resorcinol caused ochronosis and myxoedema in a patient who received 12 % in ointment on the leg ulcers for 13 years before dying. In a case of resorcinol poisoning in a young child (7 weeks) the compound gave severe haemolytic anaemia with haemoglobinuria and a generalized papullo-squamous eruption. Resorcinol (3-10-35 % in vaseline) caused urticaria in 4 patients treated dermally: 3/4 fever and headache; 2/4 showed articular pain. One person died after cutaneous application of an ointment with 20 % of resorcinol.

6. Teratogenicity

Resorcinol administered by gavage (0, 125, 250, 500 mg/kg in propylene glycol, 10 ml/kg) to pregnant Sprague-Dawley rats (13 females for each group) on days 6 to 15 of gestation, did not produce neither a significant reduction in maternal weight and in fetal external, nor visceral and skeletal anomalies.

Groups of 23 pregnant female rats received 0-40-80-250 mg/kg b.w. of resorcinol in distilled water (10 ml/kg b.w.) on days 6 to 15 of gestation. A positive control (23 females), Vitamin A was included (15 mg/kg, 10 ml/kg suspension in rape oil).

The animals were sacrificed on day 19 of gestation. The results showed a slightly lower maternal b.w. gain at 250 mg/kg. The dose of 80 mg/kg b.w. represents the NOAEL.

New Zealand white Rabbit (18-26 mated females/group) received by oral gavage 0-25-50-100 mg/kg day resorcinol in distilled water (10 ml/kg) on days 6 to 18 of gestation. A positive control group (22 mated females) was included (Vitamin A, 6 mg/kg b.w. in rape oil, 10 ml/kg b.w.). At the dose of 100 mg/kg b.w. a slightly lower maternal b.w. gain was noted. At the dose of 50 mg/kg, b.w. was observed. 25 NMRI mice received 0 or 28.35 mg/kg by s.c. application of resorcinol in water (0.2 ml/30g b.w.) on days 5 to 7, 8 to 10 or 11 to 14 of gestation. No teratogenic effects were found in females killed on day 18 of gestation.

In mouse, rat and rabbit which received by dermal topical applications a formulation containing resorcinol (from 0.2 % to 2.0 %, 1:1 with 6 % of hydrogen peroxide) no indication of teratogenic effects was observed. Only the following differences were noted: (a) mouse (1.7 %): significant decrease in b.w. and increased number of unossified caudal and vertebral centra, as well as unossified bones in feet; (b) Rat (1.7 %): significant increase of skeletal anomalies (notched and short ribs); (c) Rabbit (1.7 %): no-sign of maternal toxicity apart from focal alopecia until the last third of gestation.

Embryotoxicity: HET test: It had no-effect-level, including systemic effects at 1-5 ppm doses ca. In a second HET test on 3-day old Chicken embryos, the following results were observed: ED_{50} = 2.4 µmol/egg; LD_{50} = 2.7 µmol/egg; and 5 % malformed fetuses vs. 3 % control (0.9-7.3 µmol/egg).

Multireproduction study: Charles River rats (F_0 generation: 40 males and 40 females) received 0.5 ml of dyes/rat (0.2 % to 2.0 % of resorcinol in formulations; initially 0.2 ml dye/rat increased of 0.1 ml/rat), 2 times a week, for 3 generations. The results showed that neither changes, nor gross and microscopical lesions related to the formulations, were observed for the parental rats or pups.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human skin absorption: In commercial hair dye (1.225 % of resorcinol) applied for 25-28 min 0.076 % of applied dose penetrated (as % of total dose excretion). Resorcinol 2 % in hydroalcholic vehicle, dermal topical applications (2 time/day, 6 day/week x 4 weeks, i.e. 48 applications) on 3 human volunteers (0.30 mg/cm^2, 12 mg/kg/day): 2.87 % (i.e. 0.34 mg/kg/day), flux rate 0.37 µg/cm^2/h. *In vitro*: 0.86 µg/cm^2/h.

Dermal absorption: 0.177 % of the applied dose of Resorcinol contained in a commercial hair dye (1.225 %) to three Rhesus monkeys was shown to be absorbed.

12 female hairless Sprague-Dawley rats received on back skin for 30 min Resorcinol (1.5 % and 3.0 %) and p-TDA (1.5 % and 3.0 %), mixed 1:1 with H_2O_2 before application. The following values of absorption for Resorcinol were obtained for 1.5 % and 3.0 % concentrations, respectively: 2.77 % (i.e. 75.55 nM/cm^2, 332.5 µg/kg) and 4.58 % (i.e. 62.46 nM/cm^2, 270 µg/kg) after 96 h.

Thyroid and Liver fixation: 12 female hairless Wistar rats received dermal topical applications (30 min., intervals of 30-40 d.) of 20 mg/cm^2 (i.e. 2.72 µM/cm^2) hair dye solution

(mixed 1:1 with H_2O_2) containing 1.5 % of radiolabelled resorcinol (i.e. 136 nM). Traces of resorcinol (2.2×10^{-3} µg) were found in liver and no fixation has been observed in thyroid 4 days after treatment.

Metabolism: Male Sprague-Dawley rats which received single s.c. injections of resorcinol (10-50-100 mg/kg in water) showed a peak in plasma after 15 min. Two or three rats were sacrificed at 1, 3, 6 and 24 hours. About 90 % of the compound equivalents were eliminated during the first two hours; the half-lives were 23 min and 8.6 h for 50 mg/kg and 18 min and 10.5 h for 100 mg/kg. Peaks in liver and kidney were found at 1 h (0.2 % - 0.3 %). At the 10 mg/kg dose, 7 % of resorcinol was found in gastrointestinal tract after 1 h; 1.4 % in gastrointestinal tract and feces after 24 h, and 93.6 % in urine after 24 h. Resorcinol was essentially found as the glucuronide conjugate. In a multiple study on male Sprague-Dawley the animals received daily s.c. injection of resorcinol (2 x 50 mg/kg, 6 h interval) and after 14-30 days of treatment 50 mg/kg of compound with trace ^{14}C-Resorcinol). Then three rats were sacrificed 1, 3, 6 and 24 hours after injection. The results showed that after 2 h the plasma level declined to ca. 90 % at 15 and 30-day; the half-lives were: 32 min for fast phase and 5.0 h (14 day) and 7.0 h (30 day) for the slow phase.

8. Mutagenicity

The compound was tested for gene mutation and found positive in L5178Y mouse lymphoma cell line. The compound induced chromosome aberrations on CHO cells in human lymphocytes and SCE on CHO cells *in vitro*.

The compound has been tested for the induction of gene mutations *in vitro* and found negative in *Salmonella* (spot test and plate test), in *E. coli* and in *Drosophila melanogaster* male germ cells (SLRL, feed).

Other *in vitro* studies for chromosome aberrations on CHO cells and human fibroblasts and SCE on CHO cells, human lymphocytes and V79 cells, showed negative results. Commercial preparations containing resorcinol tested for the induction of chromosome aberrations and SCE in human lymphocytes *in vitro* showed negative results. The compound did not induce: SCE *in vivo* on rat (up to 100 mg/kg i.p. or peroral; or up to 3 x 100 mg/kg epicutan). The compound was unable to induce UDS in primary rat hepatocyte cultures.

Resorcinol was found inactive for the inhibition of testicular DNA synthesis (100 mg/kg oral) and sperm-head abnormality (0.5-2.0 mmoles/kg i.p.) *in vivo* on mice. Resorcinol (1 %) containing formulation (1:1 with 6 % H_2O_2) tested for heritable translocation *in vivo* on Sprague Dawley rats (0.5 ml/application) showed negative results. The compound did not induce micronuclei in mice treated oral with 500 mg/kg in two equal oral doses separated by an interval of 24 hours, or intraperitoneally with doses up to 2 x 220 mg/kg b.w., 24 h interval, or 0.5-2.0 mmol/kg, or up to 300 mg/kg in distilled water.

A mixture of resorcinol and p-Phenylendiamine was able to induce gene mutation *in vitro*/*in vivo* assay (*Salmonella typhimurium* TA 98 microsome test in the urine of rats: 300 mg p-PD/Resorcinol conjugates, urine concentrate: 50, 100, 200 µl/plate).

9. Carcinogenicity

Mice received by skin painting throughout life span (50 animals/group) 0-5-25-50 % of resorcinol (0.02 ml in acetone solution). These skin tumors were observed: 1 squamous cell papilloma on dorsal skin (dose: 5 %); 1 squamous cell carcinoma on ear (dose: 25 %). The compound did not have a carcinogenic or toxic potential in comparison with negative control.

New Zealand Rabbits received cutaneously 0.02 ml of 5-10-50 % of resorcinol (5 animals/ group), 2 time/week during life-time. No adverse signs or tumors were observed.

Mice (50 or 28 animals/sex/group) and rats (50 males and 50 females/group) which received dermal topical applications of resorcinol (0.2 % to 2.0 % in formulations) showed no difference between treated and control group.

Groups of 60 male F344/N rats and male and female B6C3F1 mice received 0, 112 or 225 mg/kg resorcinol in deionized water by gavage, 5 days per week for up to 104 weeks. Groups of 60 female rats received initially the same doses as male rats, but by week 22 of the study 16 of the high-dose females had died. Consequently the female rats study was restarted using doses of 0, 50, 100 or 150 mg/kg. After 15 months of exposure interim evaluations were performed on 10 animals from each group. Decreased survival in high-dose groups of rats was attributed to chemical-related toxicity (male rats: -10 % to -15 %, from week 87 to study termination; female rats: -11 % to -14 %, from week 95 to study termination). Clinical signs suggesting a chemical-related effect on the central nervous system, including ataxia, recumbency, and tremors, were observed in rats and mice. Neither chemical-related changes in clinical pathology parameters, nor incidence of neoplasms and nonneoplastic lesions, were found during the 15-month interim evaluation. Under the condition of the study, no evidence of the carcinogenic activity was observed in F344/N rats and B6C3F1 mice.

10. Special investigations

Immunosuppressive effect: The ability of resorcinol to suppress humoral and cellular immunological responses was investigated on the following systems: 1) *in vivo*: inhibition in the rabbit of the production of circulating antibodies to sheep red blood cells; 2) *in vitro*: inhibition of the response in mixed lymphocytes cultures, using lymphocytes from non-compatible primates. The compound was negative for the induction of these immunosuppressive effects.

Thyroid: Antithyroid effects might be ascribed to inhibition of thyroid peroxidase.

11. Conclusions

The SCC does not consider the use of Resorcinol in hair dyes to be linked to any particular toxic risk for consumers.

Classification: A

12. Safety evaluation

CALCULATION OF SAFETY MARGIN

A 11-RESORCINOL

OXIDATION OR PERMANENT

Based on a usage volume of 100 ml, containing at maximum 1.25 % of Resorcinol

Maximum amount of ingredient applied:	**I (mg)= 1250 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)=0.076 %**
Dermal absorption per treatment:	**I (mg) x A (%)=1250 x 0.076/100= 0.95 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg b.w.**
	= 0.95 mg/60 kg b.w.=0.0158 mg/kg b.w.
No observed adverse effect level (mg/kg):	**NOAEL = 20 mg/kg b.w. rat oral, 90 days**
Margin of Safety:	**NOAEL / SED = 20 mg/kg b.w./0.0158 mg/kg b.w. = 1.200**

CALCULATION OF SAFETY MARGIN

A 11-RESORCINOL

SEMI-PERMANENT

Based on a usage volume of 35 ml, containing at maximum 0.5 % of Resorcinol

Maximum amount of ingredient applied:	**I (mg)= 35 x 500 mg/100=175 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)=0.076 %**
Dermal absorption per treatment:	**I (mg) x A (%)= 175 x 0.076/100=0.133mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg b.w. = 0.133 mg/60 kg b.w.=0.0022 mg/kg b.w.**
No observed adverse effect level (mg/kg):	**NOAEL = 20 mg/kg b.w. rat oral, 90 days**
Margin of Safety:	**NOAEL / SED = 20 mg/kg b.w./0.0022 mg/kg b.w. = 9.000**

A 12: *4-CHLORORESORCINOL*

1. General

1.1 Primary name

4-chlororesorcinol

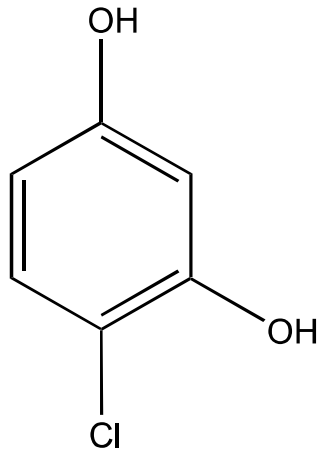
1.2 Chemical names

4-chlororesorcinol
1,3-dihydroxy-4-chlorobenzene

1.4 CAS no.

95-88-5
C.I.: 76510

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_5O_2Cl$
Mol weight: 144.56

2. Function and uses

Oxidative hair dye; max. use: 3%, 1.5% upon application.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: rat, oral 369 mg/kg.

3.7 Subchronic oral toxicity

Rat, 20 mg/kg b.w. /day per os for 12 wks. (daily/5 times a week): no effects. Rat, 40 mg/kg b.w./day for 3 wks. (5 day/wk) oral gavage: slight activation of thyroid epithelium; slight decrease in the triiodothyronin in the serum.

3.8 Subchronic dermal toxicity

Rabbit, 2 % in formulation with 6 % H_2O_2 for 13 wks.: no effects.

3.10 Chronic toxicity

Mice, skin painting, 2 % in formulation, 21 months: neither toxicity nor carcinogenicity effect.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit, 2.5 % w/v for 24 h: negative (reading at 72 h).

4.2 Irritation (mucous membranes)

Rabbit, 2.5 % w/v into one eye: negative, mild conjunctival irritation only (2/3 animals).

5. Sensitization

Guinea pig: 3 % epicutaneously, daily (6 d./wk.) for 3 weeks: no reaction.

6. Teratogenicity

Rat, 2 ml/kg b.w. in formulation on day 1-4-7-10-13-16-19 of gestation. Rat, 50, 100, 200 mg/kg by oral gavage (6 to 15 of gestation), 200 mg/kg: significant decrease in maternal weight gain, embryolethal (increase in resorptions), slight increase in fetal anomalies (not statistically significant). Minor anomalies (wavy ribs) and variations (incomplete ossification of the sternebrae, rudimentary 14th ribs on both sides of vertebral column) of skeletal; 100 mg/kg: no effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: Rat, 1 %, 2 % and 3 % in formulations (1:1 H_2O_2) for 30 min. (clipped skin): 0.216 %, 0.217 % and 0.367 % in 72 h. Rat, 2 % in formulation (1:1 H_2O_2): 0.006 % as CO_2 in expired air after 8 h. Rat, 1 % in formulation (1:1 H_2O_2) for 30 min. (unclipped skin): 0.088 % in 72 h. Rat, 300 mg in aqueous sol. for 30 min. (clipped skin): 5.47 % (0.11 mg/cm^2) in 72 h.

Organ distribution: Rat, 2 % in formulation, cutaneous appl.: No strong affinity for any particular tissue (highest conc. found after 35 min 1 h). Rat, oral 50 mg/kg in aqueous sol.: No special affinity to any organ.

Excretion: Rat, 50 mg/kg b.w. s.c.: >96 % (urine and feces) in 24 h. Rat, 50 mg/kg oral: predominantly in urine in 24 h.; 19.3 % of oral dose in bile within 3 h.

8. Mutagenicity

The compound was tested for the induction of gene mutations *in vitro* and found negative in *Salmonella* at spot test, in several studies at plate test and in *E.coli*. The compound did not induce micronuclea in mice treated with a total dosage of 600 mg/kg in two equal oral doses separated by an interval of 24 hours; the compound was negative for the induction of chromosome aberrations on human lymphocytes treated *in vitro.*

9. Carcinogenicity

A long term study is in progress at the National Toxicology Program.

10. Special investigations

Immunosuppressive effects: 4-Chlororesocinol was found negative for the induction of the immunosuppresive action evaluated by plate test and hemagglutination test when s.c. administered to 6 NMRI mice four times at the maximal tolerated dose of 1.5 mg.

11. Conclusions

The SCC is aware of the ongoing long-term study for carcinogenicity of NTP-USA and it will consider the chemical for its final evaluation when this study will be completed.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

A12

4-CHLORORESORCINOL

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 4-Chlororesorcinol)

Maximum amount of ingredient applied	**I (mg) = 1,500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 0.367 (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 5.5 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 5.5 mg /60 mg = 0.09 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 20 mg (rat: 90 days oral study)**
Margin of Safety	**NOAEL / SED = 20 mg/kg b.w. / 0.09 mg/kg b.w.= 220**

A 15: *M-AMINOPHENOL*

1. General

1.1 Primary name

m-aminophenol

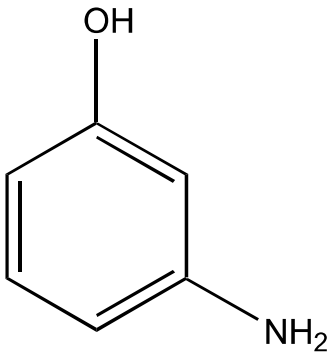
1.2 Chemical names

m-aminophenol
1-hydroxy-3-amino-benzene
3-amino-phenol
1,3-aminophenol
m-hydroxy-aniline

1.4 CAS no.

591-27-5
C.I. 76545

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_6H_7NO
Mol weight: 109.129

2. Function and uses

Oxidative hair dye; max.use: 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} rat, oral: 1,000 mg/kg
1,660 mg/kg
812 mg/kg
mouse, oral: 330 mg/kg

3.7 Subchronic oral toxicity

m-aminophenol was administered by oral intubation to SPF Wistar rats (20 males and 20 females) at the dose of 50 mg/kg b.w. (10 ml/kg of water), 5 times a week, for 12 weeks. No clinical, histological or cumulative toxicity was found. The dose of 50 mg/kg represents the NOAEL.

3.8 Subchronic dermal toxicity

Formulations containing m-aminophenol (0.04 % - 0.7 %), mixed 1:1 with 6 % H_2O_2, were dermally applied for 13 weeks (twice weekly) both on abraded and intact skin of 12 adult New-Zealand rabbits (6 males and 6 females). No evidence of systemic toxicity were observed.

3.10 Chronic toxicity

Chronic toxicity and carcinogenicity (Dermal-topical application): Four oxidative formulations (7403, 7406, P-25, P-26, mixed 1:1 with 6 % H_2O_2) containing 0.7 %, 0.7 %, 0.09 % and 0.04 % m-aminophenol, respectively, were tested on groups of 50 male and 50 female Swiss Webster mice by dermal-topical application (0.05 ml/cm^2) for 21-23 months. No statistically significant differences for tumours or other parameters were observed between controls and treated groups.

m-aminophenol contained in two formulations (0.7 %, 1:1 with H_2O_2) was tested on Charles River rats (F_0 generation) from the time of weaning to the weaning of their litter (F_{1a} generation) by dermal-topical application (0.2 ml increased by 0.1 ml to 0.5 ml) 2 times a week for 2 years. No compound-related gross lesions were observed in all treated groups. Two formulations containing m-aminophenol (0.09 % and 0.02 %) were tested with the same aforesaid treatment schedule on rats. No gross lesions related to the treatment were observed. In several males and females hyperkeratosis and/or acanthosis of stomach mucosa were found. In the liver of several rats, especially males treated with a 0.09 % formulation, hepatocellular hypertrophy/ hyperplasia or hyperplastic/hypertrofic nodules were noted (possibly compound-related).

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound (2.5 % (w/v) in 0.5 % aqueous gum tragacanth with 0.05 % sodium sulfite) applied both on abraded and intact skin of three New Zealand albino rabbits resulted mildly

irritating. Readings were performed at 24 and 72 h. At 72 h one animal showed a very slight oedema both in intact and abraded sites.

4.2 Irritation (mucous membranes)

The compound (2.5 % (w/v) in 0.5 % aqueous gum tragacanth with 0.05 % sodium sulfite) instilled into one eye of 3 New Zealand White albino rabbits resulted non irritating. A minimal conjunctival irritation was observed 1 hour after instillation in all rabbits.

5. Sensitization

m-aminophenol (3 % in water with 2 % Natrosol, 2 % Tween 80 and 0.05 % sodium sulfite) showed no allergic reaction in 20 guinea pigs by open epicutaneous method. A further study with m-aminophenol by dermal topical applications (0.1 ml, dose not reported) on 10 Guinea pigs showed no sensitized animals after challenge reactions.

Human: m-aminophenol resulted negative in a patch test on a sensitized man (positive to IPPD and PPD) and it was unable to produce cross-reactions.

6. Teratogenicity

Syrian golden hamsters (number of animals not reported) received i.p. injection (100, 150, or 200 mg/kg in 10 % DMSO aqueous solution) of compound in the morning of day 8 of gestation (8 days after the evening of breeding). The females were killed at 13 days of gestation for foetuses analysis. The compound induced some malformations (type not reported) at 150 mg/kg (6/84 = 7.1 % malformed foetuses), without maternal toxicity, not statistically significant.

25 of 35 females previously exposed to m-aminophenol in the 90-day oral study on Sprague-Dawley rats were further treated in the diet with the compound at same dose levels (0, 0.10, 0.25 or 1.00 %, i.e. 600, 150 and 60 mg/kg) from days 0 to 20 of gestation. Dams were killed on day 20 of gestation for foetuses analysis. The 1 % dose produced maternal toxicity: during gestation the body weight gain of the high-dose group continued to differ significantly from the control.

m-aminophenol based formulations (0.04 % - 0.7 %, 1:1 with H_2O_2) were applied (2 mg/kg) to the shaved skin of rat (0.7 % (2 formulations), 0.09 % and 0.02 %, on day 1-4-7-10-13-16-19 of gestation, 20 females for each group), mouse (0.7 %, 0.05 ml/mouse, 2 times a week from 4 weeks before mating to day 18 of gestation), and rabbit (0.7 %, 2 ml/kg, 2 times a week from 4 weeks prior to mating through 30 day of gestation). In rat a significant reduction of the mean live fetal weight was noted. In mouse a retarding effect of ossification process (bones of feet and of cervical and caudal vertebral center) and slightly lower fetal weights were found. No signs of maternal toxicity were found in rabbits. Focal alopecia until the last 3rd of gestation and a reduction of fetal survival were observed in rabbits as possible embryotoxic effects. Those results are not significant for teratogenicity potency.

6.2 Two-generation reproduction toxicity

In a multireproduction study on Charles River rats, the animals (F_0 parents: 40 males and 40 females, F_1 and F_2: 20 males and 20 females) received formulations containing m-aminophenol (0.02 % - 0.9 %) by dermal topical application (0.5 ml/rat). The treatment was performed twice

a week during, growth, mating, gestation, and during lactation and weaning of the F_{1b}, F_{2b} and F_{3c} litters of respective generations. No treatment-related gross or microscopic lesions were found.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: m-aminophenol HCl (ring ^{14}C, radiochemical purity >98 %) administered dermally (2 mg/kg as 0.3 ml of an 8 % aqueous solution) on shaved skin (9 cm^2) of OFA Sprague Dawley rats (5 males and 5 females) for 30 min. before shampooing and rinsing, showed that 0.41 % (8.1 µg/cm^2) of the applied dose penetrated in the skin. 89-95 % of the dose absorbed was revealed in the urine after 24 hours. The maximum level of compound in the organs was found after 35 min. The labelled compound (ring [^{14}C]) containing formulations (1 %, 2 %, and 3 %, 1:1 with 6 % H_2O_2) applied on the shaven skin of OFA Sprague-Dawley rats for 30 min. before shampooing and rinsing, showed the following values of dermal absorption: 0.14 % (1 % of m-aminophenol), 0.16 % (2 % m-aminophenol), 0.15 % (3 % m-aminophenol), and 0.053 % (2 % m-aminophenol) when skin was not shaven. After rinsing 92-96 % of the radioactivity was found in the rinsing water.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have demonstrated that m-aminophenol does not induce gene mutation in *Salmonella*, *E.col*, S.pombe and *S.cerevisiae in vitro* and *in vivo* (Sperm-head abnormality) on mouse and on Drosophila (SLRL)*;* chromosome aberrations *in vitro* on CHO cells and *in vivo* on bone marrow cells by micronucleus test on rat (2x25000 mg/kg oral, 24 interval), and mouse (up to 2x225 mg/kg i.p., 24 h interval) or up to 2.0 mmol/kg i.p.; genotoxicity effects *in vitro* on *E.coli* (DNA repair by plate-test), *S.cerevisiae* (mitotic gene-conversion) and V79 (SCE); genotoxicity effects *in vivo* on Chinese hamster (SCE, 5.0 mg/kg i.p.). Positive results were obtained in one study on Salmonella TA1538 with co-factors for gene mutation *in vitro*. SCE were found slightly positive (x1,4 the control) in bone marrow cells of Sprague-Dawley rats treated by i.p. with doses of 30 and 90 mg/kg, but not when treated orally up to 900 mg/kg. The compound resulted positive in the ability of producing aneuploid products of meiosis in Neurospora. Formulations containing m-aminophenol resulted negative: (a) for heritable translocation test on rat (1 % mAP, 1:1 with 6 % H_2O_2, 0.5 ml/cm^2 on shaved back skin, 2 times a wk x 10wks); (b) for chromosome aberrations and SCE in human volunteers by lymphocytes analysis (1:1 with 3-6 % H_2O_2 solution, dyed the hair 13 times at 3-6 wks intervals, 30 min of application, the dye washed out with shampoo).

9. Carcinogenicity

See 3.10.

10. Special investigations

Haemoglobin effects: m-aminophenol did not cause metahaemoglobin formation neither in fetal haemoglobin nor in adult haemoglobin at different values of pH (6.35 - 7.20).

Immunosuppressive: The compound (2.5 mg, 4 times with 1/4 maximal tolerated dose, sc. inject.) resulted negative for immunosuppressive action on mice.

11. Conclusions

However, the committee points out that the subchronic toxicity test by the oral route carried out with a single dose, was but of little significance and that it would have been preferable to have an adequate study and to know the no-effect dose.

Approved by the SCC on April 12th, 1988.

The committee was aware that CTFA has additional data on a 90 days study and this has been requested and should be assessed in due course; a modification to SCC opinion would be made if necessary.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

A 15 M-AMINOPHENOL

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1% of m-Aminophenol)

Maximum amount of ingredient applied	**I (mg) = 1000 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 0.16% (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 1000 x 0.16/100 = 1.6 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. = 1.6 mg /60 kg b.w.= 0.026 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 50 mg/kg b.w. (rat oral, 90-days)**
Margin of Safety	**NOAEL / SED = 50 mg/kg b.w. / 0.026 mg/kg b.w. = 1800**

A 17: *1-HYDROXYNAPHTALENE*

1. General

1.1 Primary name

1-hydroxynaphtalene

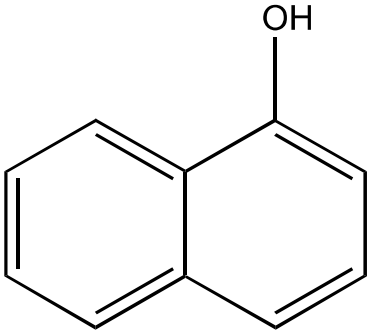
1.2 Chemical names

1-hydroxynaphthalene
1-naphthalenediol
1-naphthol
α-naphthol

1.4 CAS no.

90-15-3

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_8O$
Mol weight: 144.16

2. Function and uses

Oxidative hair dye; max.use 1.0 %; 0.5 % in combination with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	Rats, oral	2300 (1700 - 3300) mg/kg b.w.
	Rats, oral	2590 mg/kg

3.7 Subchronic oral toxicity

1-naphthol orally administered to rats (20 males and 20 females) for 12 weeks (5 times a week) showed that the dose of 20 mg/kg b.w./day (10 ml/kg) does not represent a toxic cumulative dose.

In a 30-day repeated dose study in mice treated with 200, 100, and 50 mg/kg b.w. (five animals/sex/group; controls included undosed and solvent groups) gastric lesions related to the treatment were observed only at the dose of 200 mg/kg in male mice. No other sign of toxicity was observed.

3.8 Subchronic dermal toxicity

A formulation containing 1-naphthol (0.5 %), mixed 1:1 with hydrogen peroxide, topically applied for 13 weeks (twice weekly) on abraded and intact skin of rabbit showed no evident toxic effect.

3.10 Chronic toxicity

Chronic toxicity and carcinogenicity: One oxidative formulation (7403, mixed 1:1 with 6 % hydrogen peroxide) containing 0.5 % 1-naphthol was tested on Swiss Webster mice by dermal application (0.05 ml/cm^2 x 21 months). No adverse effects were reported.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was applied to intact and abraded skin of rabbit at doses of 2.5 % (0.5 % aqueous gum tragacanth solution with 0.05 % sodium sulphite, pH = 7); it resulted not irritating after reading at 24 and 72 hours (primary irritation index = 0). No signs of irritancy were noted.

4.2 Irritation (mucous membranes)

The compound was instilled into one eye of 12 rabbits at concentrations of 0.5 % - 1.5 % -2.0 % - 2.5 % w/v (0.5 % in aqueous gum tragacanth with 0.05 % sodium sulphite, 3 animal/doses) and the eyes were washed out 10 sec after treatment. The results (ocular reaction evaluated at 1 h and 1-2-3-4-7 days) showed the minimum irritant level, between 1.5 % and 2.0 %: positive reactions were observed in 2/3 of the rabbits at 2.0 % w/v and 1/3 of the rabbits at 2.5 % w/v.

5. Sensitization

1-naphthol (3 % in water with 2.0 % Natrosol, 2 % Tween 80, 0.05 % Sodium sulphite and 10 % isopropanole) showed no allergic reaction in guinea pig by open epicutaneous method.

Sensitization was induced in 20 guinea pigs by simultaneously intradermal injections in the shoulder region of 0.1 ml of Freund's Complete Adjuvant (FCA), 0.1 ml 1-naphthol (0.1 % in water) and a 1:1 mixture of test compound and 0.05 ml Adjuvant at day 0. The test compound was dermally applied (0.1 % in water) 7 days later, under occlusion, on the injection site for 48 hours. 14 days later the guinea pigs were challenged by dermal application on the flank with

0.1 % and 0.05 % of 1-naphthol (aqueous solutions), under occlusion for 24 hours. The results evaluated after 24 and 48 hours of challenge showed that 1-naphthol was not a sensitizer in guinea pigs.

6. Teratogenicity

A formulation containing 1-naphthol (0.5 %, 1:1 with hydrogen peroxide) was topically applied (2 mg/kg/day) to the shaven skin of rats on day 1-4-7-10-13-16-19 of gestation. Only a significant reduction of the mean number of corpora lutea was observed between treated and two control groups (12.85 vs. 15.35 or 13.55). There was no evidence of any teratogenic or other adverse effect in the developing embryo/fetus.

7. Toxicokinetics (incl. Percutaneous Absorption)

Metabolism: 1-naphthol was administered to 6 male and 6 female white rats (20 % w/v in corn oil, 0.67 ml/rat, total amount of the compound = 6.4 g) by injection under the skin of the back for 4 days after the feeding period. The urine analysis, after extraction and using chemical methods, showed the following data (percentages of 1-naphthol administered are indicated by brackets): p-toluidine 1-naphthylglucuronidate: 2.8 g (14.7 %), 2.0 g (15.2 %) and 3.2 g (16.8 %); p-bromoaniline 1-naphthylsulphate: 0.063 g (0.4 %), 0.087 g (0.5 %), 0.008 g (0.6 %). These results showed that 1-naphthol was excreted in urine as 1-naphthylglucuronidate and 1-naphthylsulphate after subcutaneous injections. The study was performed in 1950.

Human absorption: An ointment containing 1-naphthol-[1-^{14}C] (3 g, 50 % soft soap and 50 % white soft paraffin) was applied in the interscapular region (10 cm, circular area) of the skin of 3 subjects, under occlusion for 8 hours. The percutaneous study showed a rapid and efficient absorption of the compound (3 days): 65.0-23.8-48.1 % (mean = 45.6 %) of the applied dose not recovered from the skin. The estimation of total urinary radioactivity was calculated only in one subject: 88.55 % (day 1), 5.2 % (day 2) and 2.8 % (day 3) of the dose not recovered from the skin (ca 97 %). The analysis of the major metabolites showed the following results (percentage of the dose not recovered from the skin): Subject 1: glucuronide fraction (day 1: 31.0 %; day 2: 1.0 %; day 3: 0.8 %), sulphate fraction (day 1: 1.3 %; day 2: 1.0 %; day 3: 1.2 %); acid hydrolysable fraction (day 1: 2.6 %; day 2: 0.2 %; day 3: 0.9 %); Subject 2: glucuronide fraction (day 1: 1.3 %; day 2: 1.0 %; day 3: 1.2 %), sulphate fraction (day 1: 0.8 %; day 2: 0.0 %; day 3: 0.03 %); acid hydrolysable fraction (day 1: 0.26 %; day 2: 0.04 %; day 3: 0.04 %); Subject 3: glucuronide fraction (day 1: 2.6 %; day 2: 0.3 %; day 3: 0.9 %), sulphate fraction (day 1: 0.0.8 %; day 2: 0.03 %; day 3: 0.0 %); acid hydrolysable fraction (unmeasurable). In the end, the radiolabelled compound, when applied topically under occlusion for 8 hours, shows an absorption value of 45.6 %; ca. 97 % of the absorbed dose is found in the urine during 3 days of analysis.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have demonstrated that 1-naphthol does not induce gene mutation *in vitro* in *Salmonella* and in mouse lymphoma L5178Y cells, and *in vivo* on *Drosophila* (recessive lethals, Basc test); chromosome aberrations *in vivo* on bone marrow cells by micronucleus test on mice (2x144-288 mg/kg i.p. = 2x1-2 mmoles/kg; 2 doses with

an interval of 24 h; analysis 30 h after the second dose) and rats (2x3000 mg/kg intragastric intubation, 2 doses separated by an interval of 24 h, analysis 6 h after the second dose); genotoxicity effects *in vitro* by DNA repair test on *E.coli* (3 strains) and *B.subtilis* (2 strains). Positive results were obtained for DNA repair test in one strain of *E.coli* (JC5547) using the spot test technique.

9. Carcinogenicity

See 3.10.

11. Conclusions

The SCC requires cutaneous absorption study in more realistic experimental conditions. However it was recommended that the B classification be maintained for one year.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-NAPHTHOL

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 0.5% of 1-naphthol)

Maximum amount of ingredient applied	**I (mg) = 500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 65%**
Dermal absorption per treatment	**I (mg) x A(%) = 500 x 65/100 = 325 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 325 mg / 60 kg b.w. = 5.416 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 20 mg/kg (rat: 90 day oral study)**
Margin of Safety	**NOAEL / SED = 20 mg/kg b.w / 5.416 mg/kg b.w. = 3**

This is clearly too low. However, the absorption was based on the use of a formulation in soft soap and white paraffin under occlusion for 8 hours and may grossly over estimate absorption in use. It was also noted that the 20 mg/kg NOAEL was based in a single dose level 90 day study. In a 30 day study the only effect seen at 200 mg/kg were local effects to the gut. The compound did appear to have relatively low toxicity.

Classification: B

A 18: *1,5-DIHYDROXYNAPHTHALENE*

1. General

1.1 Primary name

1,5-dihydroxynaphthalene

1.2 Chemical names

1,5-dihydroxynaphthalene
1,5-naphtalenediol

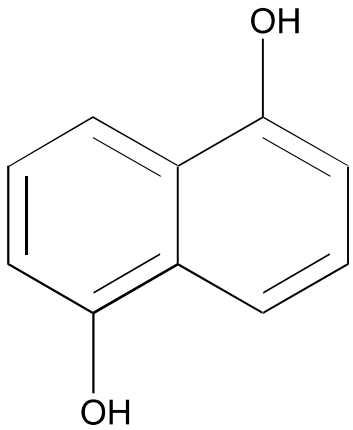
1.3 Trade names and abbreviations

Ro 576

1.4 CAS no.

83-56-7

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_8O_2$
Mol weight: 160.18

2. Function and uses

Oxidative hair dye; max. use: 1 %; 0.5 % with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Male mice, oral 680 (543-851) mg/kg

3.7 Subchronic oral toxicity

The compound was administered to 20 male and 20 female rats (Wistar strain, MuRa Han 67 SPF) by oral gavage 5 times a week for 12 weeks at a single dose of 50 mg/kg b.w./day in water suspension: no adverse effects were reported. The dose of 50 mg/kg represents the NOAEL for 1,5-dihydroxynaphthalene after oral treatment of rats.

4. Irritation & corrosivity

4.1 Irritation (skin)

Application of 0.5 ml of a 10 % aqueous suspension (in 2 % carboxymethylcellulose, pH 9.0) to the clipped intact skin of rabbit under occlusion for 4 hours. No signs of irritation were observed after 4, 24, 48 and 72 hours.

The compound as a 10 % (w/v) olive oil suspension, applied (2 droplets) to adult male hairless mice (strain hr hr) twice a day for 5 days to the same skin area showed no skin irritation.

4.2 Irritation (mucous membranes)

The compound as a 5 % carboxymethylcellulose solution (2 %, pH 9), instilled into one eye of albino rabbits of both sexes at doses of 0.1 ml (aqueous suspension) without rinsing off, resulted not irritating after 2, 6, 24, 48 and 72 hours.

5. Sensitization

In a study on female guinea pigs (20 females) induction doses consisted of simultaneous intradermal injections of 5 % (w/v) aqueous suspension of the test compound, 0.1 ml of Freund's Complete Adjuvant (FCA) and a 1:1 (v/v) mixture of FCA and 5 % water suspension of the test substance on day 0. Seven days later 5 % (w/w in vaseline) of test substance was dermally applied, under occlusion, on the same area for 48 hours. On day 21 the guinea pigs were challenged by dermal application at a new skin site of a 25 % (w/w in vaseline), under occlusion for 24 hours. The results were evaluated after 24 and 48 hours. There was no evidence of any sensitization.

7. Toxicokinetics (incl. Percutaneous Absorption)

Cutaneous absorption: The ^{14}C-1,5-dihydroxynaphthalene (1,5-DHN, labelled at the C-1 of the naphthalene molecule, in a cream formulation) applied on 8 cm^2 of intact and clipped skin of 7 male and 7 female Wistar rats (SPF-TNO) for 48 hours (1 % in formulation: 6.0 mg ^{14}C-1,5-DHN, 54.7 mg 1,5-DHN, 422,1 mg distilled water and 173.0 mg ammonia conc.,

cream: 5.34 g; the formulation saturated the exposed air of the skin) showed the following values of cutaneous resorption: 7.73 % (= 28.6 mg/cm^2, for males), and 9.49 % (= 25.7 mg/cm^2, for females) of the applied compound equivalents. The radioactivity was eliminated within 24 hours after application. In the expired air practically no radioactivity was observed (0.026 % males; 0.065 - 0.072 % females).

The same study with radiolabelled compound contained in a cream (ca. 1 %) with developer and hydrogen peroxide when applied on 8 cm^2 of the intact clipped skin for 30 min., showed, after 48 hours, the following results of cutaneous resorption: 0.486 % (1.02 mg/cm^2, males) and 0.981 % (2.09 mg/cm^2, females). The radioactivity was excreted mostly with the urine in the first 24 hours after application. In the expired air the following values were revealed: 0.293 mg/cm^2 (males) and 0.358 mg/cm^2 (females).

Organ distribution and placental transfer: ^{14}C-1,5-dihydroxynaphthalene was administered to 5 pregnant and 1 non-pregnant Wistar rat, by tail vein injection at a single dose of 15 mg/kg b.w. (at 19 days of gestation) for evaluating by whole body autoradiography, the organ distribution and placental transfer of the test compound, 30 min, 1, 2, 6 and 24 hours after treatment. Significant amounts of radioactivity were revealed in small intestine and kidney 30 min after application. The blood, the lungs and the placenta resulted distinctly labelled. The placenta barrier protected the fetal tissues as confirmed by the autoradiographic analysis in the punched out portion. The radioactivity in the placenta and in the fetuses decreased in the course of the study. A temporary labelling of the bones and the eyes in the maternal body was observed 6 hours after application. No selective retention in the fetal organs was observed. Low retention of radioactivity was revealed in mammary tissue 24 hours after treatment. No difference in the distribution of radioactivity was observed between pregnant and non pregnant rats (1 hour after treatment). The excretion was very rapid in the urine (1 hours: 46.6%; 24h: 81%); in the faeces 12.1% of the dose was excreted after 24 hours.

Excretion: ^{14}C-1,5-dihydroxynaphthalene was subcutaneously applied to 6 male and 6 female Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expired air and in the carcass was evaluated after 8, 24, 48 and 72 hours of observation period. The following results were obtained as percentage of the administered radioactivity after 3 days (main values): 84.1 % (72 h, males, urine); 78 % (72 h, female, urine); 8.42 % (72 h, males, faeces); 8.07 % (72 h, females, faeces); 0.292 % (expired air, male); 0.123 % (expired air, female); >1 % (carcass). Radio-Thin layer chromatography study of the urine showed that the parent compound was completely metabolized. At the end of the study 95.8 % of the administered radioactivity was recovered.

^{14}C-1,5-dihydroxynaphthalene was orally administered to male and female Wistar rats at a single dose of 10 mg/kg b.w. and the excretion in the urine, faeces, expired air, carcass and gastrointestinal tract was evaluated after 72 hours of observation period. A value of ca. 94.6 % of the administered dose was found for the intestinal absorption. Within 8 hours 59.5 % (males) and 65.1 % (females) of the applied dose were excreted in the urine. The following results for excretion were obtained (percent of the applied dose, 72 h): 86.5 % (urine, males); 83 % (urine, females); 5.57 % (faeces, males); 6.83 % (faeces, females); 0.061 % (carcass, males); 0.106 % (carcass, females); 0.027 % (gastrointestinal tract, males); 0.036 % (gastrointestinal tract, females); 0.025 % (liver, males); 0.016 % (kidney, males); 0.0086 % (blood, males); 0.0069 %

(plasma, males); 0.021 % (liver, females); 0.010 % (kidney, females); 0.006 % (blood, females); 0.005 % (plasma, females); negligible (expired air).

8. Mutagenicity

The compound was tested for gene mutation and found negative in the *Salmonella* assay.

In the micronucleus test performed by oral gavage on mice (2 equal doses separated by an interval of 24 h, 10 ml/kg) at doses of 2x75-150-300 mg/kg b.w. negative results were obtained.

11. Conclusions

The SCC requires a study on the chromosome aberration on mammalian cells grown *in vitro*.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1,5-DIHYDROXYNAPHTHALENE

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 0.5% of 1,5-dihydroxynaphthalene)

Maximum amount of ingredient applied	**I (mg) = 500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 1%**
Dermal absorption per treatment	**I (mg) x A(%) = 500 x 1/100 = 5 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 5 mg / 60 kg b.w. = 0.083 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 50 mg/kg b.w. (rat: 90-day oral study)**
Margin of Safety	**NOAEL / SED = 50 mg/kg b.w. /0.083 mg/kg b.w. = 600**

This was acceptable.

However, since data requested by the SCC were still outstanding, it was recommended that the B classification be maintained for 1 year, due to the requirements made by SCC.

Classification: B

A 19: *2,7-DIHYDROXYNAPHTHALENE*

1. General

1.1 Primary name

2,7-dihydroxynaphthalene

1.2 Chemical names

2,7-dihydroxynaphthalene
2,7-naphthalenediol

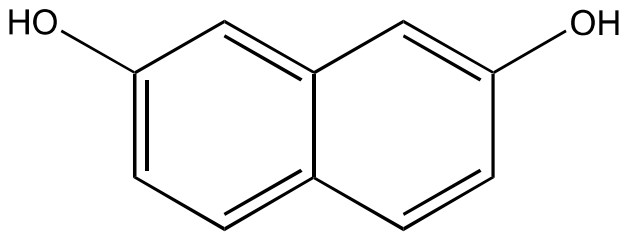
1.3 Trade names and abbreviations

Ro 575

1.4 CAS no.

582-17-2
C.I.: 76645

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_8O_2$
Mol weight: 160.2

2. Function and uses

Oxidative hair dye; max. use: 1 %; 0.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: mice CD1, oral: 720 (655-792) mg/kg b.w.
rat, oral: > 5000 mg/kg b.w.
(1 % of formulation containing 2,7-dihydroxynaphthalene)

3.7 Subchronic oral toxicity

2,7-dihydroxynaphthalene was administered daily by oral gavage, over a period of 12 weeks to 15 male and 15 female Wistar rats (Mu Ra Han 67 SPF) for each group, at dose levels of 0-20-60-180 (5.5 weeks)/360 (6.5 weeks) mg/kg b.w./day (10 ml/kg in aqueous suspension). The highest test dose produced weight increase in liver, spleen and kidney, liver pigmentation, increased hematopoiesis in the spleen, and hyaline deposition in the kidney. The other doses (20 and 60 mg/kg/day) did not show clinical, biochemical and pathological-anatomical signs of a systemic cumulative toxicity. The dose of 60 mg/kg/day represents the dose with the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied (500 µl in gauze patches) as a 10 % (w/v) solution in 2 % carboxymethylcellulose (pH = 8-10) for 4 hours on the clipped skin of rabbits resulted mildly irritating.

The compound applied twice daily for 5 days, as 10 % (w/v) aqueous solution, on the same back skin area of male hairless mice resulted not irritating.

A formulation (1 %) containing the compound resulted not irritating to rabbit skin when applied under occlusion for 4 hours.

A formulation (1 %) containing the compound resulted not irritating to mouse skin when applied daily (30 min per application) for 5 days.

4.2 Irritation (mucous membranes)

The compound applied as a 5 % (w/v) water solution (100 µl) on rabbit eyes resulted not irritating for the cornea and iris in all animals. The conjunctiva 2 hours after instillation showed mild or severe redness in all animals, with mild oedema (1 rabbit) and exudation (2 rabbits), disappearing 72 hours after treatment.

A formulation (1 %) containing the compound resulted slightly irritating for the eyes of the rabbit.

5. Sensitization

It was induced in guinea pigs by intradermal injection of 5 % (w/v) test compound in propylene glycol, Freund's complete adjuvant (FCA) and 1:1 (v/v) mixture of the above solution on day 0, and 7 days later by dermal application of 5 % (w/w) test compound in Vaseline, under occlusion, for 48 hours. 14 days later the guinea pigs were challenged by a dermal application, under occlusion at a new skin site, of the 10 % (w/v) test compound in propylene glycol. The compound resulted non-sensitizer in guinea pigs.

The formulation (1 %) containing the compound resulted non-sensitizer in guinea pigs after two different challenge exposures (open epicutaneous at day 21, and dermal administration at day 28).

6. Teratogenicity

2,7-dihydroxynaphthalene administered daily by oral gavage to groups of 30 pregnant CD-Sprague Dawley rats from day 5 to 15 of gestation at doses of 0-20-60-360 mg/kg showed at the highest test dose a slight retardation of average body weight during the treatment. No other difference was observed for other teratogenicity and embryotoxicity parameters. The dose of 60 mg/kg resulted the dose with the NOAEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

0.93 % of 2,7-dihydroxynaphthalene equivalents was absorbed through the skin of rats for over a period of 24 hours after 30 min of dermal application to intact and clipped skin of male and female rats with a formulation containing ^{14}C-2,7-dihydroxynaphthalene (21.76 mg).

Metabolism: ^{14}C-2,7-dihydroxynaphthalene applied subcutaneously (20 mg in distilled water) or oral (60 mg in distilled water) to male and female Wistar rats (SPF-TNO) showed that the radioactivity was excreted within 24 hours: in urine (partly as glucuronide or sulphate) and feces after subcutaneous treatment (more than 95 %) and in urine after oral administration. In the expired air no radioactivity was found after subcutaneous test. In the subcutaneous test no parent compound was revealed in the urine. In the oral treatment the test substance was completely absorbed by the intestine.

8. Mutagenicity

Embryotoxicity: The compound tested in the Hen's Egg Test resulted moderately toxic:

LD_{50}: 5.1 mg/egg (1 day) and 2.05 mg/egg (5 days). The compound did not show evidence of teratogenic potential in this system.

Mutagenicity and genotoxicity studies have shown that the 2,7-dihydroxynaphthalene does not induce: (1) gene mutations on five strains of *Salmonella typhimurium* in the absence and in the presence of Phenobarbital or Aroclor Induced rat liver enzymes; (2) micronuclea in CD-1 mice (bone marrow cells) treated by oral gavage (2 equal doses separated by an interval of 24 hours) with total doses of 0-60-300-600 mg/kg b.w.

11. Conclusions

The SCC requires a cytogenetic and a mouse lymphoma gene mutation *in vitro* study with full specifications of the compound tested and the nature and quantity of impurities eventually present, including mono, di, and trioxide naftalene.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2,7-DIHYDROXYNAPHTHALENE

A 19

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 0.5% of 2,7-dihydroxynaphthalene)

Maximum amount of ingredient applied	**I (mg) = 500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.93%**
Dermal absorption per treatment	**I (mg) x A(%) = 4.65 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 4.65 mg/ 60 kg = 0.077 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 60 mg/kg b.w. (rat: 90 days oral study)**
Margin of Safety	**NOAEL / SED = 60 mg/kg b.w./0.077 mg/kg b.w. = 770**

The B classification is maintained for 1 year, due to the requirements made by SCC.

Classification: B

A 22: *P-METHYLAMINOPHENOL*

1. General

1.1 Primary name

p-methylaminophenol

1.2 Chemical names

p-methylaminophenol
1-hydroxy-4-methylamino-benzene
Phenol, p-(methylamino)-benzene
N-methyl-p-aminophenol
4-(methylamino)-phenol
N-(methyl-4-aminophenol)
p-hydroxy-N-methylaniline
N-methyl-p-hydroxyaniline
N-methyl-4-hydroxyaniline
4-hydroxy-N-methylaniline

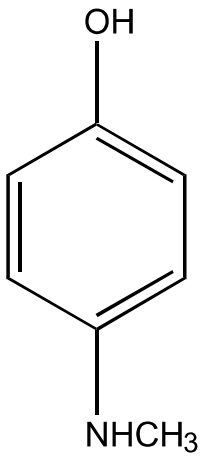
1.3 Trade names and abbreviations

IFG 62/78

1.4 CAS no.

150-75-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_3NO
Mol weight: 123
134 (as sulphate ½ H_2O)

1.7 Purity, composition and substance codes

The compound is generally used as sulphate.

2. Function and uses

Oxidative hair dye; max. use 3 %, 1.5 % with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD50: male mice, oral: 380 mg/kg (320-440 mg/kg).

3.4 Repeated dose oral toxicity

The compound was administered daily (7 days/week) for 30 days (males) and 31 days (females) by gastric intubation to 10 male and 10 female Sprague-Dawley OFA rats per group at doses of 0, 10, 30, 90 mg/kg b.w. (as sulphate) in 10 ml sterile water/kg b.w. The macroscopical histopathological analysis showed discoloration of spleen in 9 females (90 mg/kg) and acute tubular necrosis (30 and 90 mg/kg). Pigments and cells in urines (30 and 90 mg/kg) were observed at the urinary analysis. The hematology examination revealed signs of anaemia in females (90 mg/kg). No adverse effects have been revealed at the doses of 10 mg/kg/day. It is concluded that the dose of 10 mg/kg represents the NOEL for p-methylaminophenol after oral treatment of rats.

3.8 Subchronic dermal toxicity

N-methyl-*p*-aminophenol sulphate in two formulations (0.05 % and 0.1 % in water) were tested on shaven intact or abraded skin of New Zealand rabbits by topical applications: no toxic effects at 3, 7 and 13 weeks were observed after treatment by means of histopathological analyses.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied, under occlusion, to intact (left flank) and abraded (right flank) skin of 3 male and 3 female albino Bouscat rabbits, as 2 % sulphate in 0.5 ml aqueous solution for 24 hours, resulted slightly irritating after a reading at 24 or 72 hours: primary cutaneous irritation index = 0.74/8.

4.2 Irritation (mucous membranes)

The compound instilled into the conjunctival sac of one eye, without rinsing, of 6 male albino rabbits, as 2 % sulphate salt in aqueous solution (0.1 ml/animal) resulted practically not irritating after a reading at 1 day, 2, 3, 4 and 7 days after treatment.

5. Sensitization

It was tested in 10 male and 10 female Albino Hartley Guinea pigs treated with 0.5 g of the pure compound by topical occlusive applications behind the right shoulder blade, 3 times/week, with a 2-day interval for 3 weeks (treatments of 48 h) and once at the beginning of the 4th week. The animals received also an intradermal injection of 50 % saline Freund's complete adjuvant on days 1 and 10 of induction phase. At challenge phase, 12 days after induction, the untreated left flank received 0.5 g of test compound for 48 hours under occlusion. The compound showed no reaction after macroscopical and histological examinations at 1 hour, 6, 24 and 48 hours after the removal of the patch.

6. Teratogenicity

The compound (as sulphate) administered orally to pregnant rats on days 6-15 of gestation at the doses of 0, 10, 30, 70 and 150 mg/kg/day (0.5 ml/kg b.w. in sterile water) did not show embryotoxic or teratogenic activity at doses up to 70 mg/kg/day; the dose of 150 mg/kg/day gave adverse clinical signs and mortality in the dams.

No teratogenicity effects were observed on rats dermally treated with formulations containing the compound (0.05 % and 0.1 % in water) as sulphate.

A multigeneration reproduction study on rats with a formulation containing the compound (1.0 % in water) has produced negative results.

7. Toxicokinetics (incl. Percutaneous Absorption)

In vitro **absorption:** It was studied on abdominal human epidermis with finely cut human hair (10 mg) with a commercial hair dye formulation (1.5 g N-methyl-p-aminophenol: 1.34 g Resorcinol), containing the test compound (0.2475 mg), mixed 1:1 with hydrogen peroxide. After the application of 33 mg of test solution on 1.65 cm^2 and rinsing off after 30 min, no amount of the test compound was revealed by HPLC in the resulting chamber (4 ml NaCl 0.9 %, detection limit = 20 x 10^{-9} g/ml) during 4 h and 30 min observation period, thus indicating a value of absorption of less than 0.05 mg/cm^2: the absorption percent calculated is less than 0.033 %.

8. Mutagenicity

The compound was tested for gene mutations and found negative in the *Salmonella* (spot and plate tests), in the yeast *S. pombe P1* (forward mutation assay) and in *Drosophila melanogaster* (sex-linked recessive lethals test SLRL). The compound has been also evaluated for the induction of chromosome aberrations *in vitro* on CHO cells with negative results. In the micronucleus test performed by i.p. injections on mice (2 doses separated by an interval of 24 hours, 10 ml/kg) at doses of 2x 50 -75 -100 mg/kg b.w. negative results have been obtained.

9. Carcinogenicity

Long term study was carried out with two formulations containing the test compound (0.05 and 1.0 % in water, as sulphate) by dermal topical applications on mice once a week for 21 or 23 months (0.5 ml per application): no biologically significant differences were observed between treated and controls groups.

Another study, performed on rats treated dermally, by topical applications (0.2 ml, increases by 0.1 ml to 0.5 ml, 2 times/week per 2 years) from the time of weaning to the weaning of their litter with two formulations containing 0.05% or 0.1% of test compound as sulphate, produced negative results.

11. Conclusions

The SCC requires an *in vitro* mouse lymphoma gene mutation study and a dermal absorption study on rats. Data on contamination of this compound are also required (with nitrosamine?)

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

(P-METHYLAMINOPHENOL)

A22

(OXIDATION OR PERMANENT)

Based on a usage volume of 100 ml, containing at maximum 1.5 % of P-methylaminophenol

Maximum amount of ingredient applied:	**I (mg) = 1500 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.033 % (*in vitro*: human epidermis)**
Dermal absorption per treatment:	**I (mg) x A (%) = 1550 x 0.033/100 = 0.52 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg 0.52/60 kg b.w. = 0.009 mg/kg b.w.**
No observed adverse effect level (mg/kg):	**NOAEL = 10 mg/kg (rat: 30 days oral study)**
Margin of Safety:	**NOAEL / SED = 10 mg/kg b.w./0.009mg/kg b.w. = 1111.1**

This was acceptable.

However since further data were required it was recommended that the B classification be maintained for 1 year.

A 25: *6-HYDROXYBENZOMORPHOLINE*

1. General

1.1. Primary name

6-hydroxybenzomorpholine

1.2. Chemical names

6-hydroxybenzomorpholine
Hydroxy-6-phenomorpholine

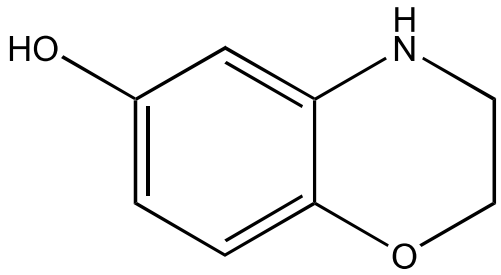
1.3. Trade names and abbreviations

Imexine OV (Chimex)
N°2164 E
Compound n°2164
IFG 58-78

1.4. CAS no.

977067-94-9

1.5. Structural formula



1.6. Empirical formula

Emp. formula: $C_8H_9NO_2$
Mol weight: 151

2. Function and uses

Oxidative hair dye; max use 2.0 %; 1.0 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1. Acute oral toxicity

LD_{50}: Mice, oral (gastric intubation): 860 (720-1020) mg/kg b.w.

3.7. Subchronic oral toxicity

The compound was orally administered to groups of 10 male and 10 female Sprague Dawley rats at doses of 40 mg/kg b.w. (2 % in propylene glycol, 5 ml/kg as water suspension) for 3 months. One rat died on the 40th day without correlation with the treatment. Treated male rats showed a slight decrease of the mean body weight gain at the end of treatment when compared with control male rats (174 g vs 222 g; mean absolute weight gains: 377 g vs. 430 g). Only 1/20 of the rats died after 40 days. The hematological, biochemical and urine analyses, as well as the anatomopathologic exams (macroscopic or histological) did not show significant differences between treated and control group. The compound produced very slight toxic effects (hepatocytes vacuolisation in one rat) like isolated lesions revealed after histopathological examinations.

The compound (as suspension in hydrogel with 2 g polysorbate 80 per 100 ml sterile water for injectable preparation) was administered by oral intubation to groups of 10 male and 10 female Sprague-Dawley OFA rats at doses of 0, 10, 100 or 1000 mg/kg/day for 30 days (males) or 31 days (females). No treatment-related abnormalities were observed at gross necropsy. The microscopic examination of the cortical tubules of the kidney of males (100 or 1000 mg/kg/day) revealed the following changes: epithelial necrosis, anhistic acidophilic substance deposits, cytoplasmic basophilia and dilatation. The severity of the changes was dose-dependent. No histopathological lesions were observed in the low-dose (10 mg/kg/day) group.

3.8. Subchronic dermal toxicity

A formulation containing the compound (coded as P-25) at dose level of 1.1 % (1:1 with 6 % hydrogen peroxide), was topically applied (1 ml/kg) on abraded and intact skin of rabbits for 13 weeks (twice a week). Hematologic and clinical chemistry were performed at 0-3-7-13 weeks. In females a statistically significant decrease of the mean haemoglobin values (11.87 ± 0.59 vs. 12.54 ± 0.68 g, $P < 0.05$), was observed between treated and combined control groups, at the end of treatment. Such differences were not considered to be of toxicological significance (in the range of historical control values). No evidence of systemic toxicity was observed.

3.10. Chronic toxicity

Chronic toxicity and carcinogenicity: *Dermal topical application.* One oxidative formulation (coded as P-25, 1:1 with 6 % hydrogen peroxide), containing 1.1 % of the compound was tested on Swiss Webster mice by dermal topical application (0.05 ml/cm on interscapular area) once a week for 23 months. The following remarks were noted: mortality, behaviour and dermal changes (daily); skin lesions (weekly) and gross appearance (continuously). Gross and microscopic examinations were performed in mice found dead or sacrificed during the study, and in all surviving animals at the end of the study. Negative results were obtained.

4. Irritation & corrosivity

4.1. Irritation (skin)

The compound was applied, under an occlusive patch, on the abraded and intact skin of 3 male and 3 female albino Bouscat rabbits as 1 % solution in propylene glycol for 24 hours. The compound resulted “slightly irritating” (primary irritation index = 0.45).

4.2. Irritation (mucous membranes)

The compound was instilled into one eye of 3 male and 3 female albino rabbits as a 1 % solution in propylene glycol (0.1 ml) without being rinsed off after instillation. The compound resulted "practically not irritating" to the eye of rabbit at reading, 48-72 hours and 4-7 days after treatment.

5. Sensitization

Sensitization was induced in 20 guinea pigs by topical occlusive applications of 0.5 g of the compound (3 times a week, with 2 days of interval, for 3 weeks and one at the start of the 4th week; 10 applications, patch test for 48 hours, right shoulder blade) and an intradermal injection of Freund's complete Adjuvant (0,1 ml^{3} diluted to 50 % in sterile isotonic solution) on days 1 and 10. The treatment was suspended for 12 days (from day 24 to 35 of the experiment). On day 36 the guinea pigs were challenged by topical application (0.5 g) under occlusion for 48 hours on left untreated flanks. Evaluation of sensitizing reaction was done at 1, 6, 24 and 48 hours after removal of the occlusive patches. The compound showed no skin reaction.

Photoallergenicity: The test was performed at a concentration of 0.4 % (w/w) of the compound in propylene glycol, using 25 albino Hartley guinea pigs. The compound was applied to the shaved skin on day 2 and then 20 animals (group 2) were immediately exposed to UVA (1.32 mW/cm^2 at 360 nm) and UVB (1.32 mW/cm^2 at 310 nm) radiation (2 lamps at 5 cm from the back of the animal) for 20 min. Five animals received no irradiation (group 1). The test sites were scored at 1 and 6 h, and on day 3. On day 4 and 9 the same procedure of day 2 was repeated. The test sites were scored on day 5 and 10 and shaved on day 3 and 8. The induction phase was performed 13 weeks after the third application applying the compound on a previously untreated area with the substance or with irradiation. The animals (group 2) were irradiated only with the UVA lamp for 5 min. (20 cm from the back) and then for 15 min. (5 cm from the back). Photoallergic reactions were evaluated 1, 6, 24 and 48 hours after the treatment with the compound. No edema was observed in both groups of guinea pigs. No evidence of allergic reaction (group 1) or photoallergic reactions (group 2) was seen at the microscopic examination. In this study the compound was not photoallergen in guinea pigs.

6. Teratogenicity

The formulation containing the compound (1.1 %), coded as P-25 (1:1 with 6 % of hydrogen peroxide), was applied topically to the shaven skin of Charles River rats at the dose of 2 mg/kg on days 1-4-7-10-13-16-19 of gestation. The results did not show embryotoxic and teratogenic effects.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human - Dermal absorption in vitro: The penetration of the compound through human epidermis placed on Franz type diffusion cells was studied in four separate assays. The section of epidermis of human mammary skin (lower layer) was in contact with 0.625 % of the dye solution of the compound (9 % sodium chloride, 0.01 % sodium ascorbate) for 30 min and then the skin was rinsed off by an aqueous solution (2 % sodium lauryl sulfate and 10 ml distilled

water). The amount of the compound which penetrated the epidermis (evaluated after 4 hours) averaged 0.05, 0.048, and 0.06 % of the applied dose in each of the four assay, respectively.

8. Mutagenicity

Mutagenicity / Genotoxicity studies demonstrated that the compound was found negative *in vitro* for: gene mutations by the reverse system analysis on *Salmonella* by plate and spot test (with 2 % NH_4OH and 1:1 H_2O_2) and forward mutation on *Schizosaccharomyces pombe* P1 (10); chromosome aberration *in vivo* by micronucleus test on mice (400 mg/kg i.p., analysis at 24, 48, 72 and 96 hours) (12); genotoxicity by the UDS assay on Hela human cells line using two different methodologies (scintillation count and autoradiography).

9. Carcinogenicity

See 3.10.

11. Conclusions

The possibility of nitrosamine formation of this compound should be considered.

The SCC requires a chromosomal aberration test in mammalian cells grown *in vitro*.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

6-HYDROXYBENZOMORPHOLINE

(A25)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1% of 6-hydroxybenzomorpholine)	
Maximum amount of ingredient applied	**I (mg) = 1000 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.1%**
Dermal absorption per treatment	**I (mg) x A(%) = 1000 x 0.1/100 =1 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 1 mg / 60 kg b.w. = 0.017 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 10 mg/kg b.w. (subchronic rat study)**
Margin of Safety	**NOAEL / SED = 10 mg/kg b.w. / 0.017 mg/kg b.w = 580**

This was acceptable.

However since further data were required (an *in vitro* chromosome aberration study) it was recommended that the B classification be maintained for 1 year.

Classification: B

A 27: *1-METHYL-2-HYDROXY-4-AMINO-BENZENE*

1. General

1.1 Primary name

1-methyl-2-hydroxy-4-amino-benzene

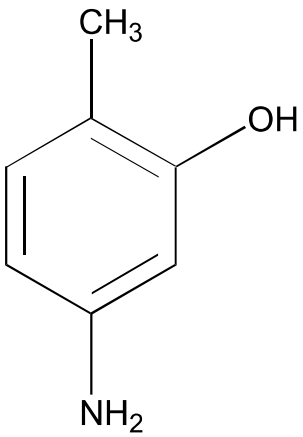
1.2 Chemical names

1-methyl-2-hydroxy-4-amino-benzene
2-hydroxy-4-aminotoluene
p-amino-o-cresol

1.4 CAS no.

2835-95-2

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123.1

1.9 Solubility

Solubility: slight in cold water. Freely in hot water, ethanol, ether.

2. Function and uses

Use: In oxidative hair dye formulations at 3 %; or at 1.5 % with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat: Following a preliminary range-finding test, five male and 5 female animals were given a 10 % suspension of a. i. by gavage; the volumes of the doses ranged from 16 to 64 ml/kg b.w. A control group was treated with 64 ml/kg b.w. of vehicle alone. The LD_{50} was estimated at 3.6 g/kg b.w., with 95 % confidence limits of 3.1 to 4.0.

Five male and four female rats were tested with 4050, 5400, 8100 and 10800 mg/kg b.w. of a.i. orally. The LD_{50} was estimated to lie between 9000 and 10000 mg/kg b.w.

3.4 Repeated dose oral toxicity

Subacute toxicity. As part of a range-finding study for a proposed 90-day study, groups of 5 male and 5 female rats were given 0, 100, 300, 900 and 2700 mg/kg b.w./day of a.i. by gavage, 5 days a week, for 2 weeks. The following observations were made. Animals of the high-dose group were apathetic for a few hours after dosing, and it was noted that they offered little resistance to the gavage after the first administration. The urine of these animals was coloured dark brown. No obvious clinical signs were noted in animals of the other groups. In the top-dose group body weight gain was reduced, and food consumption was also reduced in this group. Changes were noticed in other groups, some of which were statistically significant, but they were not judged to be of biological significance, and were not dose-related. There was no difference in the levels of methaemoglobin between the groups, and Heinz bodies were not seen.

Autopsy: Certain variations are common in the strain of rat used, such as diaphragmatic hernia and widening of the renal pelvis; the following findings were made: Most frequently disseminated white lesions in the pancreas were found; these seemed to be dose-related. Otherwise, although several abnormal findings were noted, they did not appear to be dose-related, or due to administration of the material.

As a result of these studies it was decided to conduct the 90-day study with doses of 900 to 2700 mg/kg b.w./day; however, it should be noted that the doses chosen for the 90-day study were in fact 300, 900 and 2700 mg/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. A thirteen-week study was carried out using 4 groups of animals, each consisting of 10 males and 10 females. The doses used were 0, 300, 900 and 2700 mg/kg b.w./day, and were administered as a finely-ground suspension by gavage, 5 days a week for 13 weeks. The following were recorded: body weight; food consumption; water consumption (at first for groups 1 and 4 only, but later for all groups); daily clinical examination and weekly veterinary examination; ophthalmoscopy at the beginning and end of the experiment.

Haematology: The animals were bled at the beginning and during the fifteenth week of the experiment. The usual haematological investigations were made, and the plasma tested for

thromboplastic activity, and urea, glucose, total protein, alkaline phosphatase, alanine aminotransferase, sodium and potassium.

Urine examination: Rats were placed in metabolic cages, fasting but with free access to water, before the experiment and on the twenty-eighth day. A test of ability to concentrate and dilute urine was carried out on days 82 and 83. Additional tests were appearance, smell, volume, specific gravity, pH, and tests for albumin, glucose, ketone bodies, urobilinogen, bilirubin and blood.

Autopsies. All animals were examined. The following organs were weighed: kidneys, adrenals, spleen, testicles, heart, liver, brain and hypophysis. From the animals of groups 1 & 4 (i.e., the control and the top dose) these organs, and numerous others, were fixed and prepared for histological examination. From the remaining animals sections were prepared from liver, kidney, pancreas and stomach.

Results. Two animals died - one on day 78, and one on day 84. Both these deaths were attributed to gavage accidents.

Clinical observations: After dosing, animals showed somnolence or even loss of consciousness, tremor, writhing, and inhibition of respiration. These changes were more marked and lasted longer in the early stages of the investigation; later they became less severe, so that after a month even the top-dose animals showed somnolence for only a few minutes.

Body weight: The top-dose male animals showed a reduced body weight compared with the controls. The other two experimental groups showed no change. The female animals showed little change; the only significant reduction in weight development was in group 3, and that on two occasions only, days 36 and 64.

Food intake. No significant difference occurred in the males. There was a significant increase among the female animals at two points: days 35-42 in group 3 and days 53-70 in group 4.

Water intake: In the top dose groups, the females had a significantly increased intake throughout the experiment, while in the male top-dose group the increase was confined to the first 58 days.

Haematological changes: On the thirtieth day, the white cell counts in the top dose animals were reduced, but this reached significance only in the females. The level of eosinophils in the top-dose males was also reduced. On the last day, both male and female animals showed a dose-related decrease in red blood cells, haematocrit, and haemoglobin. These did not always reach significant levels, except that in female rats the haematocrit showed a significant decrease at all dose levels. The eosinophil count of the top-dose females also showed a significant decrease.

Biochemical analyses. In the last week there was a dose-related increase in alanine aminotransferase levels, which, however, only reached significance in the top-dose group. Protein levels similarly showed a tendency to a dose-related increase, but this did not reach significance.

Urine analysis. The urine was stained dark brown. On the twenty-eighth day the volume of urine was increased, in line with the increased water consumption. An increased specific

gravity was found in the top-dose group, but this was attributed to the amount of a.i. being excreted. The capacity of the kidney for dilution and concentration was not affected. The urine in the top-dose animals was more acid than in the controls.

Ophthalmoscopy. Two animals were affected: one had a corneal erosion and one a cloudy anterior chamber.

Autopsy. The strain of rats used showed a number of abnormalities even in the control groups. Excluding these, the autopsy findings that seemed to be of significance seemed to be due to dosing were as follows.

Stomach: in the higher-dose groups, there were brownish-red deposits in the crypts of the glands; commonly, there was also a thickening and hardening of the mucosa, and increased rugosity of the superficial layers.

Organ weights. Absolute: there was a dose-related increase in the weights of the liver in groups 3 and 4. Females of groups 3 and 4 also showed a dose-related increase in the weights of the kidneys and adrenals as well. Males of group 4 showed a fall in the weights of testicles and heart.

Relative weights: Males of groups 2, 3 and 4, and females of groups 3 and 4, showed increases in the relative weight of the liver. Males and females of groups 3 and 4 showed increases in the relative weight of the kidneys. Both male and female animals of group 4 showed increased relative weight of the adrenals. Males of group 4 showed an increase in relative weight of the spleen. While all these changes appeared to be dose-related, statistical significance was, in general, achieved only in the higher-dose groups.

Histopathology. A preliminary examination of sections from numerous organs of half of the control animals and all of the group 4 animals suggested that certain changes were common to both groups, and so histological examination was carried out in animals of groups 1 and 4 only, and was confined to those organs in which dose-related changes were probable. The spleen was examined in the top-dose group. In summary, the following changes were found to be significant. Liver: necrosis, presence of vacuoles (group 4). Kidney: deposits in tubules (groups 3 and 4, but also present in groups 1 and 2). Hyaline cylinders in collecting tubules (group 4). Tubular nephrotic changes (groups 3 and 4). Vacuolation and cloudy swelling of tubular epithelium (group 4). Stomach: hyperkeratosis (groups 3 and 4), erosion of mucosa (group 4). Pancreas: localised vacuolisation (group 4).

Sudan staining was carried out on liver and kidney. There was probably a dose-related increase in sudanophilic material in the kidney in females of group 3 and males of group 4.

Prussian blue staining was carried out in the spleen. There were statistically significant increases in iron content in males of groups 3 and 4, and in females of group 4.

In summary, this investigation showed that even at the lowest dose (300 mg/kg b.w./day) there were dose-related adverse effects: symptoms referable to the nervous system, a fall in the erythrocyte and haematocrit values, and an increase in the relative weight of the liver. High doses produced other abnormalities, many of which seemed to be dose-related, but which did not reach significance. The author comments that the liver enlargement might be due to increased detoxification activity, and that this view was supported by the progressive reduction

in adverse clinical findings as the experiment was continued. The renal enlargement might be related to the large amounts of substance that had to be excreted. The enlargement of the adrenals, together with the lowered eosinophil count, raised the question of whether a general adaptation syndrome might not be present in these animals as well.

Rat: In view of the failure to find a dose which was tolerated in the previous investigation, a supplementary study was carried out in which doses of 0, 20, 60 and 180 mg/kg b.w./day were given by gavage to groups of 10 male and 10 female rats, 5 days a week, for 13 weeks. The conduct of the experiment was similar to that of the preceding one, but the observations and measurements that were carried out were a little different. All animals surviving the experiment were subjected to autopsy. It is stated that large numbers of tissues were prepared for histological examination, but there is no report of any such examination in the literature surveyed.

Two animals died before the end of the experiment, and deaths were attributed in one case to a gavage accident, and in the other to an overdose of ether.

Clinical observations. Immediately after the gavage, animals of all groups (including the control) showed some exhaustion and reluctance to move. These signs diminished as the experiment proceeded, and continued for not more than about one third of the period of the investigation. They are not further mentioned. Local hair loss occurred in all groups in the early stages, and may have been due to rodent bites or to dermatitis; in any event, it cleared up in about 2 weeks. Many of the abnormal findings are linked with the taking of blood by puncture of the retrobulbar venous plexus, and are found equally in all groups.

Body weight. There was no significant change in the body weight in any of the test groups compared with the control, except an increased weight in male rats of group 3 in the fourth week.

Food consumption: There was a significant increase in males of group 4 in the sixth and seventh weeks; in females of group 2 and 3 in the fifth week; and in females of group 4 in the twelfth week. No consistent trend, however, was seen. Water consumption: There were no important differences between control and test groups.

Haematological investigations. Despite the fact that these investigations were carried out on all the animals, no significant differences were found. The reticulocytes were more extensively examined than in the previous study, but yet showed no adverse trends.

Urine analysis. Some significant changes were found: a lower specific gravity in females of group 4 before the experiment began and a fall in males of group 3 on the eighty-third day; and a rise in pH in females of group 4 on the thirty-sixth day. These changes were not regarded as having biological significance.

Autopsy. Abnormalities known to occur in this strain of rat, and which were equally distributed over the groups, were not recorded here. No dose-related changes were found, and in particular no dose-related changes in the stomach were found, except that one male animal of group 3 showed signs suggestive of gastric ulceration.

Organ weights, absolute as well as relative, showed no dose-related changes.

Histological examinations - see above.

In summary, the findings suggest that under the specified experimental conditions, 1-methyl-2-hydroxy-4-amino-benzene in a dose of 180 mg/kg b.w./day does not produce adverse effects in the rat.

4. Irritation & corrosivity

4.1 Irritation (skin)

This test was carried out in accordance with the recommendations of the Consumer Product Safety Commission of the USA. A 2.5 % solution in gum tragacanth was applied to the shaved skin of 3 rabbits. Presumably the application areas were both abraded and non-abraded, but this is not stated. The treated areas were covered with an occlusive dressing and allowed to remain for 24 hrs. Readings were made at the end of the exposure and 48 hrs later. No abnormalities were seen. The substance was considered not to be an irritant.

Another report (which may be a fuller version of the previous one), suggested that the substance was a mild irritant.

Supplement :

40 human volunteers received on the skin, under occlusion, 1 % compound in water for 24 hours. The readings were made 24, 48 and 72 hours after applications. No skin reactions were revealed in any of the subjects.

4.2 Irritation (mucous membranes)

The compound was tested by the method prescribed by the FDA. The material was made up in a strength of 2.5 % in aqueous gum tragacanth. It was instilled into one eye of each of 3 rabbits and the eye was rinsed after 10 seconds exposure. There was mild inflammation of the conjunctiva one hour after instillation, but not thereafter. The test was regarded as negative.

5. Sensitization

The compound was made up in a strength of 3 % in a vehicle containing hydroxyethyl-cellulose and “Tween 80”. Nineteen guinea-pigs were used for the test, and 20 for the control. The solution was applied daily, with a glass rod, to the shaved skin over an area of 6 cm^2, 6 days a week for 3 weeks. Two weeks later, a similar application was made to the previously untreated skin of the opposite side. Four animals showed weak sensitization.

6. Teratogenicity

Supplement, December 10, 1993:

The compound in 0.5% Carboxymethylcellulose was orally administered by intragastric intubation to 25 pregnant female Sprague Dawley albino rats at doses of 0, 20, 60 or 180 mg/kg b.w. (10 ml/kg) day from day 6 to 15 of gestation. A positive control group received 15 mg/kg Vitamin A in rape oil, during the same period. The rats were killed on day 19 (positive control) or 20 (treated or negative control) of gestation and the dams and fetuses were analysed. No

embryotoxicity, embryolethality or teratogenicity effects were observed. The dose of 180 mg/kg b.w. day represents the NOEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

Supplement, December 10, 1993:

Dermal absorption: A formulation containing 1.0 % of compound (specific activity: 5.7 mCi/mMol), mixed 1:1 with 6 % H_2O_2, was applied on 10 cm^2 intact clipped skin of 6 male and 7 female of SPF-TNO Wistar rats for 30 min. At 48 hours 3.62 µg/cm^2 compound for the males or 5.64 µg/cm^2 compound for the females penetrated in the skin. In the first 24 hours the compound was excreted in the urine. Extrapolated to human use the amount absorbed was 0.18-0.28 % of the applied dose of compound.

The same formulation, mixed 1:1 with 6 % H_2O_2, was applied on 10 cm^2 of the clipped skin of 12 male and 12 female of SPF-NNO Wistar rats for 30 min at dose of 200 mg (i.e. 2 mg of compound). The organ distribution was evaluated 2, 6, 24, 48 h after treatment in 3 males and 3 females for each time, respectively. No significant accumulation of radioactivity in any organ was found.

The partition coefficients of hair dye between octanol/H_2O and stratum corneum (guinea-pigs)/H_2O were: 25.4 (Octanol/H_2O), 8.0 (intact stratum corneum/ H_2O), 21.1 (delipidized stratum corneum/H_2O).

Supplement :

Metabolism: A suspension (1 g) of radiolabelled and unlabelled compound was administered by stomach intubation in a single oral dose (9.5 mg/kg b.w.) to 5 male SPF-TNO Wistar rats. At 96 hours, 88.7 % of the applied dose was revealed in the intestine. Radioactivity in gastrointestinal tract was 0.035 %, and in the carcass 0.267 %. 87.5 % of the radioactivity was excreted in the urine and 11.1 % in the faeces.

A 2 % aqueous suspension (0.9 g ca.) of radiolabelled and unlabelled compound was subcutaneously injected (10 mg/kg b.w.) into the nape of 5 male SPF-TNO Wistar rats. At 24 hours 80.7 % of radioactivity was excreted in the urine, and more than 83 % at the end of observation period (96 hours). 17.6 % of applied dose was revealed at 96 hours in the biliary tract. The radioactivity in the faeces showed great variability. Negligible radioactivity was found in the expired air.

Supplement :

Human absorption: A formulation containing 0.69 % of compound, 6 % H_2O_2 (1:1), and ^{14}C-labelled compound (specific activity: 159.1 µCi/mg) was applied to human scalp for 25-28 min. under normal condition of use. The dye mixture at lotion/hair ratio of 1.5-2 was worked into the hair of 3 human volunteers for 5-8 min. and left on for other 20 min. The human absorption evaluated as urinary excretion was 0.2 % of the applied dose. The half time of urinary excretion was 24 hours. The flux of the hair dye through human scalp was 4.5×10^{-10} mol/cm^2/h.

8. Mutagenicity

In vitro.

An Ames test using strains TA 1535 and 1538, with and without activation, at 1 to 100 µg/plate, showed a two-fold increase in revertants in strain TA 1538 with activation, in the top dose plate only. The test was considered to be negative.

A study was carried out on the mutagenicity of a number of hair dyes, including the present one, by the Ames technique, using the TA 98 strain. The amounts used were 15, 50 and 150 µg/plate, and activation was employed. There was no evidence of mutagenic activity of the a.i.

An Ames test using strains TA 1535, 100, 1537, 1538 and 98 was carried out at levels of 0.8, 4, 20, 100, 500 and 2500 µg/plate. Without activation, there was a doubling of revertants at 4 µg/plate with TA 1535. Higher concentrations were negative. After activation, no increase in revertants was seen. The test was considered to be negative.

An Ames test was carried out using strains TA 1535, 1537 and TA1538. Amounts used were 1, 10, 100, and 1000 µg/plate. There was a marked increase (about thirty-fold) in revertants at the top dose with activation. The compound was considered to be a frame-shift mutagen.

An Ames test was carried out using amounts of 8, 40, 200, 1000 and 5000 µg/plate, and the strains TA 1535 and 1538, with activation. It is not clear whether aroclor induction was used. Tests were carried out with and without the addition of a NADPH-generating system. There was a marked increase (about ten-fold) in the number of revertants in strain TA 1538 at 200 µg and 1000 µg/plate, and about half as big an increase at 5000 µg/plate, when the NADPH-generating system was included. In the absence of this system, an increase of about two-fold was found at 1000 µg/plate, and about 5-fold at 5000 µg/plate. The compound was considered to be a frame-shift mutagen.

A bacterial test for mutagenicity was carried out using *E.coli* strain 343/113. This strain can give rise to two forward and two backward mutations if exposed to mutagens. The bacteria were exposed to the substance at concentrations of 1, 10 and 100 µg/ml. There is no report of a positive control. The compound was considered to be non-mutagenic.

In vivo.

Micronucleus test. Five male and five female rats were given 2 doses of a.i. by intubation, the doses being separated from each other by 24 hrs. The dose was chosen after a preliminary test to find one which would be toxic, and might kill some of the animals. A similar group was given vehicle only. Six hours after the second dose the animals were sacrificed and the femoral bone marrow examined for micronucleated red cells. No significant increase was found.

Supplement :

The compound did not induce chromosome aberrations in cultured human lymphocytes *in vitro.*

Five formulations containing the compound, tested for the induction of SCE *in vivo* in lymphocytes of 10 human volunteers produced negative results. Before application the

products were mixed 1:1 with 3-6 % H_2O_2. The hair was dyed 13 times at intervals of 3-5 weeks. Formulations contained other dyes too.

10. Special investigations

Test for immunosuppressive activity. Several substances, including the present one, were tested. Six mice received 4 subcutaneous injections of one quarter of the maximum tolerated dose (calculated to be 7.75 mg) on 4 occasions. At the time of the first dose, the animals were also immunised against $2x10^8$ sheep red cells. Control animals, which did not receive the drug, were also used. On the fifth day the animals were sacrificed and the degree of immunity produced tested in two ways: (a) cell-mediated immunity was tested by harvesting spleen cells and testing for plaque formation according to the method described by Jerne. (b) Humorally mediated immunity was tested by a haemagglutinin test according to the method described by Middlebrook-Dubos, as modified by later workers. Both tests were stated to have been negative.

11. Conclusions

Comment: Several investigations that might have been expected have not, apparently, been carried out. Perhaps in view of the ambiguous results of the tests for mutagenicity a test for carcinogenicity might be required. Tests in man for sensitization, photosensitivity and percutaneous absorption might be desirable. Other tests, such as for teratogenicity, or reproduction tests, might or might not be thought desirable.

SCC requires a study on chromosome aberration test in mammalian cells *in vitro***.**

Classification: B

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-METHYL-2-HYDROXY-4-AMINOBENZENE

(A27)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 1-methyl-2-hydroxy-4-aminobenzene)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.28 (rat) (see additional information)**
Dermal absorption per treatment	**I (mg) x A(%) = 1500 x0.28/100= 4.2 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. = 4.2 mg / 60 kg b.w. = 0.07 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 180 mg/kg b.w. (13-weeks oral rats study)**
Margin of Safety	**NOAEL / SED = 180 mg/kg b.w./ 0.07 mg/kg b.w.= 2500**

A 42: *2,4-DIAMINOPHENOXYETHANOL DIHYDROCHLORIDE*

1. General

1.1 Primary name

2,4-diaminophenoxyethanol dihydrochloride

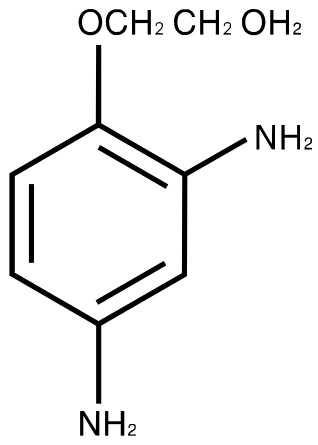
1.2 Chemical names

2,4-diaminophenoxyethanol dihydrochloride
2-(2',4'-diaminophenoxy) ethanol dihydrochloride
(Diamino-2',4'-phenoxy)-2-ethanol dichloridrate
1-ß-hydroxyethyloxy-2,4-diaminobenzene

1.4 CAS no.

66422-95-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{13}N_2O_2$
Mol weight: 241

2. Function and uses

Oxidative hair dye; maximum use 4%; 2% in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}	rats, oral:	1160 mg/kg
	male rats, oral:	1191 mg/kg
	female rats, oral:	1739 mg/kg
	male mice, oral:	1760 mg/kg
	female mice:	1739 mg/kg

3.7 Subchronic oral toxicity

The compound, as a 5 % Tween 80 solution, was administered orally to groups of 10 males and 10 females at doses of 0 and 56 mg/kg b.w. day for 3 months. No significant differences were found in body weight gain. At histological, clinical or anatomopathological exams no significant differences between treated and control group were found. The dose of 56 mg/kg b.w. represents the dose of NOAEL.

A preliminary subacute toxicity study on rats and mice was carried out in order to select the doses for a carcinogenicity study. Groups of 10 male and 10 female Crj:BDF_1 mice received 0, 0.01, 0.03, 0.05, 0.1 or 0.2% of 1-(2-hydroxy-ethyloxy)-2,4-diaminobenzene in drinking water for 12 weeks. The body weight gain was reduced in male mice treated with 0.1 and 0.2 % of compound. The dose of 0.05 % represents the NOAEL for mice. Similarly, groups of 10 males and 10 females of F344/DuCrj Charlers River rats received 0, 0.01, 0.03, 0.05, 0.1 or 0.2 % in drinking water for 12 weeks. A dose-related increase in mean body weight gain rate was observed. The dose of 0.1 % represents the NOAEL for rats.

3.10 Chronic toxicity

Chronic toxicity and carcinogenicity: Groups of 50 male and 50 female F344/DuCrJ rats received 0 %, 0.05 % (mean intake: 20.9 mg/kg day for males and 22.8 mg/kg day for females) or 0.1 % (mean intake: 35.5 mg/kg day for males and 60.9 mg/kg for females) of 2 (2',4'-diaminophenoxy)ethanol in drinking water for 104 weeks. Similarly, groups of 50 male and 50 female Crj:BDF_1 mice received 0 %, 0.04 % (mean intake: 35.8 mg/kg day for males and 44.6 mg/kg day for females) or 0.07 % (mean intake: 62.8 mg/kg day for males and 81.4 mg/kg for females) of the compound in drinking water for 104 weeks. No significant difference between treated and control group for type and target organs or incidence of tumors, was found in rats and mice. Pigment deposits in epithelial cells of thyroid follicles were found both in mice (0.04 % and 0.07 %) and in rats (0.1 %), but their distribution did not show any correlation with the occurrence of tumors. The results showed that the treatment had no carcinogenic effect in rats and mice.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as a 4 % solution in propylene glycol, was applied both on the intact and abraded skin of 3 male and 3 female albino Bouscat rabbits for 24 hours. The compound resulted mildly irritating for the skin of rabbits.

4.2 Irritation (mucous membranes)

The compound as a 4 % aqueous solution (pH 2.5) was instilled into one eye of 3 male and 3 female albino Bouscat rabbits. The eyes of the animals were not rinsed after treatment. The ocular reactions were evaluated after 1, 2, 3, 4 and 7 days. The compound resulted "practically not irritating" to the eyes of rabbits.

5. Sensitization

The compound in the presence of 50 % Freund's complete adjuvant, was applied epicutaneously, under patch-test, on 8 cm^2 of abraded skin of 5 male and 5 female Hartley guinea pigs for 48 hours. A second epicutaneous application was carried out after 8 days from the end of the first treatment. After 15 days the challenge reaction was carried out using a 25 % solution of compound in petrolatum, applied, under occlusion for 24 hours. The skin reactions were evaluted 24 and 48 hours later. 3 out of 10 animals showed allergic reactions. The compound had a medium potential of allergenicity.

6. Teratogenicity

The compound was administered by gastric intubation to 20 female (CL: COBS CD (SR) BR) Charles River rats on days 6 to 15 of pregnancy at doses of 0, 50, 100, 200 mg/kg b.w. day. The dams were killed on day 20 of gestation. The body weight gain was retarded at 200 mg/kg and to a slightly lesser extent at 100 mg/kg. A dose-related increase in the incidence of minor skeletal anomalies and the proportion of litters containing such foetuses were observed. At the dose of 200 mg/kg significant increases in the proportion of foetuses with skeletal variants extra rib ($P < 0.001$) and variant sternebrae ($P < 0.01$) were observed. These differences were not revealed at the dose of 100 mg/kg b.w. No statistically significant difference from control for litter and mean foetal weights were found at the dose of 200 mg/kg, althought lower values for both parameters were observed. The dose lower than 50 mg/kg b.w. days represent the NOAEL.

The compound was applied topically on the shaved skin of groups of six pregnant C57B1/6 female mice on days 6 to 15 post-fertilization at doses of 0 (16 females), 15, 150 and 1,500 mg/kg in corn oil. Benzo[a]pyrene as positive control was applied by i.p. injections to 19 females at the dose of 150 mg/kg b.w. on day 10 1/2 post-fertilization. No teratogenic effects were observed in mice both for the positive control and treatment with the compound.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: The radiolabelled compound, in a commercial vehicle or in a commercial formulation with other dyes, at the concentration of 0.40 % (i.e. 23.65 nM), mixed (1:1) with 20 vol. H_2O_2, was applied on 25 cm^2 of the dorsal region of 6 female hairless Wistar rats at the dose of 20 mg/cm^2 for 40 min. The following values of skin absorption were obtained:

5.05 ± 0.79 nM (i.e. 0.84 ± 0.13 µg/cm) for the compound alone in commercial vehicle; and 2.83 ± 0.49 nM (i.e. 0.47 ± 0.08 µg/cm) for the compound in commercial formulation.

In a second study, the radiolabelled compound, in a commercial vehicle at the concentrations of 0.40 % (i.e. 23.65 nM), 0.80 % (i.e. 47.30 nM) and 1.20 % (i.e. 70.95 nM), mixed (1:1) with 20 vol. H_2O_2, was applied on rats in the same way of the first study. The following values of absorption of compound at 0.40 %, 0.80 %, or 1.20 % concentrations were obtained after 4 days, respectively: 5.03 ± 0.79 nM (i.e. 0.84 ± 0.13 µg/cm); 7.96 ± 0.97 nM (i.e. 1.34 ± 0.16 µg/cm); 9.42 ± 0.84 nM (1.58 ± 0.14 µg/cm).

Commercial vehicle (1:1 with 20 ml H_2O_2) for 40 min (20 mg/cm^2):
0.40 %, 23.65 nM

1. Compound in vehicle
   20 x 0.40 = 0.08 mg/cm^2 applied
   penetrated: 0.84 µg/cm^2, 5.05 nM i.e. 0.84/80 = 1.05 %

2. Compound in complete formulation (mixture of several other hair dyes):
   20 x 0.40 = 0.08 mg/cm^2 applied
   penetrated: 0.47 µg/cm^2, 2.83 nM i.e. 0.47/80 = 0.58 %

8. Mutagenicity

Mutagenicity/Genotoxicity: The compound was tested for gene mutations *in vitro* in several experiments on *Salmonella typhimurium* and found negative on 5 studies with only one positive study. Negative results were also obtained in *Salmonella* with an urinary assay on rats treated orally (100 mg/kg), i.p. (100 mg/kg) or by topical applications (120 mg for 20 min), and mice treated dermally (15, 150, 1500 mg/kg b.w.). Negative results for the induction of gene mutations *in vitro* were obtained by reversions systems on *E.coli* (2 studies) and *S.cerevisiae* XV185-^{14}C, and forward mutation assays in *S.pombe* P1 and V79 hamster cell line (HPRT). Negative results were also obtained *in vivo* on *D.melanogaster* and in mouse spot-test (15, 150, 1500 mg/kg day for 3 days). The compound was unable to induce chromosome aberrations *in vitro* on CHO cell line and in human lymphocytes, and *in vivo* by micronucleus (2 x 250, 2 x 500, or 2 x 1000 mg/kg oral gavage, doses separated by an interval of 24 h) and dominant lethals test (dermal application, 15, 150, 1500 mg/kg/day x5 days) on mice. The compound did not induce gene conversion on *S.cerevisiae* D4, and UDS on HeLa human cell line.

9. Carcinogenicity

See 3.10.

11. Conclusions

The SCC does not consider the use of 2,4-diaminophenoxyethanol in hair dyes to be linked to any particular toxic risk for consumers.

Classification: A

CALCULATION OF SAFETY MARGIN

2,4-DIAMINOPHENOXYETHANOL HYDROCHLORIDE

(A 42)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 2 % of 2,4-diaminophenoxyethanol)

Maximum amount of ingredient applied	**I (mg) = 2000 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 1.05 % (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 2000 x 1.05/100 = 21 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A % / 60 kg b.w. = 21 mg / 60 kg b.w. = 0.35 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 56 mg/kg b.w. (90-day oral rat)**
Margin of Safety	**NOAEL / SED = 56 mg/kg b.w. /0.35 mg/kg b.w. = 160**

Classification: A

A 44: *2-METHYLRESORCINOL*

1. General

1.1. Primary name

2-methylresorcinol

1.2. Chemical names

2-methylresorcinol
1,3-dihydroxy-2-methyl-benzene
1,3-benzenediol-2-methyl
2,6-dihydroxytoluene

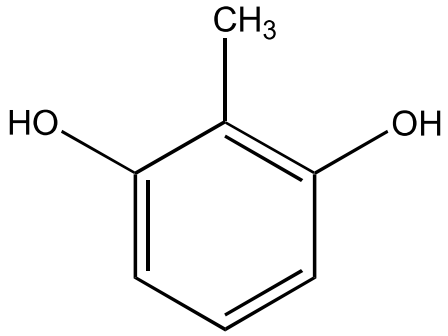
1.3. Trade names and abbreviations

Ro 261

1.4. CAS no.

608-25-3

1.5. Structural formula



1.6. Empirical formula

Emp. formula: $C_7H_8O_2$
Mol weight: 124

2. Function and uses

Oxidative hair dye; max. use 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1. Acute oral toxicity

LD_{50}: mice, oral 390 (360-420) mg/kg
rats, oral > 5,000 mg/kg (0.2 % in hair dye basic cream)

3.7. Subchronic oral toxicity

20 male and 20 female Wistar rats received by oral gavage 0, 20, 60 or 180 mg/kg b.w. in aqueous solution of 2-methylresorcinol, 5 times a week, for 12 weeks. The compound did not produce any exposure-related toxic effect in rats. Only marginal statistical differences were observed for biochemical parameters at all doses. The dose of 180 mg/kg b.w. represents the NOAEL.

3.8. Subchronic dermal toxicity

Six males and six females of adult New Zealand Rabbit were treated by dermal topical application twice a week for 13 weeks with a formulation containing 1 % of 2-methylresorcinol and mixed (1:1) with 6 % H_2O_2 before use. No evidence of systemic toxicity was found. The treated skin showed slight thickening after 26 applications.

4. Irritation & corrosivity

4.1. Irritation (skin)

Six adult male albino New Zealand Rabbits which received a 10 % (w/v) water solution of 2-methylresorcinol for 2 hours on clipped skin, showed a mild reversible dermal irritation.

A basic cream containing 2 % of 2-methylresorcinol, mixed (1:1, v/v) with 6 % H_2O_2, was applied on the clipped skin of five adult male rabbits (New Zealand) for 4 hours under occlusion. The results showed that the formulation resulted not irritating for the skin of the rabbits.

Five adult male hairless mice which received 5 µl of a 10 % (w/v) aqueous solution of 2-methylresorcinol (2 appl./day for 5 days) on the same skin area showed a mild skin redness after 5 applications.

Human dermal irritation: 5 human volunteers received a 10 % aqueous solution of 2-methylresorcinol on forearm skin for 30 min. with 30 sec. intervals, using the open method. No irritation was found. 5 human volunteers who received a 10 % aqueous solution on upperarm skin for 2 h under occlusive conditions did not show irritation within 24 h.

4.2. Irritation (mucous membranes)

One eye of six New Zealand rabbits received into the conjunctival sac 100 µl of 5 % w/v water solution of 2-methylresorcinol. The results showed a mild conjunctival irritation after 24 hours, disappearing after 7 days. No irritation of cornea and iris was found.

A basic cream containing a 2 % of 2-methylresorcinol, mixed 1:1 with 6 % H_2O_2, instilled into one eye of five adult male New Zealand rabbits for 10 sec. resulted slightly irritating.

5. Sensitization

The test was carried out on 20 female Pirbright White guinea pigs using a 5 % (w/v) aqueous solution of 2-methylresorcinol. The challenge exposure consisted in a dermal treatment for 24 h of the test compound with the same dose on a new skin site 14 days later. The compound did not produce dermal sensitization on guinea pigs.

A basic cream containing 2 % 2-methylresorcinol, mixed 1:1 with 6 % H_2O_2, was tested on 20 female Pirbright white guinea pigs in the maximization assay. No positive skin reaction was observed within 72 hours after the two challenge treatments performed with a 1 % basic cream on day 21st and 28th.

6. Teratogenicity

Rats (20 pregnant females) received 2 ml/kg topical applications of a formulation containing 1 % of 2-methylresorcinol on days 1-4-7-10-13-16-19 of gestation. The study resulted inadequate because the treatment was performed every 3 days and only one dose was tested.

Sprague-Dawley rats were treated (25 females for each dose) with 0, 0.1, 0.4 or 1.5 % of 2-methylresorcinol in the diet during the period of major organogenesis. The analysis were carried out on day 20 of gestation. At the doses of 0.4 % and 1.5 % a slight increase (not significant statistically) was observed in the mean post-implantation loss with a corresponding decrease in the mean number of viable fetuses and implantation sites. This result was considered as a biological variance. The compound was neither embryotoxic nor teratogenic. The dose of 1.5 % (i.e. 900 mg/kg) represents the NOAEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

Excretion study: 6 male and 6 female rats treated subcutaneously with 20 mg 2-methyl-resorcinol in water showed that more than 90 % of the administered dose was excreted in urine and feces within 24 hours. The radioactivity was excreted as glucuronide or sulphate. No radioactivity was found in expired air. In the oral study on rat (8 males and 8 females) more than 90 % of the administered dose (40 mg in water) was excreted in the urine. The major part of radioactivity was eliminated within 8 hours of treatment. The 2-methylresorcinol was almost completely absorbed by the intestine.

Dermal absorption: 8 male and 8 female Wistar (SPF-TNO) rats received 6.25 g of basic cream containing 16.9 mg (= 0.136 mMol) of 2-methylresorcinol, mixed with 6 % H_2O_2 (1:1), on intact clipped skin for 30 min. The results showed a maximum skin absorption of 0.48 % of 2-methylresorcinol equivalents in a 24 hours period.

8. Mutagenicity

The compound was tested for gene mutation *in vitro* and found negative on *Salmonella.* The compound did not induce chromosome aberrations *in vitro* on CHO cells and was unable to induce micronuclei *in vivo* on mice with oral doses up to 2 x 350 mg/kg.

Embryotoxicity: 2-methylresorcinol resulted moderately toxic in the HET test performed on Chicken embryos of White-Leghorn: LD_{50} = 6.1 mg/egg (day 1), LD_{50} = 1.08 mg/egg (day 5). No evidence of teratogenicity was found in dead or hatched chickens.

11. Conclusions

The SCC requires a teratogenicity study.

Classification: B

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2-METHYLRESORCINOL

A 44

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1 % of 2-methylresorcinol)

Maximum amount of ingredient applied	**I (mg) = 1000 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.48% (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 1000 x 0.48/100 = 4.8 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. = 4.8 mg / 60 kg b.w. = 0.8 mg/kg b.w.**

No observed adverse effect level (mg/kg) NOAEL = 180 mg/kg b.w. (rat oral, 90-days)

Margin of Safety	**NOAEL / SED = 180 mg/kg b.w./ 0.8 mg/kg b.w. = 225**

Classification: A

A 70: *1-METHYL-2,6-DIAMINOBENZENE*

1. General

1.1 Primary name

1-methyl-2,6-diaminobenzene

1.2 Chemical names

1-methyl-2,6-diaminobenzene

2,6-diaminotoluene

1,3-benzenediamino-2-methyl

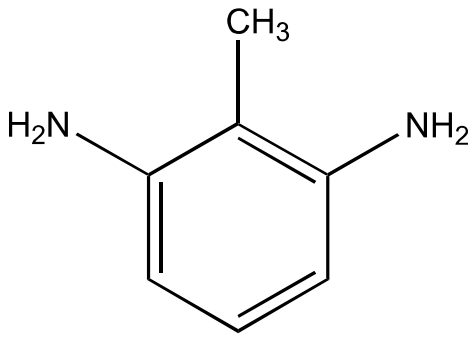
1,3-diamino-2-methylbenzene

Toluene-2,6-diamino

1.4 CAS no.

823-40-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_7H_{10}N_2$

Mol weight: 122

2. Function and uses

Oxidative hair dye; max. use 1 %; 0.25 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} rat, oral gavage: The results were evaluated according to three different methods :

a) 230 mg/kg b.w. (Kärber method)
b) 190 mg/kg b.w. (Litchfield/Wilcoxon method)
c) 190 (140-260) mg/kg b.w. (graphical method).

3.7 Subchronic oral toxicity

Groups of 5 male and 5 female rats (Wistar Bor: Wissw SPF/TNO) were treated by oral gavage with 0-0.3-3-30 mg/kg b.w./day of test compound (1 ml/100 g, in deionized water) for 91 days. The dose of 0.3 and 3 mg/kg did not show significant biological effects. A slight reduction in body weight gains of males (7-13 wks., statistically significant differences: 80.4 % vs 100.0 % control, $P < 0.05$) was seen in rats treated with 3.0 mg/kg. Such body weight gain reduction was not biologically significant because, at the end of the 13-week study, no difference between treated (3.0 mg/kg) and control group was obtained for the mean body weights in male rats: 289.2 ± 29.7 g (treated at 3.0 mg/kg) vs. 308 ± 37.2 g (control). The dose of 30 mg/kg gave the following toxicological effects: reduction of activity and hyporeflexia (10-120 min. post-treatment); decrease of body weight gains (1-13 wks) in males; reduction of food consumption in males (1-13 wks) and females (1-6 wks); significant increase of glucose levels (males and females; presence in the urine of erythrocytes and proteins (3/5 males); significant increase of liver and kidney weights. No significant differences for the histological changes were observed between control and treated at 30 mg/kg (10 males and 10 females/group). The "No Effect Level" (NEL) in this study was at 3.0 mg/kg b.w. /day.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound was applied to the intact and abraded skin of 6 White New Zealand rabbits at doses of 1 % in water (0.5 ml. pH = 7.3) under occlusive conditions for 4 hours. The skin reactions were evaluated 30 and 60 min. after the end of treatment and after 24-48-72 hrs. The results showed that the test compound did not produce any skin reaction.

4.2 Irritation (mucous membranes)

The compound was instilled into the conjunctival sac of one eye of 9 white New Zealand rabbits at doses of 1 % in water (0,1 ml, pH = 7.3). The eyes of 6 rabbits were rinsed out (4 sec or 30 sec after applications in two equal groups) while the eyes of the other 3 treated animals were not. Observations were done at 1-2-8-24 h and daily up to 7 days after treatment. Rabbits whose eyes were not rinsed out, showed a slight reddening of the conjunctiva up to 8 h after treatment. 1 % solution of compound in this study did not produce any significant irritant effect.

5. Sensitization

20 guinea pigs were treated topically (once a week for 3 weeks) with an 0.1 % water solution of test compound under occlusive conditions. The method of Buehler, occlusive patches without Freund's Adjuvant, was applied. The patches were removed after 6 hrs and the animals challenged (0.5 ml of test compound solution on shaven untreated skin) after an interval of 2 weeks. The reaction, evaluated 24 and 48 hours later, showed no skin sensitization. The low concentration used for the induction in this non-adjuvant technique was noted.

Human sensitization: Data not available.

8. Mutagenicity

Mutagenicity and genotoxicity studies have shown that 1-methyl-2,6-diaminobenzene is mutagenic in *Salmonella* and it is able to induce cell transformation in secondary hamster embryo cells (HEC); it enhances the transformation of primary HEC by Simian adenovirus 7 (SA 7) when given after virus.

Negative results were obtained for micronucleus test on mice treated orally by gavage (25-50-100 mg/kg in 1 % methylcellulose, 2 equal doses separated by an interval of 24 hrs., analysis 6 hours after the second dose).

The compound does not induce UDS *in vivo* on male rats (Fisher-344) treated orally by gavage with a dose of 150 mg/kg in corn oil (analysis at 2 and 12 hours).

9. Carcinogenicity

Long-term studies were carried out on B6C3F1 mice and F344 rats in a NCI bioassay, the compound (as dihydrochloride) being fed in the diet at 250 or 250 ppm for rats for 103 weeks (observed for 1 additional week) and at 50 or 100 ppm for mice for 103 weeks (observed for 1 additional week). The compound was considered to be not carcinogen for both sexes and both species.

In male rats, islet cells adenomas of the pancreas (P = 0.025) and neoplastic nodules or carcinomas of the liver (P = 0.037; 4/50 (8 %) vs. 2/334 (0.6 %) showed a significant dose-related trend using Cochran-Armitage test, but not with Fisher's exact test. The incidence of neoplastic nodules or hepatocellular carcinomas in male rats in the highest treated group is 4/50 (8 %) vs. 2/344 (0.6 %) of the historical control of NCI laboratory and the 36/2.230 (1.6 %) across all laboratories. The incidence of islet-cell adenoma of the pancreas in males of the highest dose group is 4/45 (ca. 9 %) in comparison with 2/35 (5.7 %) observed in one group of vehicle control male rats or 0/344 of historical control (NCI laboratory).

11. Conclusions

Classification: D

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-METHYL-2,6-DIAMINOBENZENE

(A70)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 0.25 % of 1-Methyl-2,6-diaminobenzene)

Maximum amount of ingredient applied	**I (mg) = 250 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 100% (in absence of data)**
Dermal absorption per treatment	**I (mg) x A(%) = 250 x 100/100 = 250 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 250 mg / 60 kg b.w. = 4.167 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 3 mg/kg b.w. (rat: 90-day oral study)**
Margin of Safety	**NOAEL / SED = 3 mg/kg b.w. / 250 mg/kg b.w. = below 1**

This is clearly unacceptable. Actual data on skin absorption was needed as a matter of urgency. However, even then it was considered unlikeky that there would be an adequate safety margin.

Classification: D

A 74: *1-HYDROXY-3-METHYL-4-AMINO-BENZENE*

1. General

1.1 Primary name

1-hydroxy-3-methyl-4-amino-benzene

1.2 Chemical names

1-hydroxy-3-methyl-4-amino-benzene
4-amino-3-methyl-phenol
4-amino-m-cresol
2-methyl-4-hydroxy-aniline
2-amino-5-hydroxy-toluene
6-amino-3-hydroxy-toluene

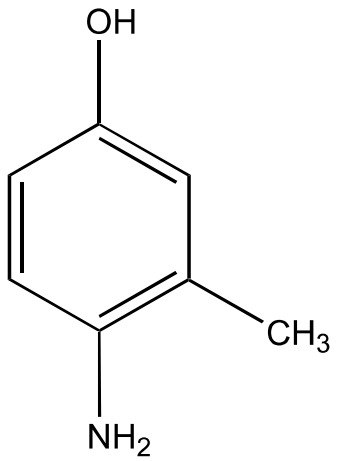
1.3 Trade names and abbreviations

Oxyrot

1.4 CAS no.

2835-99-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123

1.7 Purity, composition and substance codes

It exists as free base, hydrochloride and hemisulfate.

2. Function and uses

Oxidative hair dye; max.use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: female rats, oral 1010 mg/kg
male rats, oral 870 mg/kg
female mice, oral 908 mg/kg

3.7 Subchronic oral toxicity

1-hydroxy-3-methyl-4-aminobenzol-sulfat in water was administered daily by stomach intubation to Wistar rats (20 males and 20 females for each group) for 13 weeks at dose levels of 15, 60 and 120 mg/kg/b.w. (10 ml/kg). No specific finding was revealed at dose of 15 mg/kg b.w. At doses of 60 and 120 mg/kg b.w. dark discoloured urines were found on weeks 8 to 13 of treatment in both sexes. At dose of 120 mg/kg b.w. increases of the spleen weights (males and females) and the creatinine values (females) were observed after 13 weeks. No histopathological findings were revealed. The dose of 60 mg/kg represents the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as 3 % aqueous suspension, was applied on clipped skin (3 x 4cm) of 15 female Pirbright guinea pigs, once a day, for 5 consecutive days. The site of application was not washed. No erythema or oedemas were found at the application site 5 hours after each treatment. In this assay the compound resulted non-irritanting for guinea pigs.

Human skin-irritation: 40 human volunteers were treated with 1 % aqueous solution of compound for 24 h with a soaked patch. No skin reaction was observed after reading at 24, 48 and 72 h.

4.2 Irritation (mucous membranes)

The compound, as 1.5 % (0.1 ml) in 50 % ethylene glycol, was instilled without washing, into one eye of 5 female guinea pigs. The reading (examinations with 0.1 % fluoroscein sodium solution) carried out 24 and 48 h after treatment did not show any pathological lesions on conjunctiva, iris, cornea and the found of the eyes. The compound resulted non-irritating for guinea pigs.

5. Sensitization

3-methyl-4-aminophenol-hemisulfat (recrystallized) was intradermal injected two times in craniodorsal area of 10 male and 10 female guinea pigs. The first injection was carried out using Freund's Adjuvant complete (FCA), diluted (1:1) in water and the second with 3 % of compound diluted in 0.05 ml water. The animals were pretreated on the clipped shoulder area with 10 % sodium laurylsulfat one day after the first two injections. 6 to 8 h later the compound was applied, under occlusion, by dermal topical application (3 % in 0.5 ml white Vaseline), on the same skin area. 48 hours after the two injections, the bandages were removed, and the third intradermal injection was carried out with 3 % of the compound in 0.05 ml in FCA diluted (1:1) in oleum arachidis. The challenge reaction was carried out by closed patch test on day 14 after the last exposure with 1 %, 2 % and 3 % of compound in 0.05 ml FCA diluted (1:1) in oleum arachidis. The allergic reactions were evaluated 24 and 48 hours later. Any signs of erythema and edema were found after the challenge. The compound did not cause no delayed contact hypersensitivity in guinea pigs.

6. Teratogenicity

1-hydroxy-3-methyl-4-amino-benzene sulphate was orally administered by stomach intubation to groups of 24 pregnant BOR:WISW-SPF rats from day 5 to 15 of gestation at doses of 10, 40 and 80 mg/kg b.w. in deionized water (1 ml/100 g b.w.). No signs of maternal toxicity or adverse effects to the fetal development after autopsy of dams were revealed on day 20 of gestation. The dose of 80 mg/kg b.w. represents the NOAEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: A hair dye product containing [14C]-4-amino-3-methyl phenol hemisulfate (radiochemical purity 96 %) was applied on dorso-lumbar region of 3 male and 3 female PVG rats for 30 min (1 g per animal). Similarly, the compound in DMSO solution (150 mg/ml, 0.1 ml per animal) was applied for 24 h. In both treatments each animal received 15 mg ca. of compound (1,667 mg/cm^2, 200 µCi). 0.42 % (i.e. 0.25 % in urine, 0.02 % in faeces and 0.15 % in expired air) of the applied dose of compound in hair dye product or 7.47 % (i.e. 6.54 % in urine, 0.42 % in faeces, 0.38 % in cages washing and 0.13 % in expired air) in the solutions of DMSO were excreted after 72 hours. 87.77 % (hair product) or 89.24 % (DMSO solution) of the administered radioactivity was recovered from the dressing, the application site washing and the application site. No significant radioactivity levels were found in tissues at 72 hours in either treatment.

8. Mutagenicity

The compound was tested and found negative: (1) for the induction of gene mutation *in vitro* on *Salmonella typhimurium* performed with and without hydrogen peroxide*;* (2) for chromosome aberrations *in vivo* by the micronucleus tests on CD1 (2 x 20, 2 x 100 or 2 x 500 mg/kg day, oral gavage) and NMRI mice (100, 333, 1000 mg/kg b.w. in DMSO by stomach intubation); (3) for sister chromatid exchanges *in vivo* in male chinese hamster (tested as hemisulphate, 10, 30, 100 and 400 mg/kg i.p. or 100, 300, 1000, 1500 and 2000 mg/kg oral); (4) for UDS *in vitro* (colourimetric method) on primary rat hepatocytes cultures; (5) for UDS

in vivo on male Wistar rats treated orally (1000 mg/kg b.w. for 4 h, 60 and 600 mg/kg for 16 h).

11. Conclusions

In the absence of the carcinogenicity data and due to the structural similarity to known mutagens, the SCC requires the submission of data from *in vitro* cytogenetic lymphocytes and gene mutation on mouse lymphoma studies.

Classification: B

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-HYDROXY-3-METHYL-4-AMINO-BENZENE

(A74)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 1-hydroxy-3-methyl-4-amino-benzene)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.42% (rat, hair dye formulation)**
Dermal absorption per treatment	**I (mg) x A(%) = 1500 x 0.42/100 = 6.3 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A % / 60 kg b.w. = 6.3 mg / 60 kg b.w. = 0.105 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 60 mg/kg b.w. (90-day oral rat)**
Margin of Safety	**NOAEL / SED = 60 mg/kg b.w. / 0.105 mg/kg b.w. = 570**

Classification: A

A 75: *2-AMINO-5-METHYLPHENOL*

1. General

1.1 Primary name

2-amino-5-methylphenol

1.2 Chemical names

2-amino-5-methylphenol
1-hydroxy-2-amino-5-methyl-benzene
2-hydroxy-4-methyl-aniline
4-amino-3-hydroxy-toluol
6-amino-m-cresol
4-amino-5-methyl-benzene

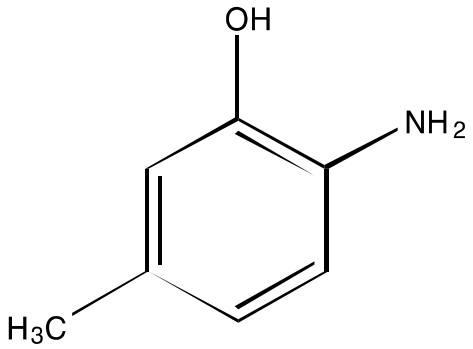
1.3 Trade names and abbreviations

Oxygelb

1.4 CAS no.

2835-98-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
Mol weight: 123

1.7 Purity, composition and substance codes

It exists as free base and hemisulfate.

2. Function and uses

Oxidative hair dye; max. use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}:	female rats, oral	1225 mg/kg
	male rats, oral	1375 mg/kg
	female CF 1 mice, oral	1225 mg/kg
	male CF 1 mice, oral	1020 mg/kg
	female CBL mice, oral	750 mg/kg

3.7 Subchronic oral toxicity

The compound (98 % purity) as 10 % suspension in 5 % gum Arabic was administered orally by stomach intubation for 90 days to 10 male and 10 female albino rats at dose of 800 mg/kg/day b.w. reduced at 500 mg/kg/day after 5 weeks (5 ml/kg). 2 rats died during the treatment. Tyrosine crystal were revealed in urine, and liver, kidney and spleen weights were reduced. Increased in Bilirubin and iron concentrations in males, reduction in T4 with no histopathological change in thyroids. The NOAEL < 500 mg/kg.

Oxygelb as 0.5 % in carboxymethylcellulose administered orally by stomach tube at doses of 0, 50, 250 and 500 mg/kg/day to 15 males and 15 females rats per dose (1 ml/100 g b.w.) for 4 weeks showed these results: 250 mg/kg: slightly increased activity for 10 min. post treatment during 3rd and 4th week; increased urine excretion (yellow-orange discolored); significative alterations of hematology and clinical chemistry values (reduction in erythrocytes and hemoglobin in males and females and iron in females; increase in reticulocytes and hematocrit in males and females); increase in liver, kidney and spleen weights. 500 mg/kg: moderate reduced activity during the 1st treatment week and later moderated increased activity for 10 min. post treatment; significant increase in water consumption; increased urine excretion (yellow-orange discolored); significant alterations of hematology and clinical chemistry values (reduction in erythrocytes, hemoglobin, hematocrit and iron in males and females; increase in reticulocytes in males and females and MCV and Prothrombin time in females; significant increase in liver, kidney and spleen weights; dark discolored spleens at autopsy. No significant histopathological alterations were observed at all doses. The oral dose of 50 mg/kg/day x 28 days represents the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 1 % aqueous solution (thickened with methyl cellulose) was applied on abraded skin area (3 x 4 cm, washed out after 20 min.) of albino guinea pigs 3 times daily on two consecutive days. A negligible erythema on the first day, not recognizable (only skin area stained) on the second day, was observed; no edemas and crusts were revealed, during further observation.

4.2 Irritation (mucous membranes)

The compound as 1 % aqueous solution instilled into one eye (0.1 ml) of 10 female Pirbright white guinea pigs, resulted not irritating after 24 hours observation period (eye reactions evaluated at 0.5, 1, 2, 3, 4, 6, 7 and 24 hours).

5. Sensitization

Sensitization was tested in 15 females Pirbright white Guinea pigs treated with 3 % in aqueous test suspension of test compound applied epicutaneously without occlusion on abraded flanks, once a day on 5 days/week for 3 wks, using the method of Magnusson and Kligman. The compound did not show any erythemas or edema 24, 48 and 72 hours after challenge reaction.

6. Teratogenicity

1-hydroxy-2-amino-5-methylbenzene administered oral by gastric intubation to 23-26 pregnant Sprague-Dawley rats from day 8 to 15 of gestation at doses of 5, 50 and 200 mg/kg b.w./day in distilled water (10 ml/kg b.w.) not showed embryotoxicity and no sign for embryolethality or teratogenicity. NOAEL > 200 mg/kg b.w.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: [^{14}C]-2-amino-5-methylphenol hemisulfate (radiochemical purity 96 %) in DMSO (150 mg/ml, 0.1ml animal for 0.5 h) and as ingredient of hair dye products (133.14 mg, 1 g mixture animal for 24 h) applied on dorso lumbar region of PVG rats under occlusion (15 mg animal, 1.667 mg/cm^2, 190 mCi) showed after 72 h that 0.58 % (0.41 % urine, 0.09 % faeces, 0.15 % expired air and 0.02 % cage washing) of the applied dose as the hair dye product and 14.25 % (12.83 % urine, 0.82 % faeces and 0.60 % cages washing) of the solutions in DMSO were excreted and that 82.78 % as the hair dye product and 74.48 % in DMSO solution were recovered from dressing, washing and application sites. No significant radioactivity level was found in tissues.

Human-skin absorption: 1-hydroxy-2-amino-5-methylbenzene (mean = 54.1 mg, i.e. 0.06 %) containing in hair dye product was epicutaneously applied (mean = 90.02 g) on five healthy female volunteers by professional hairdresser for 29-31 min. and blood samples were taken at 0, 10, 20, 30, 45 and 60 min and 2, 3, and 24 h. after applications. The results showed within the range of the sensitivity of method (10 ng/ml, HPLC technique and fluorescence photometer) neither the hair dye nor a possible metabolites was detected in the serum; therefore the volunteers (64.70 kg mean b.w.), presuming a whole body distribution and absorption of at least 0.647 mg (on the bases of method sensitivity) per volunteers, absorbed nothing or less than 1.198 % of the applied dose of test compound.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have demonstrated that 1-hydroxy-2-amino-5-methylbenzene does induce gene mutations *in vitro* in *Salmonella* (+S9mix ± H_2O_2; -S9mix -H_2O_2). The compound (tested as hemisulfate in *in vitro* test) have been found negative for: (1) gene mutations on mouse lymphoma L5178Y (Na^+/K^+ ATPase and HPRT loci, fluctuation test)

in vitro; (2) chromosome aberrations *in vitro* on human peripheral lymphocytes and (3) *in vivo* by micronucleus test (up to 2 x 750 mg/kg oral; increase in the frequency of micronuclei when compared with negative control (up to 0.6 % mean per 2000 cells vs. 0.2 %), neither significant nor dose-related) on bone marrow cells of CD-1 mice: this study was however inadequate, because the positive control (Cyclophosphamide 100 mg/kg) was not able to induce a significant increase in percentage of micronuclea in this *in vivo* test (mean per 2000 cells: 0.6 % vs. 0.2 % negative control); several genetic damage *in vitro* on *S.cerevisiae* D7 (mitotic crossing over, mutation, gene conversion or aneuploidy) and sister chromatid exchange *in vivo* on mouse (up to 600 mg/kg oral).

11. Conclusions

Since several studies have shown that this compound has produced positive results in *in vitro* mutagenicity studies, the SCC requires a study for the *in vivo* induction of UDS.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2-AMINO-5-METHYLPHENOL
(A 75)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 2-amino-5-methylphenol)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.58 %**
Dermal absorption per treatment	**I (mg) x A(%) = 1500 x 0.58/100 = 8.7 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 8.7 mg/ 60 kg b.w. = 0.14 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 50 mg/kg b.w. (rat: 28 days oral study)**
Margin of Safety	**NOAEL / SED = 60 mg/kg b.w./0.14 mg/kg b.w.= 350**

This was acceptable.

However since further data were required it was recommended that the B classification be maintained for 1 year.

Classification: B

A 79: *1,3-BIS-(2,4-DIAMINOPHENOXY)-PROPANE*

1. General

1.1 Primary name

1,3-bis-(2,4-diaminophenoxy)-propane

1.2 Chemical names

1,3-bis-(2,4-diaminophenoxy)-propane
4,4´-1,3-propanediylbis(oxy)-bis-2,4-benzeneamine

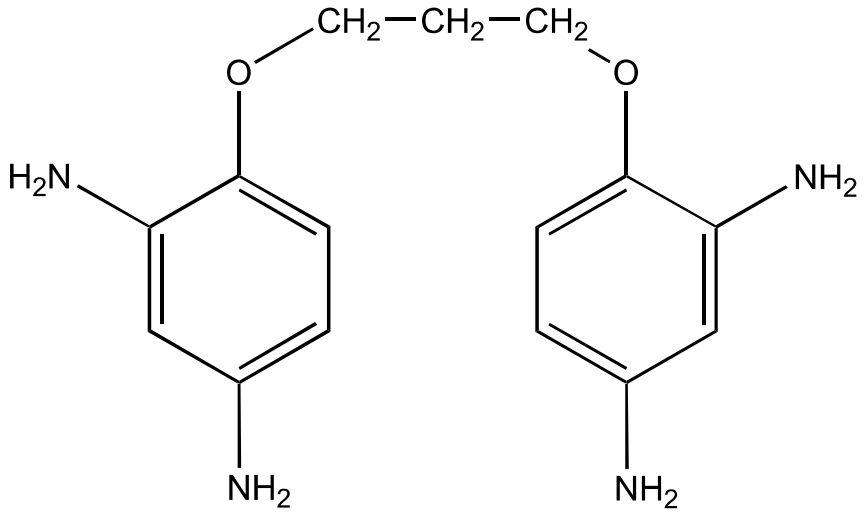
1.3 Trade names and abbreviations

Ro 463

1.4 CAS no.

74918-21-1

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{20}N_4O_2$
Mol weight: 288.3

2. Function and uses

Oxidative hair dye; max. use: 2 %; 1 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: rat, oral: 3570 (3170 - 4002) mg/kg;
rat, oral: > 5000 mg/kg (2)
(2 % test compound containing formulation)

3.7 Subchronic oral toxicity

The compound was administered daily by oral gavage, over a period of 13 weeks, to male and female Wistar rats (Mu Ra Han 67 SPF) at doses of 0-5-10-15 mg/kg b.w. in aqueous suspension (10 ml/kg). The following results were obtained: 5 mg/kg d.: the thyroid glands of all rats were free of pigments; 10 mg/kg d.: slight pigmentation of the thyroid glands (in a few females) and pigmented macrophages in the small intestine (in a few females and males); 15 mg/kg d.: reddish discolouration of the thyroid gland at macroscopical level, a pigmentation of the thyroidal epithelia and pigment depositions in the small intestine (all rats). The dose of 5 mg/kg day represents the dose with the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied (500 ml in gauze patches) as a 10 % (w/v in water) solution (pH=8-10) resulted non-irritant.

The compound applied twice a day for 5 days, as 10 % (w/v) aqueous solution (10 ml), to the same skin area of male hairless mice resulted not irritating.

The formulation containing the compound (2 %) resulted non-irritating to rabbit skin.

The formulation containing the compound (2 %) resulted not irritating to mouse skin when applied daily (30 min for application) for 5 days.

4.2 Irritation (mucous membranes)

The compound applied as 5 % (w/v) water solution on the eyes of rabbits showed no irritation of the cornea and iris, and from mild to severe redness of the conjunctiva in 3 animals (2 and 6 hours) disappearing 24 hours after instillation.

The formulation containing the compound (2 %) resulted slightly irritating for the eyes of rabbits.

5. Sensitization

It was induced in guinea pigs by intradermal injection of 5 % (w/v) test compound in aqueous solution, Freund's complete Adjuvant (FCA) and 1:1 (v/v) mixture of the above solution on day 0, and 7 days later by dermal application of 5 % (w/w) test compound in Vaseline, under

occlusion, for 48 hours. Challenge exposures were carried out at day 21 (closed patch, 24 hours) and at day 28 (open dermal) at a new skin site. The compound resulted non-sensitizer in guinea pigs.

The formulation containing the compound (2 %) resulted non-sensitizer in guinea pigs.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: 0.63 % of equivalents of the test compound was absorbed through the skin of rats over a period of 72 hours after dermal application to intact, clipped skin of male and female rats with a hair dye basic cream containing 0.23 % of ^{14}C-1,3-bis-(2,4-diamino-phenoxy)-propantetrahydrochloride (17.25 mg), without a developer. The radioactivity was revealed both in urine and in the feces.

A maximum of 0.079 % of equivalents of the test compound was absorbed through the skin of rats over a period of 72 hours after 30 min of dermal application to intact, clipped skin of male and female rats with oxidative formulation containing 0.23 % ^{14}C-1,3-bis-(2,4-diamino-phenoxy)-propanetetrahydrochloride (34.5 mg). The radioactivity was revealed mainly in the feces.

Metabolic studies: ^{14}C-1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride applied subcutaneously (10 mg/kg b.w.) to 4 male Wistar rats (SPF-TNO) showed more than 88 % of the radioactivity was found in feces (65 %) and urine 24 h after treatment. The radioactivity in expired air, in the carcass, liver and kidney was very low during an observation period of 144 hours.

^{14}C-1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride administered oral at doses of 10-100-1000 mg/kg b.w. showed an excretion range of 57-79 % in the feces and 23-34 % in the urine over an observation period of 120 hours.

These studies demonstrated that the compound was eliminated with the bile.

^{14}C-1,3-bis-(2,4-diaminophenoxy)-tetrahydrochloride was applied intraperitoneally to male and female Wistar rats at single dose of 20 mg/kg b.w. and the organ distribution was evaluated by whole body autoradiography at 0.5-2-6-24-96 hours after treatment. The results showed that the compound was principally excreted by the gastrointestinal tract and a minor amount by the kidney. The decrease of radioactivity was faster in liver than in kidney. The compound was still revealed in the spleen, thymus, kidney and in the Hardarian gland 96 hours after treatment.

8. Mutagenicity

Mutagenicity/Genotoxicity studies have shown that the 1,3-bis-(2,4-diaminophenoxy)-propane-tetrahydrochloride induces gene mutation *in vitro* on *Salmonella typhimurium* in the presence of metabolic activation.

Other studies have shown that the compound did not produce: gene mutation *in vitro* on CHO-K1 and V79 hamster cells line (HPRT: 6-TG resistance), *in vivo*/*vitro* by the urinary assay (Salmonella-rat: 100 mg/kg b.w. on the clipped dorsal skin, 24-hours urine sample), and *in vivo* by SLRL test on *D.melanogaster* and spot test in mice with oral doses up to 125 mg/kg b.w.; chromosome aberrations by micronucleus test on mice at oral doses of 100-2500-5000 mg/kg

b.w. (in two equal doses separated by an interval of 24 hours); genotoxicity *in vitro* by the mitotic gene conversion on the yeast *S.cerevisiae* and UDS on rat hepatocytes and, *in vivo*/*vitro* by urinary assay (*S.cerevisiae* D4-rats: mitotic gene conversion) with oral doses up to 250 mg/kg.

Embryotoxicity: The compound administered daily by oral gavage to groups of 41-43 pregnant Wistar TNO rats from days 6 to 19 of gestation at the dose of 0-100 mg/kg b.w. (10 ml/kg in water) showed 4/352 (treatment) vs. 0/300 (control) foetuses with visible malformation at analysis of the dams on day 20 of gestation. The other fetal and maternal parameters did not reveal an embryotoxic or maternal toxic effect.

The compound was administered daily by oral gavage to groups of 20 pregnant Sprague-Dawley CD rats from day 6 to 15 of pregnancy at the doses of 0-20-60-180 mg/kg b.w. (10 ml/kg in distilled water). The results showed a slight increase in the number and type of foetal variation in all test groups not treatment related. The other maternal and foetal parameters did not show indication of maternal toxicity, embryotoxic and teratogenic effects.

11. Conclusions

In the absence of carcinogenicity data, the SCC requires an *in vitro* cytogenetic study and an *in vivo* UDS study.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1,3-BIS-(2,4-DIAMINOPHENOXY)-PROPANE (A79)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1 % of 1,3-bis-(2,4-diaminophenoxy)-propane)

Maximum amount of ingredient applied	**I (mg) = 1000 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.63 (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 6.3 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w.**
	6.3 mg / 60 kg b.w. = 0.105 mg/kg b.w.
No observed adverse effect level (mg/kg)	**NOAEL = 15 mg/kg b.w. (rat: 90 days oral study)**
Margin of Safety	**NOAEL / SED = 15 mg/kg b.w. / 0.105 mg/kg b.w. = 140**

This was acceptable.

However since further data were required it was recommended that the B classification be maintained for 1 year.

Classification: B

A 80: *2,5-DIAMINO-PHENYLETHYLALCOHOL*

1. General

1.1 Primary name

2,5-diamino-phenylethylalcohol

1.2 Chemical names

2,5-diamino-phenylethylalcohol
1-ß-hydroxyethyl-2,5-diaminobenzene
1,4-diamino-2-ß-hydroxyethyl-benzene
2,5-diamino-phenylethylalcohol

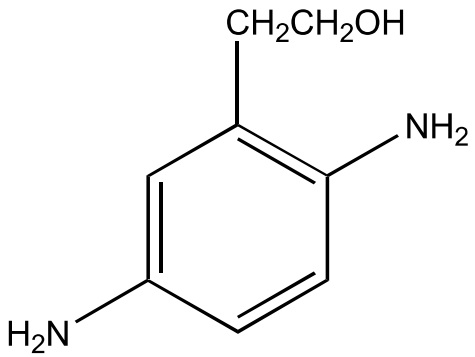
1.3 Trade names and abbreviations

Oxytol B

1.4 CAS no.

93841-25-9

1.5 Structural formula



(sulphate)

1.6 Empirical formula

Emp. formula: $C_8H_{12}N_2O$
Mol weight: 152

1.7 Purity, composition and substance codes

It exists as free base, dihydrochloride and sulphate. It is used as a sulphate.

2. Function and uses

Oxidative hair dye; max. use 3 %; 1.5 % in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: male and female rats, oral 150 mg/kg
female CD1 mice, oral 90 mg/kg

3.7 Subchronic oral toxicity

The compound, as sulphate, administered orally to groups of 10 male and 10 female Sprague Dawley rats for 90 days at dose levels of 0, 5, 25, 40 and 40 (recovery) mg/kg/day (10 ml/kg in water) showed a NOAEL at 25 mg/kg b.w. The following effects were observed: dose-related orange-coloured urine from 11th to 13th week, weight deviations and macroscopic changes of the organs, as well as an increase of the mean GOT and GTP values after 13 weeks at the highest test dose.

The compound, as hydrochloride, administered daily by stomach tube to 12 male and 12 female SPW Wistar rats for 12 weeks at dose level of 25 mg/kg/b.w. in all examens (food and water consumption; hematological, clinico-chemical changes and ophthalmoscopical changes; urine; macroscopical finding; and complementary examination of the organs of 5 males and 5 females) showed no difference between treated and control group (5 ml/kg b.w. water). The dose of 25 mg/kg/b.w. represents the NOAEL (90-day oral study on rats).

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as dihydrochloride (3 % in aqueous solution) applied daily for 5 days to the clipped skin area (3 x 4 cm), without washing off, of 15 female Pirbright White guinea pigs resulted not irritating (skin reactions evaluated daily 5 h. post treatment).

4.2 Irritation (mucous membranes)

The compound as dihydrochloride instilled (1.5 % in water, 0.1 ml) into the conjunctival sac of one eye (without washing) of 5 female Pirbright guinea pigs resulted not irritating after a 24-hour (examinations with 0.1 % fluoroscein sodium solution) observation period (eye reactions evaluated at 0.5, 1, 2, 3, 4, 5, 6, 7 and 24 hours).

5. Sensitization

Sensitization was tested in 10 male and 10 female Pirbright guinea pigs treated with 3 % intradermal injections and closed dermal topical application (including Freund's complete adjuvant FCA) of test compound on the clipped shoulder area. Challenge reaction by closed patch test on day 14 after the last exposure with 1 %, 2 % and 3 % in distilled water. The compound showed no skin reactions (reading at 24 and 48 hours).

6. Teratogenicity

1-(β-Hydroxyethyl)-2,5-diaminobenzene-sulphate administered daily by gastric intubation to 25 mated female Sprague-Dawley rats from day 6 to 15 of gestation at oral doses of 10 mg/kg/day (10 ml/kg in distilled water) did not show embryotoxicity and teratogenicity on day 20 of gestation.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human-skin absorption: 1-(β-Hydroxyethyl)-2,5-diaminobenzene (mean = 1855.20 mg, i.e. 2.4 %) contained in a hair dye product was epicutaneously applied (mean = 77.3 g) to five healthy female volunteers by a professional hairdresser for 24-32 min. and blood samples were taken from 4 volunteers at 0, 10, 20, 30, 45 and 60 min and 2, 3, and 24 h. after application. The results showed that within the sensitivity range of the method (25 ng/ml, HPLC technique and fluorescence photometer) neither the hair dye nor a possible metabolite was detected in the serum; therefore the volunteers (64.18 kg mean b.w.) - presumed a whole body distribution and absorption of at least 1.604 mg (on the bases of method sensitivity) per volunteer - absorbed none or less than 0.086 % of the applied dose of test compound.

8. Mutagenicity

The compound tested as sulphate was found negative for: (1) gene mutation *in vitro* on *Salmonella* (tested only in the presence of metabolic activation) and in mouse lymphoma 6-TG^{R} fluctuation assay; (2) chromosome aberrations *in vitro* on CHO cells and *in vivo* by micronucleus test on mice (up to 200 mg/kg oral); (3) and sister chromatid exchange *in vivo* in the bone marrow cells of rats (up to 80 mg/kg i.p. and p.o. or 5x128 mg/kg epicutaneous).

11. Conclusions

The SCC requires an adequate study for the induction of gene mutations in the Salmonella assay.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2,5-DIAMINO-PHENYLETHYLALCOHOL (A 80)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 2,5-diamino-phenylethylalcohol)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 0.086 (human)**
Dermal absorption per treatment	**I (mg) x A(%) = 1.29 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 1.29 mg / 60 kg b.w. = 0.0215 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 25 mg/kg (rat: 90 days oral study)**
Margin of Safety	**NOAEL / SED = 25 mg/kg b.w./ 0.0215 mg/kg b.w. = 1160**

This was acceptable.

However since further data were required it was recommended that the B classification be maintained for 1 year.

Classification: B

A 82: *1-HYDROXY-3,4-METHYLENEDIOXYBENZENE*

1. General

1.1 Primary name

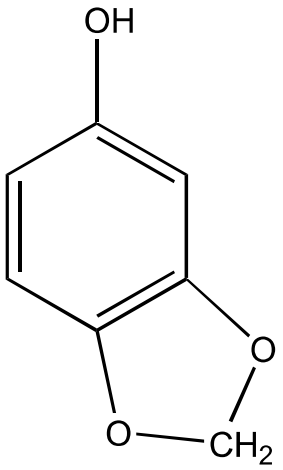
1-hydroxy-3,4-methylenedioxybenzene

1.2 Chemical names

1-hydroxy-3,4-methylenedioxybenzene

Sesamol

1.5 Structural formula



1.7 Purity, composition and substance codes

The compound is a colourless crystalline powder with a purity of 99 %.

1.9 Solubility

It is soluble in water and various organic solvents; ethanol, isopropanol, acetone, chloroform and ethyl acetate; however, no quantitative solubility data were available.

2. Function and uses

The compound is supplied as an oxidative hair dye at concentrations up to 3 % and used at a concentration of 1.5 % after dilution with hydrogen peroxide.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute oral toxicity has been investigated in rats and mice following administration in 10 % gum arabic. The following LD_{50} values were obtained, female mice 415 mg/kg, male rats 430 mg/kg and female rats 300 mg/kg. The observed signs of toxicity were decrease in activity, staggering and exitus.

3.7 Subchronic oral toxicity

Two 90 day studies have been carried out in the rat with the compound administered by gavage. Dose levels of 5, 10 and 15 mg/kg (5 days/week) and 10, 30 and 60 mg/kg (7 days/week) were used. No compound related effects were noted on weight gain, clinical chemistry, haematology or on examination of tissues at autopsy.

4. Irritation & corrosivity

4.1 Irritation (skin)

No signs of skin irritation were observed in guinea pigs following application of a 3 % solution for 4 hours under occlusion. Similarly no signs of skin irritation were observed in rabbit exposed to 50 mg/kg under occlusion for 4 days or rats given the same dose daily for 30 days.

4.2 Irritation (mucous membranes)

Eye irritation has been studied in both the rabbit and the guinea pig. The compound was added to one eye of groups of albino rabbits (1.2, 2.3 or 4.6 mg); this resulted in signs of irritancy being seen in all groups at 4 hours (slight swelling of palpebral membrane, conjunctivitis and oedema of nictating membrane). No effects were seen after 24 hours in animals given 1.2 mg of sesamol nor in the animals given 2.3 mg after 48 hours. The only effect seen at this time in the group given 4.6 mg was a slightly inflamed nictating membrane. In the guinea pig study no significant effects were seen in studies using a 1 % solution.

5. Sensitization

The ability of the compound to induce skin sensitization has been investigated in one study in guinea-pigs using small numbers of animals (4) given i.c. or topical application ten time over 20 days, followed by challenge 15 days after final application. No evidence of sensitization was observed; however in the light of the small number of animals and the use of a non-standard method no conclusions can be drawn. Limited studies in humans have been reported. In one of these, no reactions were seen when 5 subjects were treated with 1.25 mg of compound in alcohol for nine daily doses and challenged 12 days after the final dose. Although no reactions were reported the number of subjects was far too small to allow any definite conclusions to be drawn. Sensitization was seen in a high proportion (8/13) of patients sensitized to sesame oil (from therapeutic treatment of leg ulcers) and in one of 15 subjects

sensitized to p-aminoaryl compounds. In the first of these cases the relevance to individuals with normal skin is unclear in view of the frequency of contact allergies in individuals with stasis eczema and in the second case was thought to be a consequence of the polyvalent allergy in this subject. The limitations of the animal and human data preclude any conclusions being drawn regarding the sensitization potential of sesamol.

8. Mutagenicity

Negative results were obtained in studies to investigate the ability of sesamol to produce gene mutation in *Salmonella typhimurium*. Strains TA1535, TA1537, TA1538, TA98 and TA100 were investigated both in the presence and absence of an exogenous metabolic activation system at concentrations up to 30 μmol. Similar results were obtained in an investigation of gene.

11. Conclusions

Sesamol has moderate acute toxicity by the oral route, however studies suggest dermal penetration is low from hair dye formulation. The compound produced eye irritation but a 1 % solution was found to be practically non-irritating. There was no evidence of skin irritation with sesamol. There was no evidence of skin sensitization in the animal or human repeated insult study, but sensitization was seen in 2 studies on patients with allergies. In a 90 day oral study no effects were reported at doses up to 60 mg/kg. Mutagenicity data comprised negative results in both a *Salmonella* and a CHO assay for gene mutation and an *in vivo* study on sister chromatid exchange in bone marrow. No adverse effects were reported in an oral teratogenicity study at 10 mg/kg, the only dose level studied. No compound related effects were observed in a chronic study, however no conclusions can be drawn from this study due to the inadequate study design.

A further study is needed to investigate whether sesamol can induce sensitization using a more rigorous protocol to current standards. There is also a need for an *in vitro* study to investigate the clastogenicity of sesamol in mammalian cells.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-HYDROXY-3,4-METHYLENEDIOXYBENZENE (A 82)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 1-hydroxy-3,4-methylenedioxybenzene)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 1% (rat)**
Dermal absorption per treatment	**I (mg) x A(%) = 15 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 15 mg/ 60 mg b.w. = 0.25 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 60 mg/kg b.w. (rat: 90 days oral study)**
Margin of Safety	**NOAEL / SED = 60 mg/kg b.w./0.25 mg/kg b.w.= 240**

This was acceptable.

However since further data were required it was recommended that the B classification be maintained for 1 year.

Classification: B

A 84: *1-METHOXY-2-AMINO-4-ß-HYDROXYETHYL-AMINO-BENZENE*

1. General

1.1 Primary name

1-methoxy-2-amino-4-ß-hydroxyethyl-amino-benzene

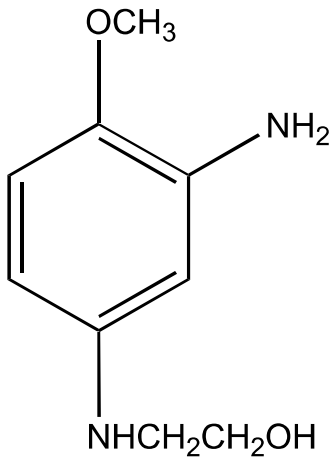
1.2 Chemical names

1-methoxy-2-amino-4-ß-hydroxyethyl-amino-benzene
2-amino-4-ß-hydroxyethyl-amino-anisole

1.4 CAS no.

83763-47-7

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{14}N_2O_2$
Mol weight: 182

1.7 Purity, composition and substance codes

The compound exists as free base (oxidizing), as hydrochloride, as dihydrochloride, and as sulphate.

2. Function and uses

Oxidative hair dye; maximum use 3 % (included as salt); 1.5% in combination with H_2O_2.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: female CF 1 mice, oral 538 mg/kg
female Wistar rats, oral 588 mg/kg
male Wistar rats, oral 475 mg/kg

3.4 Repeated dose oral toxicity

The compound as dihydrochloride (0.5 % in distilled water), was administered orally by stomach intubation, 5 days a week, for 3-4 weeks, to groups of 9 male and 9 female SPF Wistar (TNO/W.74) rats at dose levels of 0, 10, 25 mg/kg b.w. day (5 ml/kg). A slight and not clear activation of the thyroid epithelium was observed in rats treated with 10 mg/kg, but not in rats treated with 25 mg/kg. In females treated with 10 mg/kg, a slight reduction in food consumption during the first week and an increase of total number of leucocytes were observed. In rats treated with 25 mg/kg a slight lymphocytosis was revealed. In one rat treated with the highest dose discolouration of the thyroid was observed, without neither pigment sedimentation nor thyroid epithelium sedimentation. The dose lower than 10 mg/kg represents the NOAEL.

3.5 Repeated dose dermal toxicity

The compound was dermally applied to a clipped area on the back (3x4 cm) of Pirbright white guinea pigs (5 male and 5 female/group), 7 days a week, for 4 weeks at doses of 50, 150, 300 mg/kg b.w. (5, 15, 30 % in water). The treated skin did not show any sign of irritation. No adverse effects were revealed up to a dose of 300 mg/kg b.w.

3.7 Subchronic oral toxicity

The compound, as sulphate, was administered daily by stomach tube to 25 male and 25 female SPW Wistar rats for 13 weeks at dose levels of 0, 2, 50, 100 mg/kg b.w. in distilled water (1 ml/100 g b.w.). The dose of 100 mg/kg was increased until 1380 mg/kg b.w. The reversibility effects were evaluated after 4 weeks without treatment, in 40 additional rats both from the control group (10 males and 10 females) and the highest test group (10 males and 10 females). The dose of 50 mg/kg showed rough pelages, pigmentation of the thyroid gland and in the duodenum. At the end of treatment in males treated with 50 mg/kg dark discoloured urine and increased liver weights were observed. The dose of 100 mg/kg showed rough pelages, pale grey skin and mucosae, dark urine, reduction in activity and body weight (only in males). The weight of thyroid glands, livers, kidneys, spleens and suprarenal bodies (of males) was reduced at the highest test dose. The 100-1380 mg/kg dose showed pigmentation in thyroid glands, intestinal tracts, epididymides, livers and kidneys. The highest dose reduced erythrocytes, haemoglobin and heamatocrit values, and increased reticulocytes, MCV (mean corpuscolar volume of erythrocytes), MCH (mean corpuscolar haemoglobin), b-globulin and bilirubine. The 2 mg/kg b.w. dose represents the NOAEL.

3.8 Subchronic dermal toxicity

A hair dye formulation ("Koleston 2000"), containing 3 dose levels of test compound as sulphate (1.2 %, 1.8 % and 2.4 %), mixed 1:1 with hydrogen peroxide, was dermally applied (0.05 ml) to the back of mice (75 males and 75 females for each group), 3 times a week, for 12 months. Negative control received 0.05 ml of deionized water in the same way. In all treated animals alopecia and epithelial lesions of treated skin area were found. In females treated with the highest test dose the body weight gains were reduced. No morphological changes in thyroids were observed. The formulation contained other dyes too.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound, as sulphate (1 % suspended in 10 % Arabic gum) was applied, both to the clipped right (5 animals) and left flank (5 animals) of 10 female albino guinea pigs, 3 times for 2 consecutive days. Treated areas (3x4 cm) were washed off after 20 min. The skin reactions were evaluated during, and three days after treatment. 2 of 10 animals showed a very slight erythema of the clipped scarified skin. All animals were free from symptoms on the last day of the study. The compound resulted not irritant for the skin of guinea.

Human skin irritation: A hair dye formulation ("Koleston 2000", shade blue-black), containing the compound (2.25 %), mixed 1:1 with 9 % H_2O_2 and water, was applied topically to the skin of 40 persons by patch-test, under occlusive condition, for 24 hours. No irritation on the treated skin was found 24, 48 and 72 hours after application.

4.2 Irritation (mucous membranes)

The compound as sulphate, was instilled (1 % aqueous solution, 0.1 ml) into the conjunctival sac of one eye of 10 female Pirbright guinea pigs. The compound was not washed off. The eye of all animals was washed with 1 % fluoroscein sodium solution 24 hours after instillation. The eye reactions were evaluated at 0.5, 1, 2, 3, 4, 5, 6, 7 and 24 hours during treatment. The compound resulted "practically not irritating" in guinea pigs.

5. Sensitization

The compound as dihydrochloride (1 % aqueous solution) was intracutaneously injected to 15 female guinea pigs for induction phase (0.1 ml), 3 times a day for 5 days. 4 weeks later the challenge reaction was performed with different dilutions (1:10, 1:100, 1:500 and 1:1000) of 0.1 ml of compound, applied by intracutaneous injection into the untreated flank. The skin reactions were evaluated both 24 and 48 hours after the challenge procedure. After a 5-day induction period a weakly inflammatory skin reddening was observed. None of the treated animals showed allergic reactions within 24 hours. The compound resulted non-sensitizing for guinea pigs.

Photosensitization: The compound was applied, 30 µl in 30 % injectable water, on the shoulder region of 15 female Pirbright white guinea pigs. Positive control guinea pigs were treated with Hexachlorophene. Afterwards, animals were irradiated with UV-A and UV-B light

for 105 min. Such treatments were repeated 10 times. 2 weeks later the challenge was performed with 5, 1, 0.5, 0.1 % of compound applied on the shaved back of animals. The left side of the back was irradiated with UV-A for 105 min, and the right side remained unirradiated. The allergic reaction was evaluated 24 and 48 hours after. The compound resulted non-photosensitizing in guinea pigs.

6. Teratogenicity

The compound was orally administered by gastric intubation to mated female Sprague-Dawley rats (23-28 for each group) from days 6 to 15 of gestation at doses of 0, 150, 350 mg/kg day (10 ml/kg in distilled water with few drops of 23 % ammonia). The analysis were performed on day 19 of gestation. At 350 mg/kg the body weight gain of dams was below mean values during treatment period, and the rate of skeletal variation increased compared with the control group. Such differences were due to retarded ossifications of the osseous occipitale and parietale. No other adverse effects were observed in dams and foetuses. The dose of 150 mg/kg b.w. day represents the NOAEL.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: Two hair dye formulations (I and II) containing the compound (C-ring labelled), as dihydrochloride (I =1.05 % and II = 2.1 %) were epicutaneously applied for 30 min on the clipped back (3 cm^2) of HIM:OFA-Sprague-Dawley rats. For each experimental group 3 males and 3 females were considered. The rats were treated either with 1 g of the formulation I or 0.5 g of formulation II, mixed with 0.5 g of 9 % H_2O_2, in both cases corresponding to 38 mg/kg b.w. of compound. Similarly, another group of rats was treated with 0.3 ml of the 3.5 % aqueous solution of compound at dose of 37 mg/kg b.w. for 30 min. 0.13 %, 0.033 % and 0.24 % of the applied dose were absorbed, after treatment with formulation I, formulation II and aqueous solution, respectively. 0.57 %, 1.51 %, 0.75 % of the applied dose were revealed in treated skin area 3 days after treatment with formulation I, formulation II and aqueous solution, respectively. After 72 hrs of treatment low radioactivity was found in organs. The resorbed activity was quickly discharged with urine.

Human-skin absorption: A hair dye formulation "Koleston 2000 (1/0)" containing the compound (2.2 %) was epicutaneously applied (70.64 g, i.e. 1554 mg of compound) on five healthy female volunteers by a professional hairdresser for 15 min. Blood samples were taken at 0, 10, 20, 30, 40, 50 and 60 min and 2, 3, and 24 h. after applications. The results showed within the range of the sensitivity of method (16 ng/ml, HPLC technique and fluorescence photometer) that neither the hair dye nor the metabolites could be detected in the serum; therefore the volunteers (57.86 kg mean b.w.) - presuming a whole body distribution and absorption of at least 925.67 mg (on the basis of the method sensitivity) per volunteers - absorbed none or less than 0.06 % of the applied dose of test compound.

8. Mutagenicity

The compound tested as sulphate was able to induce gene mutation *in vitro* on $TK^{+/-}$ mouse lymphoma assay, both in the presence and in the absence of rat liver metabolic activation, with a clear dose-related effect. The increases in mutation frequency over the control were up to

2.26 (19.6 µg/ml, -S9mix) and 3.18 (147.1 µg/ml, +S9mix); sister chromatid exchanges *in vivo* on bone marrow cells of Sprague-Dawley rats SIV 50 treated i.p. (250 mg/kg: x1.74, $P < 0.001$ one-side test; 300 mg/kg (2/10 animals survived): x1.80).

The compound, tested also as sulphate, was found negative for:

- gene mutation *in vitro* on:
  Salmonella;
  E.coli;
  mouse lymphoma L5178Y cells (Na^+/K^+ ATP-ase and HPRT loci) after re-evaluation of data;
- chromosome aberrations in cultures of human lymphocytes *in vitro;*
- UDS (autoradiographic method) in primary culture of rat hepatocytes *in vitro*;
- UDS *in vivo* on male Wistar rats (750 mg/kg b.w. for 4 h and 75 and 750 mg/kg b.w. for 16 h);
- sister chromatid exchanges *in vivo* on bone marrow cells of Sprague-Dawley rats SIV 50 treated both orally (50, 100, 200, 300, 400, 500 mg/kg) and dermally (topical applications: 100, 200, 5x200, 1000, 2000 mg/kg).

The compound tested as dihydrochloride was unable to induce gene mutations *in vitro* in five strains of *Salmonella*.

The compound tested as free base does not induce micronuclei *in vivo* on bone marrow cells of mice treated by oral gavage at doses up to 2 x 500 mg/kg. The treatment was performed twice in two equal doses separated by a 24-hour interval, and an analysis 6 hours after the last dose.

10. Special investigations

Phototoxicity: The compound was applied on the back (2 cm^2) of 10 female Pirbright white guinea pigs on two test areas at doses of 5 % and 1 % in injectable water. Another area was treated with positive control (8-Methoxypsoralen) and one area remained untreated. The animals were then irradiated with UV-B light for 80 sec and UV-A light for 80 min. The compound resulted non-phototoxic 24 and 48 hours after the last irradiation.

11. Conclusions

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-METHOXY-2-AMINO-4-—HYDROXYETHYL AMINO-BENZENE (A 84)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 1.5 % of 1-methoxy-2-amino-4-ßhydroxyethyl amino-benzene)

Maximum amount of ingredient applied	**I (mg) = 1500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 0.06% (human)**
Dermal absorption per treatment	**I (mg) x A(%) = 1500 x0.06/100 = 0.9mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. = 0.9 mg / 60 kg b.w. = 0.015 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 2 mg/kg b.w. (rat oral, 13 weeks)**
Margin of Safety	**NOAEL / SED = 2 mg/kg b.w./0.015 mg/kg b.w. = 130**

Classification: A

B 24: *4-NITRO-O-PHENYLENEDIAMINE (4-NOPD)*

1. General

Conclusion from SCC, Second Series, EUR 8634 (op.1980):
"In view of the absence of conclusive carcinogenic effects in animals, the SCC sees no reason for prohibiting 4-NOPD at present but wishes to obtain additional information concerning percutaneous resorption and the repetition of more realistic carcinogenicity tests and in the meantime it can accept its continuing use on a provisional basis. The implementation of this recommendation will be reviewed each year." (Hair dye which is temporarily acceptable for use in cosmetic products until December, 31th, 1985: EUR 8634, p.1, 1980).

Revision 17 October 1986
-Banned in Italy and Denmark; recommended for banning in FRG.
-It is used in direct or semi-permanent hair colouring products, in combination with oxidant dyes. It produces brown, red and blonde shades on hair without any chemical reaction.

Present use: 600 kg.

1.1 Primary name

4-nitro-o-phenylenediamine (4-NOPD)

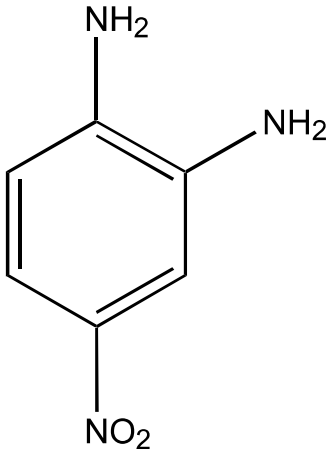
1.2 Chemical names

4-nitro-o-phenylenediamine (4-NOPD)
1,2-diamino-4-nitrobenzene
2-amino-4-nitroaniline
4-nitro-1,2-diaminobenzene
4-nitro-1,2-phenylenediamine
p-nitro-o-phenylenediamine

1.4 CAS no.

99-56-9
C.I.: 76020

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_7N_3O_2$
Mol weight: 153.1

2. Function and uses

Direct or semi-permanent hair dye; max. use 3.5 %; normal use 0.6 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: rat, oral : 2100 mg/kg; 3720 mg/kg;
rat i.p.: > 1600 mg/kg.
rat/mouse, oral: 681 mg/kg (NIOSH).

3.4 Repeated dose oral toxicity

RAT Fischer 344 & Mouse B6C3F1. NCI bioassay. 5 males and 5 females/group received in the diet for 7 weeks the following doses of 4-NOPD: 0 (2 groups) -681-1000-1430-2160-3150-4600-6800 and 10000 ppm for rats and 0 (2 groups) -1470-2160-3150-4600-6800-10000-14700 and 21500 for mice. The maximum tolerated dose was 750 ppm for rats and 7500 ppm for mice.

3.7 Subchronic oral toxicity

4-NOPD administered orally to 20 male and 20 female Wistar Rats by gavage (20 mg/kg b.w.) 5 days per week for 90 days, gave the following results: (1) slight increase of body weight gain in females; (2) slight decrease of hematocritic and erythrocytes values of blood in males and increased hemoglobin value in females; (3) slight decrease of serum glucose, calcium and protein level in males and females; (4) increase of relative adrenal, heart, kidney and liver weights in males and, in relative adrenal and liver weights in females; (6) no damage in inner organs by microscopic analysis. At this dose level, the compound showed some evidence of toxic effects on Wistar rats.

3.8 Subchronic dermal toxicity

A formulation containing 4-NOPD (0.25 % in water) tested on shaven intact skin of New Zealand white rabbits by topical applications produced no toxic effect at the histopathological analyses at 3-7-13 weeks after treatment.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied to intact and abraded skin of rabbit as a 2.5 % (w/v) preparation resulted non irritating.

4.2 Irritation (mucous membranes)

The compound as a 2.5 % (w/v) suspension in the eyes of rabbit caused a mild conjunctival inflammation and did not persist for more than 24 hours.

5. Sensitization

It was tested in Guinea (open epicutan method) pigs treated with 3 % of 4-NOPD solution containing 2 % Natrosol, 2 % Tween 80, 0.05 % Sodium sulphite and 10 % isopropanol (pH = 7) applied 6 days/week for 3 weeks. Sensitization was evaluated 2 weeks later by a single application of the compound, on the opposite untreated flank of animals. The results showed a relatively strong reaction (18/20 of the animals had an allergic effect).

In a repeated insult patch test in human (previously sensitized to p-phenylene-diamine) with the compound, no positive reactions were observed.

6. Teratogenicity

4-NOPD (suspension in sterile distilled water: 0-16-32-64-128-256-512-768-1024 mg/kg/day (once a day 1 % b.w.: 10 ml/kg s.c. injections.) administered on days 6-15 of gestation to female mice (CD-1). Teratogenic and embryotoxic effects were observed at 256 mg/kg/day and above: dose-related increase of the mean percentage of malformed fetuses, reduction of mean weight gain of the dams during pregnancy and mean fetal weight; white foci in the left ventricle of the fetal heart (512 mg/kg/day); cleft palate major malformation and major blood-vessels affected (512 - 1024 mg/kg/day); effect on the mean number of live fetuses per dam (768 mg/kg/day); and 768 (6/36) and 1024 (7/37) mg/kg/day maternal deaths were observed. The dose of 128 mg/kg/day gave no effects. A retarded effect of ossification process (bones of the feet and the cervical and caudal vertebral centre) was observed in mice topically treated twice a week for four weeks before mating and until the 18th day of gestation with a formulation containing 0.25 % of 4-NOPD to 0.125 mg/mouse. No teratogenicity effects were observed in rats and rabbits treated with formulations containing the compound (0.16 % or 0.25 %). In two reproductive studies on rats treated with a formulation containing 0.16 % and 0.25 % of 4-NOPD no negative effects were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

4-NOPD (120 (g/cm^2 as a 0.6 % (w/v), 200 (l as 3-H labelled hair dye solution) was applied on 10 cm^2 of the skin in a 50 % solution of a semi-permanent hair colorant shampoo base for 20 min. before rinsing off. Absorption was evaluated from the levels of ^{3}H present in the excreta and carcasses of the animals 48 hours after application. The results showed that 2.2 μg/cm^2 (1.83 %) of 4-NOPD apparently penetrated in the skin.

8. Mutagenicity

Since 1980, several studies have been published on the potential for mutagenicity and genotoxicity of 4-NOPD on different types of methodological approaches (gene-mutation; chromosome aberration; DNA damage) employing *in vitro* and *in vivo* assay systems and presenting positive or negative results.

Mutagenicity and genotoxicity studies have shown that 4-NOPD is mutagenic *in vitro*: (1) on *B.subtilis*; (2) on *Salmonella*, in several experiments and in different experimental conditions; (3) on *E.coli*; (4) on Mouse lymphoma test; and *in vivo*: (5) on 3 different cells lines of chinese hamster (fibroblast line CHL, prostate gland and A(T1)CI-3 cell line) for chromosome aberrations *in vitro*; (6) on *B.subtilis* (rec-assay); (7) on *E.coli* by umu-test and differential killing tests system; (8) on *S.cerevisiae* for the induction of mitotic recombination; (9) on CHO cells and in mammalian cells culture (SCE *in vitro*); (10) on *Salmonella* (urinary assay on rats) and (11) on *Drosophila* (X-recessive lethals and visible mutation tests systems by microinjections to adult and in SLRL test system by oral administration for 3 days) for gene mutation induction.

Other mutagenicity studies have shown that the compound is negative for the induction of gene mutation *in vitro* in *A.nidulans* for forward mutation in two genetic markers; in *S.cerevisiae* for the induction of reversions at three genetic loci; and in *N.crassa* in ad-3 reversion mutations system; and negative *in vivo* on *Drosophila melanogaster* in a minute loci and SLRL tests by fed for 21 hours. The compound did not induce chromosome aberrations *in vivo* on rats (5000 mg/kg, g.i., in 2 equal doses separated by an interval of 24 h, analysis 6 h after the second dose) and mice (2x75-150-225-300 mg/kg i.p., interval of 24 h, analysis 6 h after the second dose) by micronucleus test on bone marrow and dominant lethal in 2 studies on rat (20 mg/kg i.p., 3 times a week for 8 weeks, analysis 17 days after 5 days of mating; 25-50-100 mg/kg/day i.p., 3 times a week for 10 wks., analysis in females mated in the 1st and 2nd week after treatment). The compound resulted negative for genotoxicity by DNA repair test on HeLa human cell line, and in *E.coli* for the induction of SOS function; on primary rat hepatocytes culture *in vitro*, and *in vivo* for the induction of SCE in bone marrow (62.5-125-250-500 mg/kg oral, analysis 6 h after treatment) and in the intestinal epithelium of Chinese hamster cells (doses not reported).

In a recent study, the compound was tested in the *in vivo* mouse bone marrow micronucleus assay. CD-1 male mice received single i.p. injections of 125-250-500 mg/kg of test compound (0,2 ml per animal in DMSO) and were sacrificed at intervals of 24, 48 and 72 h. to determine the frequency of micronucleated-polychromatic erythrocytes. 5 animals for experimental group were used and 2000 cells of each mouse were analysed. Triethylenemelamine (TEM, 1.5 mg/kg i.p., in sterile distilled water) was used as positive control (harvest time 24 h.). The vehicle (DMSO) was sampled only at 24 h. The results showed that any dose-related response in micronuclei induction had been observed for the 3 dose levels and for 3 sampling times. In the treatment groups the PCE/NCE ratios were not significantly different from the vehicle negative control.

9. Carcinogenicity

In the 1980 report the SCC referred to NCI long-term studies developed with 4-NOPD on mice and rats. By that time other studies were available, namely: studies on formulations; studies on formulations of 4-NOPD in combination with 2NPPD.

All those studies were analyzed by the SCC. No other long-term studies have been performed with 4-NOPD since the SCC report. Carcinogenicity studies were carried out on mice and rats by a NCI bioassay (1979), the compound fed in the diet at 750 and 375 ppm for rats for 103 weeks and 7500 and 3750 ppm for mice for 102 weeks showed no significant evidence of carcinogenicity. A study with a formulation containing 4-NOPD (0.6 %) together with 2NPPD tested on A and DBAF strains of mice by repeated topical application in aqueous acetone solution (80 weeks) showed lymphoid tumours in both strains, but a weak evidence of carcinogenicity was demonstrable only in DBAF strain. This report is incomplete and the experimental protocol is not adequate; a further study does not allow the identification of the causing agent. Other 2-year studies carried out on mice and rats with a formulation 7403 containing 0.25 % of 4-NOPD, and dog with Dye/Base composite containing 0.16 % of 4-NOPD, have given negative results.

11. Conclusions

The compound tested in a long-term oral study on rats and mice of both sexes did not show any carcinogenic effect. Other chronic studies with formulations were also negative. The compound tested in a short-term oral study on rats did show some evidence of systemic toxicity. The compound was found positive for the induction of gene mutation in several organisms tested *in vitro* (bacteria, mammalian cells) and Drosophila and for the induction of chromosome aberrations in mammalian cells grown *in vitro*. However, it was negative in several independent studies for the induction of micronuclea (mice and rats) and dominant lethals (rats) *in vivo*, for UDS in HeLa cells and rat hepatocytes *in vitro* and for SCE in bone marrow and intestinal epithelium of chinese hamster *in vivo* (treated up to 500 mg/kg orally).

The SCC in its plenary meeting of October 13, 1987 requested a short-term oral toxicity study to determine the NOAEL.

A request for a short-term oral toxicity study to determine the NOAEL remains unavailable.

Classification: C

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

4-NITRO-O-PHENYLENEDIAMINE (4 - NOPD)
(B 24)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 3.5 % of 4-NOPD)

It was noted that normal use was 0.6 % but maxium use was 3.5 %; also those insufficient data were available to identify a NOAEL, althought this was lower than 20 mg/kg based on the 90 single dose level study in rats.

The following were agreed for the use of permanent hair dyes:

Maximum amount of ingredient applied	**I (mg) = 3500 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%)= 1.83%**
Dermal absorption per treatment	**I (mg) x A(%) = 3500 x 1.83/100 = 64 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 64 mg /60 kg b.w. = 0.374 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = inadequate dates but below 20 mg/kg b.w. (rat: 90-day oral study)**

Thus the SM for the maximum use is below 20 which appears unacceptable. The value at the normal use level (0.6 %) may be around 100.

Margin of Safety NOAEL / SED = below 20 mg/kg b.w. / 0.374 mg/kg b.w. = below 50

Thus although the data are inadequate for a proper calculation of safety margins, it is clear that SMs are unacceptable at the maximum use of 3.5 %. It is recommended that the maximum use concentration should be 0.6 %. Also that B 24 be given a C classification with consideration of a time limit for the sub-chronic study requested by the SCC to enable a NOAEL to be established.

Classification: C

B 25: *2-NITRO-P-PHENYLENEDIAMINE (2-NPPD)*

1. General

Conclusions of the SCC, Second Series, EUR 8634 (op.1980):

"In view of the positive carcinogenicity findings in animals, at the doses used, the SCC recommends that its use might be discontinued. Nevertheless, this decision could be modified because of the product low percutaneous absorption and because the carcinogenicity tests by the dermal route were conducted on a mixture of the substance and not with 2 NPPD alone."

(Hair dye which use should be discontinued: EUR 2634 p. 2, 1980).

Revision 17 october 1986

-Banned in Italy and Denmark, recommended for banning in F.R.G.

-It is used as a direct dye or in combination with oxidant dyes; it produces brown and red shades on hair.

-It is used at a maximum concentration of 1 % in a hair dye formulation.

Production and use: 2500 kg.

1.1 Primary name

2-nitro-p-phenylenediamine (2-NPPD)

1.2 Chemical names

2-nitro-p-phenylenediamine (2-NPPD)

1,4-diamino-2-nitrobenzene

2-nitro-1,4-phenylenediamine

2-nitro-1,4-benzenediamine

2-nitro-4-aminoaniline

2-nitro-1,4-diaminobenzene

o-nitro-p-phenylenediamine

Diaminonitrobenzene

m-nitro-p-phenylenediamine

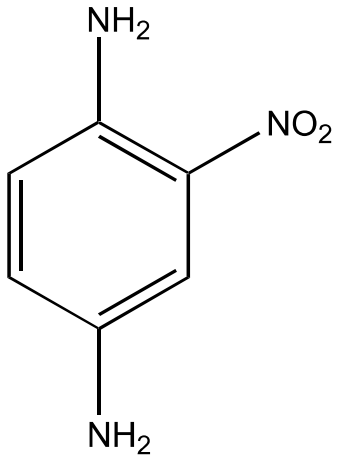
o-nitro-p-phenylenediamine

1.4 CAS no.

5307-14-2

C.I.: 76070

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_7N_3O_2$
Mol weight: 153.1

2. Function and uses

Permanent and semipermanent hair dye. Max. proposed use: 0.3 %

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} rat, oral: 1800 mg/kg; 2100 mg/kg; 3080 mg/kg
rat, i.p.: 348 mg/kg

3.4 Repeated dose oral toxicity

Subacute oral toxicity: RAT Fischer 344 & Mouse B6C3F1. NCI bioassay. 5 males and 5 females/group received in the diet for a period of 4 weeks, followed by a 2-week observation period, these doses of 2-NPPD: 0-315-680-1465-3155-6800 ppm for rats and 0-810-1180-1740-2550-3750-5550-8080-11830 ppm for mice. The maximum tolerated dose was 1100 ppm for male and 2200 ppm for female rats and 4400 ppm for mice.

On request of the SCC a 28-day oral toxicity study was conducted: 20 male and 20 female Sprague-Dawley rats were dosed by gavage with 0, 3, 30 or 100 mg/kg/day of 2-nitro-p-phenylenediamine (B 25) for 28 consecutive days.

At the end of 28 days of dosing all animals were killed and necropsied. Histopathological examination was performed on liver, heart, spleen, kidney and adrenals of all untreated and 100 mg/kg/day treated animals, as well as on liver and spleen only of animals belonging to the low and intermediate dose groups.

In this study the following toxicological effects were observed:

In animals treated with 100 mg/kg/day there were the following adverse effects (clinical and histological):
- mild centrilobular hepatocyte hypertrophy observed in 4/5 of males and 2/5 of females;
- spleen and liver weight increase;
- brown pigment present in spleen;
- increase of fine particles of pigment in the tubular cells in 4/5 of females;
- bilirubin and red cell increase;
- red cell count marginally decrease;
- red staining of coat and urines.

In animals treated with 30 mg/kg/day there were only the following adverse effects similar to those observed at the higher dose:
- mild centrilobular hepatocyte hypertrophy only in 3/5 of male animals;
- brown pigment present in 3/5 of animals;
- liver weight increase in males;
- red staining of coat only in some animals
- red staining of urines.

In animals treated with 3 mg/kg/day only a red staining of the urine was observed (*No Effect Level)*

3.8 Subchronic dermal toxicity

2-NPPD containing formulation (1.1 % in water) tested on the shaved intact or abraded skin of New Zealand rabbits by topical application produced no toxic effects at histopathological analysis at 3-7-13 weeks after treatment.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound applied as a 2.5 % (w/v) preparation (0.5 % aqueous gum tragacanth containing 0.05 % anhydrous sodium sulphite) resulted not irritant.

4.2 Irritation (mucous membranes)

The compound applied as a 2.5 % (w/v) suspension (0.5 % aqueous gum tragacanth containing 0.05 % anhydrous sodium sulphite) in the eyes of rabbit resulted “mildly irritating”.

5. Sensitization

Sensitization was tested in Guinea pigs treated with 3 % 2-NPPD solution containing 2 % Natrosol, 2 % Tween 80, 0.05 % Sodium sulphite and 10 % isopropanol (pH = 7) applied 6 days/week for 3 weeks, sensitization evaluated 2 weeks later. The results showed a weak reaction (4/20 of animals had an allergic effect).

6. Teratogenicity

2-NPPD (suspensions in sterile distilled water: 0-32-64-128-160-192-224-256 mg/kg/day) administered once a day 1 % b.w. (10 ml/kg) by subcutaneous injection on days 6-15 of

gestation of female mice (CD-1) showed teratogenic effects and maternal toxicity in the same range of doses (160 mg/kg/day and above). The compound showed a significant average weight gain reduction of the dams during pregnancy (128-256 mg/kg) and in average fetal weights (128-256 mg/kg/day); it produced a significant increase in the mean percentage of malformed fetuses: mostly cleft palate and fused ribs, white foci in the left ventricle of the fetal heart (160 and above mg/kg) and bilateral open eye (224 mg/kg). The dose without teratogenic effects was 128 mg/kg/day. A retarded effect of the ossification process (bones of the feet, and the cervical and caudal vertebral centre) was observed in mice topically treated twice a week for four weeks before mating and until the 18th day of gestation with a formulation containing 1 % of 2-NPPD equivalent to 0.5 mg/mouse. No teratogenicity effects were observed with formulations containing the compound on rat (1.1 %, 1 % or 0.24 %) and rabbit (0.24 % or 1.0 %). In two reproductive studies on rats treated with formulations containing 2NPPD (0.24 % and 1.1 %) no negative effects were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: [14-C] 2-NPPD 14-C (86 µg) in ethanol solution (40 ml) showed a high penetration to the clipped skin of rats (males: 11.7 (g/cm^2, females 24.6 µg/ml) and mice (31-36 µg/cm^2). 6.3 µg/cm^2 (4 %) of 3-H labelled compound (157 µg/cm^2 as a 0.8 % (w/v) solution) penetrated in the skin of rats at 48 hours after application of a 50 % solution of semi-permanent hair colorant shampoo base for 20 min. before rinsing off, and in a similar study 4.6 µg/cm^2 (4.8 %) of 14-C labelled compound (96 µg/cm^2, 0.5 %) penetrated in the skin of rats after 48 hours of treatment with 50 % aqueous shampoo solution for 20 min. and 6.4 % after 30 min. $^{[14\text{-}C]}$2NPPD (4 µg/cm^2 on clipped skin for 24 hours) showed a skin penetration of 29.9 % (percent of the dose applied) in monkey (ventral surface of forearm) and 17.7 % in swine (back site). Dermal absorption in rhesus monkey through the scalp was 0.55 % of the applied dose.

Metabolic Studies: 14-C radiolabelled 2-NPPD showed at 4 days after dosing to rats by different routes (oral, i.p. or s.c.) that ca. 4 % of the dose was retained in the body, mainly in the intestinal tract and less in liver and kidney. The metabolic pattern in urine showed 6 radioactive components for the rat and 7 for the mouse.

Human: The cutaneous absorption study of 2-NPPD (20 mM/l in isopropanol) *in vivo* on man showed that ca. 49 % (24 h), ca. 67 % (48 h) and 65 % (72 h) of the compound penetrates in the skin. In the normal application 0.75 % of absorption by scalp has been observed in a period of 30 days.

8. Mutagenicity

Mutagenicity and genotoxicity studies showed that 2-nitro-p-phenylenediamine is mutagenic: (1) on *Salmonella typhimurium* in the absence and presence of S9 mix + hydrogen peroxide; (2) on mouse lymphoma cells for gene mutation induction; (3) on two chinese hamster cells lines for chromosome aberration (*in vitro*); (4) on rat hepatocytes and HeLa cells for the induction of UDS, and DNA repair in *E.coli.* The mutagenicity studies showed that the compound does not induce: (1) dominant lethals in Charles River rats treated for 8 weeks (3 times a week) with a dose of 20 mg/kg i.p. (analysis 17 days after 5 days of mating) and for 10 weeks (3 times week) with 10-20-40 mg/kg i.p. (analysis in females mated in the 1st and 2nd week after treatment); (2) micronuclea in Sprague Dawley rats treated with 2 g/kg by gastric

intubation (in 2 doses separated by an interval of 24 h, analysis 6 h after the second dose); (3) SCE in bone marrow of chinese hamsters treated i.p. (75-150-300 mg/kg) or by gavage (62.5-125-250-500 mg/kg) and analysed 16 h after treatment; (4) mitotic gene conversion in yeast. The 2-NPPD induced morphological transformation in C3H/10T2CL8 and reduced lymphocite transformation.

On request of the SCC an *in vivo* UDS study on rats was carried out: An excess number of male Sprague-Dawley rats was orally treated with 2-NNPD with 400 or 2000 mg/kg. 12-hour dosed animals were killed and their liver perfused to provide a primary culture of hepatocyte. Positive control animals were dosed with 2-acetylaminofluorene (2-AAF). Also, 4-hour dosed animals with 2-NNPD were analyzed: in this case a positive control group of animals treated with N-nitroso-dimethylamine (DMNA) was analyzed. In all cases hepatocyte cultures from 5 animals were analyzed. Negative control animals gave a mean net grain count (NG) value lower than 0; 2-AAF-dosed animals gave a NG value of 19 and DMNA-dosed animals gave a NG value of 12.9. 4 and 12 treatment with 400 or 2000 mg/kg 2NPPD did not produce a mean NG value greater than 0. One can conclude that 2NPPD has no genotoxic activity in this test system.

9. Carcinogenicity

Since the SCC report in 1980 no more studies have been published. Long-term studies were carried out on mice and rats with a NCI bioassay, the compound fed in the diet at 550-1100 ppm for male and 1100-2200 for female rats for 78 weeks and, 2200 and 4400 ppm for mice for 78 weeks: they showed that 2-NPPD resulted positive in female mice producing a statistically significant increase of hepatocellular neoplasms (adenomas) and no conclusive evidence of carcinogenicity in male mice and rats was obtained. The re-evaluation of the histopathology did not confirm the presence of adenoma in one case and excluded it in a second case. According to another pathologist only an enhancement of parenchymal cell proliferation was obtained in female mice.

A formulation containing 2-NPPD (0.015 %) together with 4-NOPD tested in A and DBAf mice by repeated topical applications (80 weeks) in aqueous acetone solution showed lymphoid tumours in both strains, but a weak evidence of carcinogenicity was observed only in the DBAf strain. This report is incomplete and the experimental protocol is not adequate; also, the study does not allow the identification of the causing agent.

Other 2-year studies carried out on mouse and rat with 7401 containing 1.1 % of 2-NPPD, and dog with composite containing 0.24 %, gave negative results.

11. Conclusions

No other long-term carcinogenicity studies have been carried out since the last SCC evaluation of this compound in 1980. The compound was found in a main study carcinogenic only for female mice, in which an increase of the incidence of adenoma was observed, whose histopathology had been questioned. No carcinogenic effect was observed in male mice, as well as in male and female rats.

The compound was found mutagenic and genotoxic in an *in vitro* assay (bacterial and mammalian cells) for different genetic end points. *In vivo* mutagenicity studies (dominant

lethal, micronuclea and SCE in bone marrow cells) produced negative results. In a more recent study the 2-NPPD has been shown to be non genotoxic in an assay for the induction of liver UDS in rats treated *in vivo*.

Safety Assessment : 2-NPPD is used in both semi-permanent and permanent hair colouration. Even when the substance is included in a permanent formulation it does not participate in oxidative coupling. Depending on the required result, the mixture is left on the hair for approximately 20 to 30 minutes. After this period of time the product in excess is removed from the hair by rinsing. The concentration used depends on the wished shades. The maximum concentration never exceeds 3 % which is reached in a very small number of dark shades. Generally, the usual concentrations are below 0.3 %.

In the usual condition of application, the cutaneous absorption of 2-NPPD by the skin has been shown to be 0.75 % of the applied amount.

In permanent colouration, the formulation is applied to the hair under a volume of 50 ml mixed with 50 ml of hydrogen peroxide. The maximum concentration of 3 percent of 2-NPPD in the hair dye corresponds to the application of 1.5 g of 2-NPPD. The mixture is then rinsed off. Taking into account a penetration of 0.75 %, the penetrated amount can be evaluated as 12 mg per human (50 kg b.w.), e.g. 0.24 mg/kg b.w. Thus the safety margin (ratio between the not toxic effect level - 3 mg/kg b.w., -and the human exposure) is calculated to be 12.50.

The amount of semi-permanent formulation applied to hair is only 20 to 35 ml, without any dilution. The maximum concentration of 2-NPPD used in this formulation corresponds to the application of 1.05 g of 2-NPPD. Taking into account the same elimination and penetration ratios the penetrated amount can be evaluated as 7.9 mg per human, e.g. 0.16 mg/kg b.w. Thus, in this case, the safety margin is calculated to be 18.75.

If the same calculation is done considering the typical concentration of 0.3 % of 2-NPPD, the safety margin becomes ten times higher, e.g. 125 in permanent colouration and 187 in semi-permanent colouration.

The SCC considers that a limit for the use of this compound considered to be safe is a concentration of 0.3 %.

The SCC in its plenary meeting of June 30, 1987 asked for the following information:

An UDS test *in vivo* and a subacute oral toxicity study.

These studies have been presented to the SCC and further evaluated. On the basis of the overall available information, the use of 2-nitro-p-phenylenediamine does not present a risk for the consumer, if used at a concentration of 0.3 %.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2-NITRO-P-PHENYLENEDIAMINE (2-NPPD)

OXIDATION OR PERMANENT

(Based on a usage volume of 100 ml, containing at maximum 0.3 % of 2-NPPD)

Maximum amount of ingredient applied	**I (mg) = 300 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.75% (human)**
Dermal absorption per treatment	**I (mg) x A(%) = 300 x 0.75/100 = 2.25 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 2.25 mg / 60 kg b.w. = 0.0375 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 3 mg/kg (rat: 28 day oral study)**
Margin of Safety	**NOAEL / SED = 3 mg/kg b.w. / 0.0375 mg/kg b.w. = 80**

SEMI-PERMANENT

(Based on a usage volume of 35 ml, containing maximal 0.3% of 2-NPPD)

Maximum amount of ingredient applied	**I (mg) = 35 x 0.3 mg /100 = 105 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.75% (human)**
Dermal absorption per treatment	**I (mg) x A(%) = 105 x 0.75/100 = 0.788 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. 0.788 mg / 60 kg b.w. = 0.0131 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 3 mg/kg (rat: 90 day oral study)**
Margin of Safety	**NOAEL / SED = 3 mg/kg b.w./0.0131 mg/ kg b.w.= 220**

These safety margins were acceptable and it was agreed to recommend an **A classification**.

B 37: *N1,N4,N4-TRIS(2-HYDROXYETHYL)-1,4-DIAMINO-2-NITROBENZENE*

1. General

1.1 Primary name

N1,N4,N4-tris(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene

1.2 Chemical names

N1,N4,N4-tris(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene
1-β-hydroxyethylamino-2-nitro-4-bis-(β-hydroxyethyl)aminobenzene
2,2'-((4-(2-hydroxyethyl)amino)-3-nitrophenyl)imino)bis(ethanol)

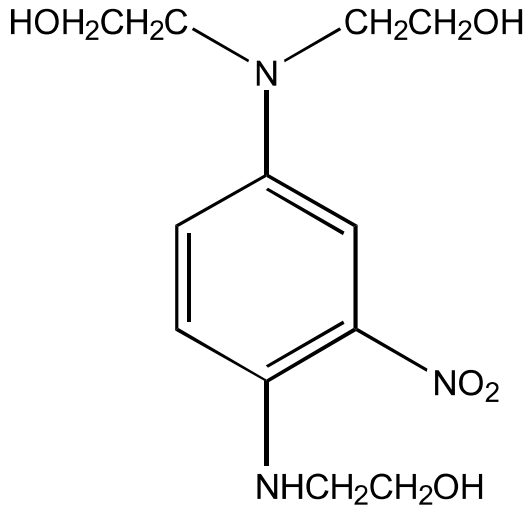
1.3 Trade names and abbreviations

Imexine FAF
HC Blue N°2

1.4 CAS no.

33229-34-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}H_{19}N_3O_5$
Mol weight: 285

1.7 Purity, composition and substance codes

Purity sample: The acute oral toxicity, the 14-day oral toxicity, and the 3-week diet studies on rats and mice: 75 % (lot. no. 513077); the 14-day oral toxicity, the 2-year carcinogenicity on rats and mice, and the NTP mutagenicity studies: 98 % (lot no. 9233); the metabolism and dermal absorption studies on mice and rats: > 98 % (TLC).

2. Function and uses

Semipermanent hair dye (nitrophenylenediamine derivative); maximum use 2.8 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}: Rat, oral > 5000 mg/kg.

The compound (1 % carboxymethylcellulose ether sodium salt saline) was administered by oral gavage both to F344/N rats (5 animal/sex/group) at single doses of 31, 62, 125, 250, 500 mg/kg, and to B6C3F1 mice (5 animal/sex/ group) at doses of 62, 125, 250, 500, 1,000 mg/kg. No animal died at the end of the observation period of 14 days.

3.4 Repeated dose oral toxicity

Two NTP 14-day repeated-exposure studies were conducted with two different samples of B 37 (75 % and 98 %) on male and female F344/N rats and male and female B6C3F1 mice. In the first study (75 % of B 37) groups of 5 males and 3 or 5 females received in the diet 0, 3100, 6200, 12500, 25000, or 50000 ppm of test compound for 14 days. The final body weight of rats and mice which received 25000 or 50000 ppm was lower than control groups. At necropsy a bluish discoloration of various tissues was observed in rats treated with 50,000 ppm. All treatment rats had dark violet urine and dosed mice had violet urine throughout the studies. The second study was conducted with B 37 98 % pure, using for each experimental group 5 males and 5 females for both species. The final body weight of male rats treated with 50000 ppm were lower than control group. Treated rats and mice had violet urine. The thymus gland of rats was red in 2/5 of males, 3/5 of females (50000 ppm) and 2/5 of males (25000 ppm). No compound-related toxic effects were observed at necropsy for rats and mice in both studies. The dose of 12500 ppm (i.e. 400 mg/kg ca.) was the NOAEL for rats and the dose of 50000 ppm (i.e. 8,523 mg/kg ca.) was the NOAEL for mice.

3.7 Subchronic oral toxicity

F344/N rats and B6C3F1 mice (10 males and 10 females of each species) received in the diet 0, 3100, 6200, 12500, 2000 or 50000 ppm of B 37 (75 % pure) for 13 weeks to evaluate the cumulative toxic effects and to determine the concentration to be used in the 2-year NTP carcinogenicity assay. Purple urine and dark feces were observed in rats after day 9. The urine of treated mice was purple. Final mean body weights were reduced in male rats treated with

6200-50000 ppm and in mice with 50000 ppm. After necropsy the thyroid glands were dark in 40-80 % of the rats in each dose group; the incidence was dose-related (8/10 of males and 8/10 of females at 50000 ppm; 7/10 of males and 4/10 of females at 3100 ppm). No compound-related histopathologic effects were observed for rats and mice. The dose of 25000 ppm (i.e. 4260 mg/kg ca.) represents the NOAEL for mice and the dose lower than 3100 ppm (i.e. 99 mg/kg ca.) represents the NOAEL for rats.

3.8 Subchronic dermal toxicity

The compound containing formulation (1.7 %) was topically applied, twice a week, for 13 weeks both on abraded and intact skin of six male and six female adult New Zealand white rabbits. Gross abnormalities in several organs, microscopic lesions, hematologic and clinical chemistry exams did not show any evidence of systemic toxicity. No dye discoloration of urines was observed at any time during the test.

3.10 Chronic toxicity

A semi-permanent hair dye composite formulation containing the compound (1.63 %) was administered in the diet of 6 male and 6 female Purebred beagle dogs at doses of 19.5 mg/kg/day or 97.5 mg/kg/day (i.e. 0.32 or 1.59 mg/kg/day of compound), 7 days a week, for 2 years. No adverse toxic effects were observed. Necropsy was performed on one male and one female of each group at 6, 12 and 18 months, and on all surviving dogs at the end of the study. The dose of 1.59 mg/kg/day represents the NOAEL for 2-year oral study on dog.

4. Irritation & corrosivity

4.1 Irritation (skin)

The compound as 3 % (w/w) extemporaneous solution in polyethylene glycol 300, applied both on intact and abraded skin (0.5 ml on 6.5 cm^2 per animal) of six male albino New Zealand rabbits, resulted non-irritant under patch-test for 24 hours.

4.2. Irritation (mucous membranes)

The compound as a 3 % (w/w) extemporaneous solution in polyethylene glycol 300 instilled into one eye of six male albino New Zealand rabbits resulted only very slightly irritant.

5. Sensitization

It was induced in 20 adult female Pirbright White guinea pigs by two simultaneously intradermal injections of 5 % test compound in distilled water, Freund's complete adjuvant and a 1:1 mixture of the above solution in a shaved intrascapular area (4 x 6 cm^2) on day 0, 3. One week later, 5 % of test substance in petrolatum was topically applied, under occlusion, on the same area for 48 h. 14 days later the guinea pigs were challenged by a single topical application of 5 % of test compound in distilled water under occlusion for 24 h on the right flank (2 x 2 cm^2). The results evaluated after 24 and 48 hours showed a slighty positive reaction on 4/20 test animals 24 hours after challenge. The compound resulted a weak sensitizer.

6. Teratogenicity

A formulation containing the compound (1.7 %) was topically applied (2 ml/kg/day, i.e. 34 mg/kg/days) to the shaven skin on 20 female Charles River rats on day 1, 4, 7, 10, 13, 16 and 19 of gestation. No embryotoxic or teratogenic effects were observed, but for a significant reduction of the mean live fetal weight.

B 37 contained in a commercial dye/base composite (1.63 %) was administered in the diet of rats from day 6 through day 15 of gestation at dose levels of 0, 1950 ppm (154 mg/kg ca., i.e. 31.785 ppm of B 37, 2 mg/kg ca.) or 7800 ppm (616 mg/kg/day ca., 127.14 ppm of B 37, i.e. ca. 10 mg/kg). Neither evidence of teratogenicity nor embryotoxic effects were observed. The doses of 616 mg/kg ca., represents the NOAEL for the formulations, corresponding to a dose of 10 mg/kg for B 37.

B 37 contained in a commercial dye/base composite (1.63 %) was administered daily by oral gavage to artificially inseminated female New Zealand white rabbits on days 6 to 18 of gestation. The animals received the composite at dosages of 19.5 or 97 mg/kg/day, the composite without the dyes at a dosage of 97.5 mg/kg/day, or the vehicle 0.5% aqueous methylcellulose. The dose volume for all groups was 1 ml/kg. For each group 12 rabbits were considered. Animal treated with 97 mg/kg/day excreted blue-brown colored urine within 1 hour after dosing. Before dosing, urine color was normal the day after. No evidence of teratogenic effect was observed.

6.1 One-generation reproduction toxicity

B 37 contained in a commercial dye/base composite (1.63 %) was administered in the diet of Sprague-Dawley rats (0, 1,950 or 7,800 ppm, i.e. 86 and 351 mg/kg in males and 124 and 554 mg/kg in females) for fertility and reproduction study. For each experimental group 20 males and 10 females were considered. The study was divided in two parts. In Part I the females received the compound from 8 week prior to mating through the weaning of their litter. The males siring these litters were treated for 8 weeks prior to, and during mating period. In Part II the males received the test diet for 8 weeks prior to, and during mating period, while the females received the compound 8 weeks prior to mating and during gestation and 21 days of lactation. Each male was mated with two females of the same test group. No abnormal pups were seen upon dissection of embryos after 13 days of gestation or upon gross examination at weaning after 21 days. The study is considered inadequate for the evaluation of potential effects of the chemical on the reproductive activity of rats.

7. Toxicokinetics (incl. Percutaneous Absorption)

[^{14}C]-B 37 (1073 µg) in ethanol solution was applied under occlusive protective patch on clipped skin of 4 male and 4 female Wistar rats (200 µl on 10 cm^2) or 4 male and 4 female C-57 black mice (40 µl on 2 cm^2) for 48 hours. The following results were obtained after topical treatment: 0.31 % (males) or 0.27 % (females) of the applied dose penetrated in the skin of rats, while 6.5 % (females) or 3.4 % (males) penetrated in the skin of mice.

50 % aqueous shampoo solution of a semi-permanent hair dye containing [^{14}C]-B37 (0.5 %) was applied under non-occlusive condition, before rinsing, for 5, 10, 20 or 30 min on clipped

skin of 3 female Wistar rats, or for 10 min on skin of 4 female C-57 black mice. The following values of skin penetration were obtained: 0.03 μg/cm^2 (after 5 min), 0.07 μg/cm^2 (after 10 min), 0.08 μg/cm^2 (after 20 min) or 0.10 μg/cm^2 (after 30 min) in rat; and 0.04 μg/cm^2 in mouse.

Different levels of [^{14}C]-B 37 (1.5 %, 0.75 %, 0.4 % 0.2 % w/v) contained in a semi-permanent hair dye (200 ml of 50 % aqueous shampoo solution) were applied, under non-occlusive condition, on clipped skin of 3 Wistar rats for 5 min before rinsing. The skin penetration in female rats increased in proportion with the increased concentration of the test compound: 0.01 μg/cm^2, 0.02 μg/cm^2, 0.10 μg/cm^2 and 0.12 μg/cm^2 at 0.2 %, 0.4 %, 0.75 % and 1.5 % dose level, respectively.

50 % aqueous shampoo solution of a semi-permanent hair dye containing 0.5 % (w/v) of [^{14}C]-B 37 (1070 μg) was applied, under non-occlusive condition, 1, 2 or 3 times (200 μl/application) on clipped skin of 4 female Wistar rats for 5 min. before rinsing. The multiple application to female rats resulted in increased penetration: 0.03 mg/cm^2 (single), 0.23 mg/cm^2 (2 applications), 0.60 mg/cm^2 (3 applications).

50 % aqueous shampoo solution of a semi-permanent hair dye containing 0.65 % (w/v) of [^{14}C]-B 37 (1295 μg) was applied (200 μl on 10 cm^2), under non-occlusive condition, on skin (clipped or not) of 4 female Wistar rats for 5 min before rinsing. The skin penetration was 0.04 mg/cm^2 for clipped skin or 0.03 mg/cm^2 in the presence of hair.

Metabolism: It was studied in Wistar rats and C-57 black mice using [^{14}C] labelled-B 37 (73.8 mg in 0.1 ml ethanol and 0.5 ml Tween 80) by oral, intraperitoneal or subcutaneous administration. For each experimental group 4 animals (2 males and 2 females) were considered, except for the i.p. study in rats (1 male and 2 females).

At the 4th day after administration to rats (0.6 ml/animal, i.e. 4431 μg of [^{14}C]-B 37) by different route (oral, intraperitoneal or subcutaneous), 5 % ca. of the applied dose was retained in the body (tissue and carcasses).

In mice a dosing s.c. with [^{14}C]-B 37 up to 2.2 % of the applied dose (0.15 ml/animal, i.e. 1108 μg of [^{14}C]-B 37) was recovered in the carcasses after 4 days.

The faecal and urinal analysis of rats (6 or 24 hs. after treatments) revealed acetylated and conjugated products of parent HC Blue No.2, Violet A isomers and HC Red 3 dyes.

Human skin-absorption: A formulation containing B 37 radiolabelled (1.77 %) applied on human hair under conditions of use (35-38 min) showed a cumulative dose absorption evaluated by means of urine radioactivity assay (1-10-20-30 days) lower than 0.1 % and a time required for 50 % excretion (T½) of 52 h.

8. Mutagenicity

The studies here presented have shown that B 37 is able to induce UDS on rat hepatocytes and sister chromatid exchange in the presence of metabolic activation system on Chinese hamster ovary cells *in vitro*. Two NTP studies (*Salmonella* with and without activation from rat and hamster liver, and mouse lymphoma with rat liver activation) have shown positive results. In the re-evaluation of NTP *Salmonella* studies using more stringent criteria the compound was

classified as negative. Negative results were obtained in the induction of chromosome aberrations on CHO cells *in vitro*; in this study the induction of SCE resulted positive.

In another reverse mutation study on *Salmonella* the compound resulted negative. The compound did not induce chromosome aberrations *in vivo* by micronucleus test on mice (2 x 750 or 1000 mg/kg i.p.). Unscheduled DNA Synthesis studies on male and female rat hepatocytes and male and female mice hepatocytes following *in vivo* treatment up to 1000 mg/kg b.w., and cell proliferation in rats and mice studies, were found negative.

Additional *in vitro* studies, requested by the SCC, performed with a sample of 99.5 % of purity, have shown that B 37 is negative in the Ames test, in mouse lymphoma L5178Y (6-TG^R) assay, and in human lymphocytes chromosome aberrations test.

Literature studies with a sample of 99.77 % of purity showed that the compound was positive in *Salmonella* assay, in mouse lymphoma L5178Y (TFT^R), and in rodent UDS *in vitro* test. This sample of compound did not induce forward mutation on *E.coli*, micronuclea on ICR and CD-1 mice bone marrow; and UDS *in vitro* on monkey primary hepatocytes.

9. Carcinogenicity

Long-term studies were carried out on mice and rats (NTP bioassay): the compound (98 % pure) fed in the diet for 103 weeks to 50 F344/N rats/sex/group and 104 weeks to 50 B6C3F1 mice sex/group at dietary concentrations of 0, 5000 or 10000 ppm to male rats (195 or 390 mg/kg/day), male mice (465 or 1000 mg/kg/day) and 0, 10000 or 20000 ppm to female rats (1320 or 2240 mg/kg/day) and female mice (2330 or 5600 mg/kg/day). B 37 caused a dose-related increase in the incidence of hyperostosis of the skull in male an female rats. A uncommon tumour (mixed mesenchymal neoplasms of the kidney) was noted for females F344/N (2/50 at high dose) and a marginal positive trend in the incidence of lymphomas in male mice (1/50; 5/48; 8/49) not significant when survival was taken into account. Under the conditions of these studies, "*no evidence"* of carcinogenicity in F344/N rats and B6C3F1 mice receiving B 37 in the diet was observed.

11. Conclusions

The SCC does not recognize any possible health risk in connection with the use of this dye.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

N1,N4,N4-Tris(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene

(B 37)

SEMI-PERMANENT HAIR DYE

(Based on a usage volume of 35 ml, containing at maximum 2.8 % of N1,N4,N4-Tris(2-hydroxyethyl)-1,4-diamino-2-nitrobenzene)

Maximum amount of ingredient applied	**I (mg) = 35 x 2800 mg/100 = 980 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.1% (human, evaluated by means of urinary radioactivity assay)**
Dermal absorption per treatment	**I (mg) x A(%) = 980 x 0.1/100 = 0.98 mg**
Systemic exposure dose (SED)	**SED (mg) = I (mg) x A% / 60 kg b.w. = 0.98 mg / 60 kg b.w. = 0.0163 mg/kg b.w.**
No observed adverse effect level (mg/kg)	**NOAEL = 99 mg/kg b.w. (rats, 13-weeks oral NTP study)**
Margin of Safety	**NOAEL / SED = 99 mg/kg b.w./ 0.0163 mg/kg b.w.= 6000**

Classification: A

P 21: *BENZYLHEMIFORMAL - COLIPA*

The toxicological properties of P21 (Benzylhemiformal; ®Preventol D2; CAS 14548-60-8) have been investigated in acute (oral, dermal, and intravenous application), subacute (oral), subchronic (dermal) studies. Genotoxicity was checked by an Ames- and micronucleus test as well as local tolerance on skin and eyes. Experimental studies assessing the allergenic potential are reported. Recently the final report of an *in vitro* penetration study applying epidermis of the pig ear became available. An embryotoxicity/teratogenicity (segment 2) study according to OECD-guideline 414 in Wistar-rats as well as an additional study on genotoxicity [*in vitro* mammalian (V79-cells) cytogenetic test] according to OECD-guideline 473 are ongoing. The final reports will be available in 1994 (see below).

A subchronic dermal tolerance study revealed a statistically significant lower pituitary weight in male, but not in female rabbits. This lower organ weight was not accompanied by a significant change in weight of thyroid and adrenal gland, indicating that the pituitary was functionally intact and capable of synthesizing and secreting normal amounts of adrenocorticotrophic hormone (ACTH) and thyrotropin (TSH). The histological examination of the thyroid gland and of the adrenal cortex did not reveal any pathological change. The pituitary gland was histologically examined by two different investigators, neither the original examination nor a re-examination revealed a pathology of the organ, in particular there was no histopathologic evidence of atrophy, degeneration, or other alteration that would explain the reduction of pituitary weight. The review of the pituitary sections indicated a marked variation in the amount of pars distalis and variable amounts of adjacent tissues such as ganglia, vessels, connective tissue, and nervous tissue. In all there is evidence that the lower pituitary weight is not related to treatment, but most probably due to normal variability and influenced by collecting procedure (see appendix D for more details). But to exclude any shadow of doubt a further experimental study may be conducted. This experiment (see appendix A for more details) should be designed based on OECD guideline 408, i.e., a 90 day subchronic oral toxicity study in rats, with special attention to the pituitary as a potential target organ. The oral route is preferred because it assures the application of a sufficient dose, the dermal application is considered to be compromised by local effects: erythematous skin changes were observed after repeated dermal application of higher concentrations, and the further treatment led to progression with ulcerations up to necrotising changes. Due to the local irritancy of high concentrations in long-term application, a systemic toxicity may therefore not be achieved by dermal application, and systemic toxicity will be concealed by secondary effects of local irritancy, respectively. It can be derived from acute studies that there is no significant difference between dermal and oral application of P21 with regard to systemic effects (see appendix B for more details). The result of this study will determine the NOAEL and set a stronger basis for final assessment.

A provisional approval for use as a preservative in cosmetics had been made for Benzyl-hemiformal/P21 with a maximum concentration of 0.2 % in the final formulation. However, on February 10, 1992, a “D”-classification was adopted by the plenary session of the SCC, because of a low NOAEL (1 mg/kg/d) in a subchronic dermal study. This classification is based on an assumption of human exposure of 1 mg/kg/d, which results from a total global exposure

to cosmetics containing 0.2 % Benzylhemiformal in the finished product. As long as additional studies for a sufficient and concluding evaluation are ongoing, the use of benzylhemiformal in cosmetics should be restricted to "rinse-off products" and a maximum concentration of 0.03 % in the final formulation. This temporary restriction is made to provide a sufficient safety margin, and is due to be revised based on a new evaluation. This document provides a safety calculation (see appendix C) for use in "rinse-off" cosmetics.

By the assessment made so far, P21 is regarded as a preservative for "rinse-off" cosmetics having a safety factor of 125 (see appendix C for safety calculation), even on the assumption of maximum concentration (0.03 %) of the preservative in the formulation, extensive use of ("rinse-off") cosmetics, and complete dermal absorption of the preservative.

In addition to the studies that have been reported and submitted the following will be conducted under good laboratory practice (GLP):

- Penetration through the epidermis of pig ear *in vitro.* The final report is available.
- *In vitro* mammalian cytogenetic test for the detection of chromosomal aberrations according to OECD-guideline 473 (V79-cells).
  The final report is expected to be available in the first quarter of 1994.
- Embryotoxicity/Teratogenicity (segment 2) study in Wistar-rats (Dosage: 0, 30, 100, and 300 mg/kg/d by gavage) according to OECD guideline 414.
  The final report is expected to be available in the third quarter of 1994.
- 90-day subchronic oral toxicity study in Wistar-rats (see appendix A for details) according to OECD-guideline 408 with special attention to the pituitary as a target organ. The study is expected to start in late 1994 and the final report to be available in the second quarter of 1996.

Appendix A

90 day subchronic oral toxicity
according to OECD guideline 408

Species: Wistar Rat
Number of animals: 10 males and 10 females per dose group
Method of administration: by gavage (gastric intubation)
Dosage, vehicle and volume: 0, 30, 100, and 300 mg/kg/d in 5 ml/kg PEG 400
Negative control: PEG 400
Dosing: once daily, 7 days per week
Duration of the study: 90 days

Appendix B

acute toxicity (LD_{50} [mg/kg]) of COLIPA P21 in rabbits and rats by route of exposure

	male rat	**male rabbit**	**female rat**	**female rabbit**
oral	1700 [5]			
dermal	2000 [6]	1429 [7]	1000-2000 [6]	2000 [7]

Appendix C

Safety Calculation

The safety calculations are based on the guideline "Estimation of consumer exposure to preservatives" issued by COLIPA (February 1993, 93/067).

The following assumption is made for "rinse off products" of cosmetics:

Maximum Concentration of P21 in the final product: 0.03 **%**
Quantity of "rinse off" cosmetics used (extensive use): 16,600 mg/d
Proportion absorbed (worst case assumption): 100 **%**
Rinse-off coefficient: 10 %
Human Body Weight: 60 kg

Thus human exposure is calculated as follows:

(0.03·0.01) · 16,600 mg/d · (100·0.01) · (10·0.01) / 60 kg = **0.008 mg/kg/d**

In accordance with the assessment of the SCC a NOAEL of 1 mg/kg/d is supposed.

NOAEL = 1 mg/kg/d
human exposure = 0.008 mg/kg/d

Factor NOAEL/human exposure (1/0.008): 125

This margin of 125 is calculated based on the assumption of maximum concentration of the preservative in the formulation, extensive use of "rinse off" cosmetics, and complete dermal absorption of the preservative, which may be considered a worst case modelling.

Appendix D

In a study on subchronic dermal tolerance male and female rabbits (n = 10 animals per sex and treatment group) were treated with P21 solutions for 90 days (5 days/week) with 6-hour occlusion/day. The concentrations used were 0.0 %, 0.2 %, 0.8 % and 3.2 %. They were selected on the basis of dermal tolerance determined in preliminary studies, and corresponded to a nominal administration of 0, 1, 4, and 16 mg/kg/day respectively. The local irritant effect of the higher concentrations proved limiting for an evaluation in this study. At the concentration level of 3.2 % P21 erythematous skin changes were observed as early as from the 1st week of treatment: after the 3rd to 5th application (females) and after the 6th to llth application (males). Further treatment led to progression with ulcerations up to necrotising changes.

Although, owing to local effects, thorough analysis or evaluation of potential systemic effects is basically not possible for this study, no substance-related systemic changes were observed. Hematological and clinicochemical investigations as well as clinical observations revealed no treatment-related influence. Body weight gain of females treated with 3.2 % P21 was noticeably, but not statistically significantly, below that of the corresponding control group. This finding can be accounted for by the local dermal effects of the highest concentration level. Male animals of the same concentration as well as all other groups did not show any unusual features with respect to body weight development. The Student's t-test produced statistically

significantly reduced pituitary weights both absolute and related to body weights for males of 3.2 % and the 0.8 % treatment group. In the Wilcoxon U-test, however, no statistically significant result could be established for the second highest dose group. These striking features gave rise to comprehensive follow-up investigations, which were performed by an expert of international reputation, namely Dr. Charles C. Capen, Professor at the Department of Veterinary Pathobiology of Ohio State University in Columbus, Ohio, U.S.A. Neither a histology-morphological nor an endocrine-functional correlate was established for the organ weight changes. There were also no unusual features in the organs depending on the pituitary, e.g., thyroid gland and adrenal (the expertise has been submitted). Dr. Capen extended the follow-up investigations to include the evaluation of pituitaries from the 4-week toxicity study (oral application of 0, 30, 100 and 300 mg/kg/day) in male and female rats. No relevant conspicuous features were observed up to the highest dose group of 300 mg/kg/d.

Neither the study on dermal tolerance of P21 in rabbits nor the follow-up investigations (including a 4-week toxicity study in rats with 300 mg/kg/day as the highest dose) gave any indication of disturbances of the endocrine system, its organs and feedback mechanisms or of a reduced capability of adaptation. The changes of pituitary weights conspicuous in this study in male rabbits are thus to be considered as a spurious observation without any relation to administration of P21.

To account for this spurious finding, attention is drawn to the topological conditions in the rabbit: due to its deep location in a bone excavation of the sella turcica the pituitary is not easily accessible and thus complete preparation is difficult. Even if dissection is performed by experienced staff, there is a wide variation of pituitary weights. An internal survey (Appendix E) of pituitary weights of 85 rabbits of the same strain from control groups of 6 studies showed a variation coefficient[1] (VC) for males (n = 42) of 35 % (absolute organ weight) and 39 % (relative organ weight) and for females of 44 % (absolute organ weight) and 49 % (relative organ weight). Despite comprehensive investigations into data bases and literature only one publication was found reporting pituitary weights of male New Zealand White rabbits. The (absolute) organ weights of the animals (n = 16 in each case) of two control groups were stated to be 15.9 ± 5.9 mg and 16.8 ± 4.9 mg respectively. The standard deviation (37 % and 29 % respectively of the mean value) indicates a remarkably wide variation. It can also be seen from these data that absolute pituitary weights below 10 mg, as were measured in 2 animals treated with P21, should not be unusual.

With the qualification that, owing to the study design, no (systemic) NOAEL value can be derived in the case of a primary irritant substance, it can be said on balance that 16 mg/kg/d P21 induced no systemic changes after dermal application.

([1]) standard deviation in per cent of the mean

Appendix E

Evaluation of pituitary weight in rabbits

- Variation in control animals of dermal toxicity studies -

1. Introduction

Measuring pituitary weights is not common in toxicity studies. Especially in rabbits the collection of this organ faces many difficulties, the main one being the topography of the pituitary, that is located in an osseous excavation of sella turcica. Thus even skilled personal will not always collect the entire organ.

This evaluation was compiled to gain a lager data-base because reference data for pituitary weight of rabbits are lacking: Only one reference was identified that gives pituitary organ weights.

2. Methods

To set up a data-base for pituitary weight of male and female rabbits (New Zealand White) aged 11-16 weeks the corresponding data derived from the controls of seven studies[2] (8109, T5027628, T3029453, T5029590, T3029589, T7029592, T6029591) were submitted to the Institute of Biometry, Bayer AG. The animals served as controls in dermal toxicity studies, in all cases only animals with intact skin were used.

Complete data were available for 43 males and 42 females, however with unequal numbers of male and female animals per study:

Study	Number of Males	Number of Females
8109	9	10
T5027628	10	5
T3029453	4	5
T5029590	9	8
T3029589	5	5
T7029592	5	5
T6029591	-	5

As usual the statistical analysis is based on the general assumption that any selection bias is absent. After entering the raw data all individual values were carefully checked for correctness and plausibility.

The variables “body weight”, “pituitary weight” as well as “relative pituitary weight” (pituitary weight/body weight) are described separately by sex and study and pooling all studies using appropriate measures for location and variability.

(2) In the raw data list only the last four digits of the study number are used for identification.

3. Results

There is an acceptable coincidence between arithmetic means and the medians both within strata as well as in total (appendix 1.).

Among strata (= studies) remarkable differences between means and standard deviations are encountered. The average body weights of both the male and female animals of the study 8109 are definitely higher than in the other studies.

Whereas the mean body weights do not show further distinguishing patterns, the mean pituitary weights may be allocated to clusters either with relatively low weights (8109, T3029453) or relatively high weights (T3029589, T6029591, T7029592). The justification for pooling the data is thus not deducible from the data at hand but must rely on the assumption that we are dealing with animals of the same husbandry and the same age range.

As anticipated the low pituitary weights in the studies 8109 and T3029453 result in low relative pituitary weights. This effect is more pronounced in 8109. There is no positive correlation between body weight and pituitary weight.

4. Discussion

The body and pituitary weights of untreated rabbits with intact skin were pooled from seven independent studies on the assumption of comparable age ranges and other potentially influential factors.

The statistical description of the data was performed separately for each sex both stratified by study and pooled over strata using appropriate measures of location and variability.

Whereas the body weight revealed only a low coefficient of variation (CV) both intra-strata (less than 10 %) and inter-strata (12.5 % in males; 16.3 % in females), the CV of pituitary weight was remarkable higher: intra-strata CV was between 15.7 % and 40.9 %, inter-strata CV was 35.0 % in male and 43.7 % in female rabbits. These results further highlight the great physiological variability of pituitary weight.

Along with the fact of no positive correlation found between body weight and pituitary weight, from all this follows that effects seen in variation of pituitary weight should be interpreted cautiously.

19.11.92

PLACEBO-VALUES FOR RABBITS (SKIN INTACT) / 002

ANIMALS

	Study Number	N	SD	MINIMUM	MEAN	MAXIMUM	2.5 %	MEDIAN	97.5 %
Body Weight (kg)	8109	9	0.35	3.31	3.84	4.56	3.31	3.80	4.56
	9453	10	0.22	2.60	3.01	3.31	2.60	3.01	3.31
	9589	4	0.01	2.97	2.98	3.00	2.97	2.98	3.00
	9590	9	0.12	2.92	3.07	3.32	2.92	3.06	3.32
	9591	5	0.12	2.77	2.94	3.09	2.77	2.98	3.09
	9592	5	0.07	3.00	3.10	3.18	3.00	3.11	3.18
	TOTAL	42	0.40	2.60	3.20	4.56	2.61	3.08	4.52
Pituitary Weight (mg)	8109	9	5.63	14.00	22.22	30.00	14.00	20.00	30.00
	9453	10	9.20	13.00	27.70	42.00	13.00	24.00	42.00
	9589	4	13.60	25.00	41.25	56.00	25.00	42.00	56.00
	9590	9	8.79	23.00	33.22	51.00	23.00	32.00	51.00
	9591	5	7.79	35.00	44.80	56.00	35.00	43.00	56.00
	9592	5	8.94	29.00	41.00	53.00	29.00	41.00	53.00
	TOTAL	42	11.43	13.00	32.62	56.00	13.07	31.00	56.00
Relative Pituitary Weight (mg/100g b.w.)	8109	9	0.17	0.34	0.59	0.85	0.34	0.55	0.85
	9453	10	0.29	0.39	0.92	1.33	0.39	0.89	1.33
	9589	4	0.46	0.84	1.38	1.87	0.84	1.41	1.87
	9590	9	0.28	0.75	1.08	1.63	0.75	0.96	1.63
	9591	5	0.23	1.17	1.52	1.81	1.17	1.52	1.81
	9592	5	0.28	0.94	1.32	1.70	0.94	1.37	1.70
	TOTAL	42	0.41	0.34	1.05	1.87	0.34	0.96	1.87

PLACEBO-VALUES FOR RABBITS (SKIN INTACT) / 002

ANIMALS

	Study Number	N	SD	MINIMUM	MEAN	MAXIMUM	2.5 %	MEDIAN	97.5 %
Body Weight (kg)	7628	5	0.18	2.58	2.85	3.09	2.58	2.86	3.09
	8109	10	0.44	3.13	4.01	4.57	3.13	4.01	4.57
	9453	5	0.10	2.94	3.05	3.17	2.94	3.08	3.17
	9589	5	0.18	2.67	2.91	3.17	2.67	2.88	3.17
	9590	8	0.11	2.89	3.04	3.19	2.89	3.06	3.19
	9591	5	0.09	2.71	2.81	2.91	2.71	2.77	2.91
	9592	5	0.09	2.83	2.96	3.07	2.83	2.96	3.07
	TOTAL	43	0.52	2.58	3.19	4.57	2.59	3.00	4.57
Pituitary Weight (mg)	7628	5	5.54	26.00	35.20	39.00	26.00	38.00	39.00
	8109	10	9.43	19.00	27.60	50.00	19.00	25.50	50.00
	9453	5	9.63	9.00	19.80	34.00	9.00	21.00	34.00
	9589	5	10.70	48.00	61.00	75.00	48.00	63.00	75.00
	9590	8	15.23	30.00	48.63	72.00	30.00	46.00	72.00
	9591	5	9.67	39.00	55.00	63.00	39.00	57.00	63.00
	9592	5	21.10	34.00	51.60	84.00	34.00	44.00	84.00
	TOTAL	43	18.09	9.00	41.35	84.00	9.40	38.00	83.10
Relative Pituitary Weight (mg/100g b.w.)	7628	5	0.21	0.91	1.24	1.47	0.91	1.26	1.47
	8109	10	0.25	0.45	0.69	1.33	0.45	0.62	1.33
	9453	5	0.32	0.28	0.65	1.10	0.28	0.71	1.10
	9589	5	0.29	1.80	2.09	2.51	1.80	2.08	2.51
	9590	8	0.56	0.95	1.61	2.49	0.95	1.47	2.49
	9591	5	0.32	1.41	1.95	2.24	1.41	1.99	2.24
	9592	5	0.76	1.15	1.75	2.97	1.15	1.47	2.97
	TOTAL	43	0.67	0.28	1.36	2.97	0.29	1.26	2.92

REPORT ON STRONTIUM PEROXIDE

Submission N° 1 for strontium peroxide requests permission for its use at a strength of 6 %, exclusively as a hairdressing product, by hairdressing professionals and with all trace of it to be subsequently rinsed away.

The data supplied, referring to the method of application, and other data supplied by people within the profession, indicate the use of a mixture of powders containing strontium peroxide (SrO_2), probably together with other peroxides and masking and thickening agents. The product is diluted and mixed with the required quantity of H_2O_2 (30 volumes) until a smooth, creamy consistency is obtained. This is then immediately applied with a brush over the full length of the hair.

It is highly alkaline (pH > 10) and the release of the reactive oxygen brings about bleaching of the darker shades of hair after approximately 30 minutes' contact. Both the hair and the scalp are then thoroughly washed with shampoo and rinsed with water.

The dossier submitted includes an acute toxicity study of topical application on rats (limit test), enabling the lethal dose to be established at over 2000 mg per kilo of body weight. Given the way the product is used, this figure may be considered acceptable.

The primary skin irritation test, carried out over 24 hours on albino rabbits using the occlusive patch test with SrO_2 at 6 % (diluted in water) resulted in a level of erythema, eschar and oedema equivalent to a primary skin irritation index of 0.7 on the Draize scale. The product should therefore be considered slightly irritating to the skin of a rabbit.

An identical study, carried out using H_2O_2 in place of water, places SrO_2 in the same category, but the index is slightly higher 1.

Bearing in mind that SrO_2 is not likely to remain in contact with the scalp for more than some 30 minutes, that the scalp is not covered in any way, and that several weeks will elapse between treatments, the risk of irritation may be considered very slight.

The sensitization study was carried out on 20 albino guinea pigs. After checking for the absence of individual reactions by means of a 48-hour topical and occlusive application of the product containing SrO_2, the sensitization protocol was applied to each guinea pig. This involved intradermic injection of Freund's adjuvant to the rib area of each animal followed (over a period of 15 days) by seven topical applications of the product containing SrO_2. There was then a rest period of 12 days before the product containing SrO_2 was applied to the abdominal region under an occlusive patch for a period of 48 hours to provoke the reaction. After removal of the occlusive patch, the application zone was examined after 6, 24 and 48 hours. These inspections identified visible macroscopic skin reactions in both the initiating and the induction zones.

It was decided that the animals should undergo histopathological examination (to assess the appearance of experimentally-induced eczema).

The inspection six hours after removing the occlusive patch revealed the need for histopathological examination of 3 of the 20 guinea pigs in the test. The result of this test showed that two animals had a "clear orthogenic reaction" and only one an "actual allergic reaction".

If this is taken to mean that the three animals were sensitised by SrO_2, this then means a class II sensitization level (i.e. a maximum of 25 % of the animals).

If we consider that the orthogenic reaction does not necessarily mask an allergic reaction, the sensitization level would be type I (no more than 10 % of the animals sensitised). These two hypotheses would rank the sensitising properties of SrO_2 as WEAK or VERY WEAK, respectively.

In contrast to the tests referred to earlier, this sensitization test was carried out using not SrO_2 but a formula (a mauve-coloured powder) of which all we are told is that it contained 11.5 % strontium peroxide.

It is difficult to regard sensitization tests carried out using a finished product, the composition of which is not fully known, as definitive, since the unknown ingredients may affect the response.

The conditions under which the sensitization test is performed require the use (in the form of an occlusive patch for at least 48 hours) of the maximum quantity before the skin becomes irritated. The slightly irritating properties of SrO_2 in a 24-hour occlusive patch were demonstrated during the skin irritation test. Under such circumstances, it is difficult to reach valid conclusions, given that the application dose might in some animals present an irritation potential which could invalidate the interpretation of the sensitization potential. For these reasons, it is not desirable to request a new sensitization test using SrO_2 (instead of the finished product).

Sr compounds are not allergenic in man. Further investigation is not necessary.

Finally, the submission document contains a study of *in vivo* penetration, carried out using rabbits and with the same formula as that used for the sensitization study. The results are to some degree contradictory and so it is difficult to interpret them properly.

The aim of the trial was to see whether application of the product to the skin, under virtually identical conditions to its normal use by hairdressing professionals, would result in an increase in SrO_2 levels in blood and accordingly presumes that SrO_2 can be absorbed through the skin.

The product contains 11.5 % of SrO_2 and 5.6 g (diluted in 12 ml of 30 volumes H_2O_2) were used. The six test rabbits were shaved the day before the trial and their blood analysed to establish the strontium content before the trial. The trial was carried out by applying the product, in the diluted form described above, to 100 cm^2 of their skin and using a semi-occlusive patch to maintain contact for two hours. At the end of this time, blood samples were taken (a double quantity so that the analyses could be repeated if necessary).

The detection limit for Sr is of the order of 25 ppb.

Blood levels before the test varied between 0.15 and 0.30 ppm, providing an average value of 0.22 ppm.

After the test, they varied between 0.15 and 0.22 ppm, yielding an average value of 0.22 ppm.

Only one rabbit showed anomalous behaviour, with a significant increase in blood Sr after application of the product: rising from 0.15 ppm before the application to 0.40 ppm after application. For this animal, the analysis was repeated with the second sample and surprising results were obtained: 0.20 ppm before and less than 0.05 ppm after the test. There is no explanation for these anomalous results.

If however the data from this animal are disregarded, it is fair to say that the absence of any increase in Sr in the blood suggests that none of the Sr present in the SrO_2 of the product tested was absorbed through the skin.

No trials other than those cited have been submitted indicating the toxicological profile of SrO_2, and for this reason it could be useful to examine some aspects of research on other strontium salts.

Acute toxicity for hexahydrated strontium chloride corresponds to an LD_{50} of 12.4 g per kilo body weight (oral pathway in the rat).

Investigation of the effect of $SrCl_2.6H_2O$ in newly-born rats.

Rats were selected with a litter of 8 young. From day 2 to day 15, during lactation, each litter received a solution of a determined dose of $SrCl_2.6H_2O$ once a day via intubation.

The elements used were Sr, Mo, Li and B. In each experiment and at each of the three doses tested, 2 or 3 litters of 8 new-born rats were used. In all case, half of each litter were used as controls and received distilled water.

The dose of 100 mg per kilo body weight did not have any adverse effects, there being no deaths, rachitis or dentine lesions, weight increase was optimal and no histopathological lesions were detected.

Short-term oral toxicity of hexahydrated strontium chloride ($SrCl_2.6H_2O$) in the rat. The test compound was administered in the diet in concentrations of 0, 75, 300, 1200 and 4800 mg/kg diet for 90 days.Growth, food intake, behaviour and mortality were recorded, extensivy haematology and clinical chemistry carried out, organ weights determined, X-ray photographs of the bones taken and complete histopathological examination was performed. In addition, the Sr-contents of blood, bone and muscles were determined. The observed effects were as follows:

- Increased thyroid weight was seen at 1200 and 4800 mg/kg in males only with histological signs indicating thyroid activity at 4800 mg/kg only. In females, no effect on the thyroid was found.
- Glycogen depletion in the liver (determined biochemically with histological confirmation) was found in males and females at 4800 mg/kg only.
- The Sr-content in bones was increased at all dose levels (increase dose-related). These increased concentrations are not considered a toxic effect. Based on these results, the NOAEL in this study is 300 mg $SrCl_2.6H_2O$ /kg diet. This level is equivalent to 300/20 = 15 mg/kg b.w./day. The NOAEL expressed as Sr is 5 mg/kg b.w./day (rounded value).

Mutagenicity using 127 metallic compounds. In an initial screening, they studied growth inhibition of bacillus subtilis strains (one without a deficiency, or rec+, the other with a recombination repair deficiency, or rec-). In each case, a precise concentration of a metal compound was used, with impregnation of filter paper disks located in the bacteria plate culture and measurement in mm of the longitude of the inhibition provoked by each strain.

When inhibition is greater in the rec- strain than in the rec+ strain, it is clear that the chemical compound in question damages cellular DNA. In this study, a variant was used which involved keeping the plates of the different strains with filter disks pre-impregnated with the metal compounds over a 24-hour period at 4°C, before proceeding to normal incubation at 37°C during the entire night. The authors report that this protocol increases test sensitivity 20 to 50-fold for many drugs.

For $SrCl_2.6H_2O$ the result was negative.

However, positive results were obtained for 44 compounds, including various compounds of arsenic, silver, barium, bismuth, celsium, chrome, platinum and rhodium. In all cases, strains of *Escherichia coli* and *salmonella* were used.

Metal-induced DNA synthesis infidelity. The study estimated the fidelity of DNA replication *in vitro* and showed that many metal ions can alter it.

The model utilised was a synthetic polynucleotide formed by deoxytimidine and deoxyadenosine monophosphates: Poly d (A-T).

This polynucleotide can be synthesised with an error of less than 2.10^6 using DNA polimerase I of *Escherichia coli*. In the protocol used the correct copy contains only dAMP and dTMP. Incorporation of dCTP and dUTP signal errors in replication.

40 metallic compounds were tested in the experiment. The authors report that Sr did not affect DNA synthesis fidelity. However, alterations did take place in the case of silver, beryllium, cadmium, cobalt, chrome, manganese, nickel and lead.

The effect of metallic ions on RNA transcription. For this experiment, they used a RNA polimerase of *Escherichia coli*, the initial model being poly d (A-T) in the presence of various metal ions, with a view to determining transcription fidelity: one incorrect nucleotide (cytidinmonophosphate - CMP) for 200 correct nucleotides, in the presence of Mg2+.

Various metal ions tested, known to be non-mutagenic or non-carcinogenic, and including Sr+, did not lead to erroneous incorporation of CMP during transcription of poly d (A-T).

Moreover, various studies provide a wide range of reliable data indicating that strontium is not teratogenic, that it is not toxic for the embryo and has no effect on the reproductive process.

The toxicity of strontium depends to a large extent on the naturalness of the anion.

Conclusions: Classification A.

Potentially irritating to the eyes and damaged skin.
Concentration : 6.0 % SrO_2 (4.5 % Sr) in preparations listed for use.

OPINIONS ADOPTED DURING THE 55TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 9 March 1994

MUSK AMBRETTE

1. General

1.1 Primary name

Musk ambrette

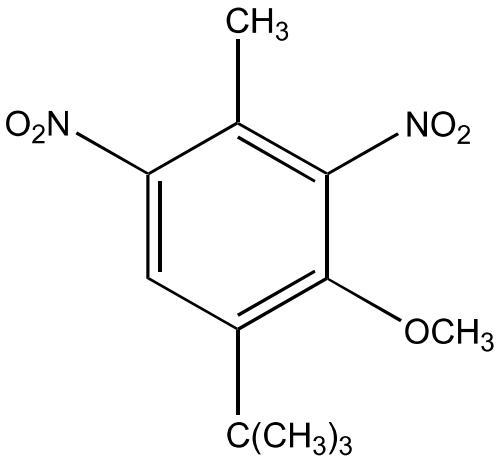
1.2 Chemical names

6-tert-butyl-3-methyl-2,4-dinitroanisole;
2,6-dinitro-3-methoxy-4-tert-butyltoluene
IUPAC name: 5-tert-butyl-1,3-dinitro-4-methoxy-2-methylbenzene

1.4 CAS no.

83-66-9

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}H_{16}N_2O_5$
Mol weight: 268.30

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}, rat, oral: 339 mg/kg b.w.
LD_{50}, rat, oral: 4.8 g/kg b.w.

Remarks: no original data available.

3.2 Acute dermal toxicity

LD_{50}, rabbit, dermal: >2g/kg b.w.

3.4 Repeated dose oral toxicity

Rats, 12 wk feeding study: NOEL 0.76 mg/kg b.w./d. Male rats, 50 wk, female rats, 20 wk, feeding study: 500, 1500, 2500, 4000 ppm; NOEL: 500 ppm

Remark: No original data available.

3.8 Subchronic dermal toxicity

7 groups of 15 male and 15 female Sprague-Dawley CD rats were treated either with 0 (control) or 1500 ppm of musk ambrette in their diet or topically with a solution of musk ambrette in phenylethyl alcohol applied to the shaven back at concentrations equivalent to 0 (control), 10, 40, 80, and 240 mg/kg for 12 wk.

Results: The study provides conclusive evidence that repeated dietary or topical treatment of rats with musk ambrette causes central and peripheral nervous system damage characterized by degeneration of myelin and selected distal axons. These toxic effects were seen in animals treated with musk ambrette at concentrations of, or greater than, 1500 ppm (diet), that is approximately 75 mg/kg b.w./d, or 80 mg/kg b.w./d (dermal).

3.10. Chronic toxicity

Long-term toxicity/carcinogenicity study: no data available.

4. Irritation & corrosivity

4.1. Irritation (skin)

Musk ambrette applied full strength to intact or abraded rabbit skin for 24 h under occlusion was moderately irritating. Tested at 20 % in petrolatum, it produced no irritation after a 48 h closed-patch test on human subjects.

Remark: no original data available.

5. Sensitization

Photosensitivity: Photosensitivity to musk ambrette was confirmed in 15 men previously photopatch tested. 6 of the recalled patients continued to react adversely to sunlight due either to unrecognized exposure to musk ambrette or their having become persistent light reactors. Musk ambrette elicited a positive patch test without light in 3 of 19 patients (15 recalled patients plus 4 patients just attending the clinic).

Photoirritation and photosensitization: Dunkin-Hartley albino female guinea-pigs were used in groups of four for preliminary photo-irritation studies, and in groups of 12 for photoallergy tests. Musk ambrette (0.1 ml) was applied to clipped and shaved interscapular skin and the guinea-pigs irradiated with 100 kJ m^{-2} UV. The procedure was repeated 24 h later.

Ten to 14 d after induction the guinea-pigs were challenged using clipped and shaved lumbar skin. Test and control animals were treated identically. Three concentrations (0.1, 1 and 10 %) of the test substance were applied (10 µl on 14 mm diameter skin). 30 min later the animals were irradiated with 100 kJ m^2- UV. After irradiation the test substance was applied to fresh skin sites to check contact sensitivity.

Results: Musk ambrette was not irritant or photoirritant under the conditions studied. There was no contact sensitivity reaction. Photoallergic reactions were elicited in 12 animals, at the 1% concentration, and in 7 animals, at the 0.1 % concentration. Reactivity tended to decrease at second challenge.

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: 37 % in rats.

Remarks: no original data available.

8. Mutagenicity

Musk ambrette was tested in the *Salmonella*/microsome test with and without metabolic activation in the strains TA 1535, TA 100, TA 1537, TA 1538, TA 98 in concentrations up to 1 µmol/plate and 2.5 µmol/plate, resp. Results: Musk ambrette was found to be mutagenic with and without metabolic activation in the strain TA 100.

Musk ambrette was tested in the Basc tests on *Drosophila melanogaster* in four independant tests. Results: There was an appearant decrease in observed mutant members, but the total of SRL mutations in the four Basc tests is significantly increased over the control.

Musk ambrette was tested in a micronucleus test in NMRI mice. Results: Musk ambrette administered to mice i.p. or orally did not produce micronuclei in bone marrow.

Musk ambrette was assayed for mutagenicity in the *S. typhimurium* strains TA 100 and TA 98 with and without a rat-liver S-9 activation system at concentrations of 50, 100, 250 and 500 µg/plate. Results: Musk ambrette caused a concentration-dependant increase in mutagenicity in the TA 100 strain, in the present of S-9.

Musk ambrette was tested using a preincubation modification of the *Salmonella*/microsome test in the absence of exogenous metabolic activation and in the presence of liver S-9 from Aroclor-induced male Sprague-Dawley rats and Syrian hamsters. Results: Musk ambrette was not found to be mutagenic under the conditions of the tests in the strains TA 100, TA 1535, TA 1537 and TA 98.

10. Special investigations

Concentration in human tissue: 22 human milk samples from 15 women and 13 human fat samples (surgery samples) were analysed for their musk ambrette content. Results: In a few samples residues of musk ambrette were detected at low levels.

391 human milk samples were analysed for their musk ambrette content. Results: In almost all samples musk ambrette was found with a mean content of 0.04 mg/kg fat, and a maximum content of 0.29 mg/kg fat.

11. Conclusions

Musk ambrette has a very low acute toxicity. It produces no irritation or sensitization in humans. Musk ambrette is a strong photosensitizer in guinea-pigs and it has been confirmed that dermatological patients are photosensitive to musk ambrette. Musk ambrette was showing neurotoxic effects in rats and can readily penetrate rat skin. The no-observed-effect-level determined in a 12 wk dermal toxicity test in Sprague Dawley CD rats was 40 mg/kg b.w./d. Musk ambrette was found to be mutagenic in the *Salmonella typhimurium* test strain TA 100 and in the Basc Test on *Drosophila melanogaster*. It was not found to be genotoxic in the micronucleus test in NMRI mice. On the basis of this data it is concluded that musk ambrette should not be used in cosmetic products.

Other recommendations: IFRA - International Fragrance Association: CODE OF PRACTICE: „Musk ambrette should not be used in fragrance compound for cosmetics, toiletries and other products which under normal conditions of use will come into contact with the skin. This includes rinse-off products.“

Classification: D

MUSK KETONE

1. General

1.1 Primary name

Musk ketone

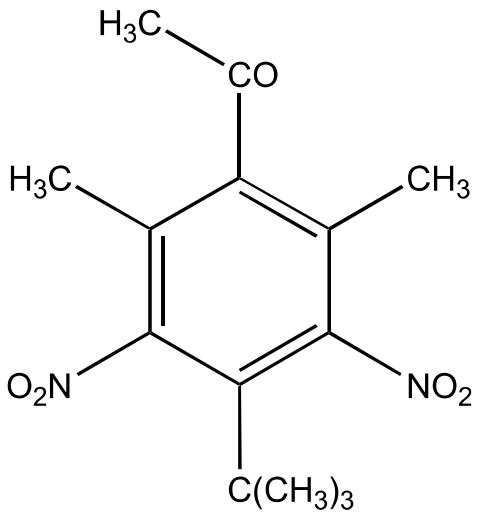
1.2 Chemical names

4-tert-butyl-3,5-dinitro-2,6-dimethylacetophenon

1.4 CAS no.

81-14-1

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{14}H_{18}N_2O_5$
Mol weight: 294

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}, rat, oral: >10 g/kg b. w.

Remarks: no original data available

3.2 Acute dermal toxicity

LD_{50}, rabbit, dermal: >10 g/kg b.w.

3.5 Repeated dose dermal toxicity

Musk ketone was tested by repeated applications once daily, 5d/wk for 3 wk, to the abraded and intact skin of albino rabbits at levels of 0 (control), 175 and 750 mg/kg b.w. Results: Slight to moderate erythema followed by slight desquamation was noted in the skin of all groups. Symptoms of disease and death in all groups occurred before termination of the study, but were not regarded to be compound-related. A variable decrease in bone marrow haematogenic activity in three of the anima on the higher dose occurred.

Remarks: No original data available.

Musk ketone was applied once daily, 5d/wk for 3 wk, to the abraded and intact skin of rabbits and levels of 0 (control), 175 and 750 mg/kg b.w. (groups of 6 rabbits). Results: There were no gross effects; cutaneous effects were minimal. Clinical chemistry studies showed a terminal compound-related increase of serum glutamic-pyruvic transaminase in five of six rabbits on the high level and in one on the low level.

Remarks: No original data available.

The abraded skin of groups of 14 albino rabbits was treated either with dimethyl phthalate in a dose of 1 mg/kg b.w./d or with musk ketone in dimethyl phthalate in daily doses of 10, 50 or 250 mg/kg b.w. on 20 consecutive days. Results: Six deaths in the high dosage group. Moderate to severe vacuolization of the hepatocytes typical of fatty change and serum glutamic-pyruvic transaminase activity was increased in the males. There were no other dose-related changes. It was concluded, that the no-effect-dose was > 50 mg/kg b.w./d.

Remarks: No original data available.

3.8 Subchronic dermal toxicity

Daily doses of 7.5, 24, 75 or 240 mg musk ketone /kg b.w./d in phenylethyl alcohol were dermally applied to 15 male and 15 female Sprague-Dawley Crl: CD^{R}(SD)BR albino rats for 90 d. The vehicle control group of 30 male and 30 female rats was treated with phenylethyl alcohol alone. Results: The body weights of males and females given the high dose of musk ketone and of the females given 75 mg/kg b.w./d were significantly lower than dose of the vehicle controls. The livers of males and females exposed to the high dose were increased in weight, but this was not associated with any clinical nor histopathological findings. The no-observed levels were 75 mg/kg b.w./d for males and females.

3.10 Chronic toxicity

Long-term toxicity/carcinogenicity study: No data available.

4. Irritation & corrosivity

4.1 Irritation (skin)

Musk ketone applied full strength to intact or abraded rabbit skin for 24 h under occlusion was not irritating.

Remarks: No original data available.

5. Sensitization

Skin: A maximization test was carried out on 25 volunteers.

The material was tested at a concentration of 3.2 % in petrolatum and produced no sensitization reactions. In another maximization test carried out on 25 volunteers, the material was tested at a concentration of 5 % in petrolatum and again produced no sensitization reactions.

Remarks: No original data available.

Photoirritation and Photosensitization: Dunkin-Hartley albino female guinea-pigs were used in groups of four for preliminary photo-irritation studies, and in groups of 12 for photoallergy tests. Musk ketone (0.1 ml) was applied to clipped and shaved interscapular skin and the guinea-pigs irradiated with 100 kJ m^{-2} UV. The procedure was repeated 24 h later. Ten to 14 d after induction the guinea-pigs were challenged using clipped and shaved lumber skin. Test and control animals were treated identically. Three concentrations (0.1, 1 and 10 %) of the test substance were applied (10 µl on 14 mm diameter skin). 30 min later the animals were irradiated with 100 kJ m^{-2} UV. After irradiation the test substance was applied to fresh skin sites to check contact sensitivity.

Results: Musk ketone was not irritant or photoirritant under the conditions studied. There was no contact sensitivity reaction. Photoallergic reactions were elicited in one out of 12 guinea-pigs at 10 and 1 % challenge concentration (second challenge).

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption: 28 % in rats.

Remarks: No original data available.

8. Mutagenicity

Musk ketone was tested using a preincubation modification of the *Salmonella*/microsome test in the absence of exogenous metabolic activation and in the presence of liver S-9 from Aroclor-induced male Sprague-Dawley rats and Syrian hamsters. Results: Musk ketone was not found to be mutagenic under the conditions of the test in the strains TA 100, TA 1535 and TA 98.

10. Special investigations

Concentration in human tissue: 22 human milk samples from 15 women and 13 human fat samples (surgery samples) were analysed for their musk ketone content. Results: The residues ranged from 0.01 to 0.09 mg/kg fat in human milk and from 0.01 to 0.05 mg/kg in human fat samples, 1 fat sample contained 0.22 mg musk ketone/kg fat. 391 human milk samples were analysed for their musk ketone content. Results: In almost all samples musk ketone was found with a mean content of 0.04 mg/kg fat, and a maximum content of 0.24 mg/kg fat.

11. Conclusions

Musk ketone has a very low acute toxicity. It is not toxic and not irritating. Musk ketone produces no sensitizing reactions. Musk ketone has only very weak photoirritant potential in guinea-pigs and causes no contact sensitivity reactions. The no-observed-effect-level determined in a 90 d dermal toxicity study in Sprague Dawley albino rats was 75 mg/kg b.w./d. Musk ketone was not mutagenic in the Salmonella Typhimurium test. There are no other mutagenicity/genotoxicity tests carried out. Musk ketone is regarded to be readily absorbed through skin. There are no other data on toxikokinetics and no data on teratogenicity.

There is no evidence that musk ketone is carcinogenic. Based on the toxicological data mentioned above no definite evaluation of musk ketone can be made. However, in view of the similar chemical structure with musk xylene and the evidence for dermal absorption and presence in human milk and fat samples, a use of musk ketone in cosmetic products cannot be accepted at present.

Classification: D

MUSK MOSKENE

1. General

1.1 Primary name

Musk moskene

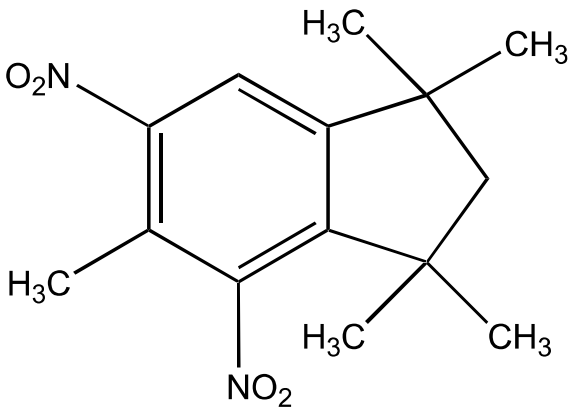
1.2 Chemical names

4,6-dinitro-1,1,3,3,5-pentamethylindane
IUPAC name: 1,1,3,3,5-pentamethyl-4,6-dinitroindane

1.4 CAS no.

116-66-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{14}H_{18}N_2O_4$
Mol weight: 278.34

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}, rat, oral: >5g/kg b.w.

Remarks: no original data available

3.2 Acute dermal toxicity

LD_{50}, rabbit, dermal: > 5g/kg b.w.

3.8 Subchronic dermal toxicity

Daily doses of 7.5, 24 or 75 mg moskene/kg b.w./d in phenylethyl alcohol were dermally applied to 15 male and 15 female Sprague Dawley Crl:CD^{R}(SD)BR albino rats for 90 d. The vehicle control group of 30 male and 30 female rats was treated with phenyl ethyl alcohol alone.

Results: There was statistically significant increases in relative, but not in absolute, weights of the liver and kidneys of males in the high-dose groups, but these were not associated with histopathological changes. The no-observed-effect-levels were 24 mg/kg b.w./d for males and 75 mg/kg b.w./d for females.

3.10 Chronic toxicity

Long-term toxicity/carcinogenicity study: No data available.

4. Irritation & corrosivity

4.1 Irritation (skin)

Musk moskene applied full strength to intact or abraded rabbit skin for 24 h under occlusion was moderately irritating. Tested at 10 % in petrolatum, it produced no irritation after a 48 h closed-patch test on human subjects. Remarks: no original data available.

5. Sensitization

A maximization test was carried out on 25 volunteers. The material was tested at a concentration of 10 % in petrolatum and produced no sensitization reactions. A guinea-pig maximization test using the Maguire method was carried out on eight guinea-pigs using moskene at 10 % and produced no sensitization reactions. Remarks: No original data available.

Photoirritation and photosensitization: Dunkin-Hartley albino female guinea-pigs were used in groups of four for preliminary photoirritation studies, and in groups of 12 for photoallergy tests. Musk moskene (0.1 ml) was applied to clipped and shaved interscapular skin and the guinea-pigs irradiated with 100 kJ m^{-2} UV. The procedure was repeated 24 h later. Ten to 14 d after induction the guinea-pigs were challenged using clipped and shaved lumbar skin. Test and control animals were treated identically. Three concentrations (0.1, 1 and 10 %) of the test substance were applied (10 µl on 14 mm diameter skin). 30 min later the animals were irradiated with 100 kJ m^{-2} UV. After irradiation the test substance was applied to fresh skin sites to check contact sensitivity.

Results: Musk moskene was not irritant or photoirritant under the conditions studied. There was no contact sensitivity reaction. Musk moskene caused photoallergic reactions in 3 out of 12 guinea pigs at concentrations of 1 and 10 % challenge concentration. Reactivity increased at second challenge, and all 3 reacted to the lowest concentration tested (0.1 %).

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

No data available.

8. Mutagenicity

No data available.

10. Special investigations

Concentration in human tissue: 22 human milk samples from 15 women and 13 human fat samples (surgery samples) were analysed for their musk moskene content. Results: In a few samples residues of musk moskene were detected at low levels (< 0.01 ppm).

11. Conclusions

Musk moskene has a very low acute toxicity. It is not irritating and produces no sensitizing reaction in a maximization test on human volunteers. Musk moskene is a weak photoallergen for guinea-pigs and causes no contact sensitivity reaction. The no-observed-effect-level in a 90 d dermal toxicity study on Sprague-Dawley albino rats was 24 mg/kg b.w./d for males and 75 mg/kg b.w./d. for females. There are no data on mutagenicity/genotoxicity, long-term-toxicity/carcinogenicity, no data on teratogenicity, no data on toxikokinetics and dermal absorption.

On the basis of this data no definite evaluation can be made. However, in view of the lacking toxicological data and based on the similar chemical structure with musk xylene, it is recommended that a use of musk moskene in cosmetic products cannot be accepted at present, especially not for use on sun-exposed skin.

Classification: D

MUSK TIBETENE

1. General

1.1 Primary name

Musk tibetene

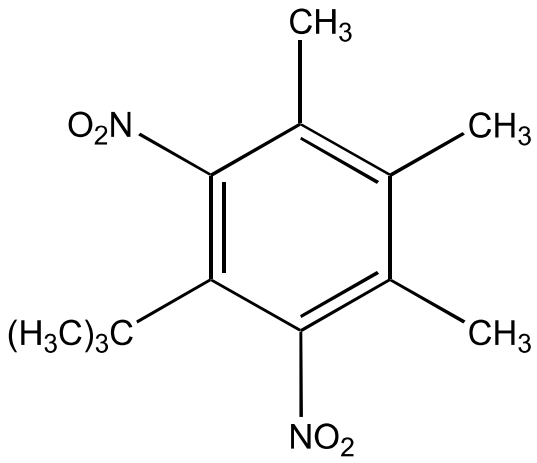
1.2 Chemical names

1-tert-butyl-2,6-dinitro-3,4,5-trimethylbenzene
IUPAC name: 5-tert-butyl-1,3,5-trimethyl-4,6-dinitrobenzene

1.4 CAS no.

145-39-1

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{13}H_{18}N_2O_4$
Mol weight: 266.33

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50}, rat, oral: >6g/kg b.w.

Remarks: No original data available.

3.2 Acute dermal toxicity

LD_{50}, rabbit, dermal: > 5g/kg b.w.

3.8 Subchronic dermal toxicity

Daily doses of 7.5, 24 or 75 mg musk tibetene/kg b.w./d in phenylethyl alcohol were dermally applied to 15 male and 15 female Sprague Dawley Crl:CD^{R}(SD)BR albino rats for 90 d. The vehicle control group of 30 male and 30 female rats was treated with phenylethyl alcohol alone.

Results: The no-observed-effect-levels were 75 mg/kg b.w./d for males and females.

4. Irritation & corrosivity

4.1 Irritation (skin)

Musk tibetene applied full strength to intact or abraded rabbit skin for 24 h under occlusion was not irritating.

Remarks: No original data available.

4.2 Irritation (mucous membranes)

Musk tibetene produced slight conjunctival irritation on the rabbit eye, which disappeared within 72 h.

Remarks: No original data available.

5. Sensitization

Photoirritation and photosensitization: Dunkin-Hartley albino female guinea-pigs were used in groups of four for preliminary photo-irritation studies, and in groups of 12 for photoallergy tests. Musk tibetene (0.1 ml) was applied to clipped and shaved interscapular skin and the guinea pigs irradiated with 100 kJ m^{-2} UV. The procedure was repeated 24 h later. Ten to 14 d after induction the guinea-pigs were challenged using clipped and shaved lumbar skin. Test and control animals were treated identically. Three concentrations (0.1, 1 and 10 %) of the test substance were applied (10 µl on 14 mm diameter skin). 30 min later the animals were irradiated with 100 kJ m^{-2} UV. After irradiation the test substance was applied to fresh skin sites to check contact sensitivity.

Results: Musk tibetene was not irritant or photoirritant under the conditions studied. There was no contact sensitivity reaction. Photoallergic reaction was elicited in one out of 12 guinea-pigs at 10 % challenge concentration.

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

No data available.

8. Mutagenicity

No data available.

9. Carcinogenicity

Long-term toxicity/carcinogenicity study: No data available.

10. Special investigations

Concentration in human tissue: 22 human milk samples from 15 women and 13 human fat samples (surgery samples) were analysed for their musk tibetene content.

Results: Musk tibetene could not be detected at all.

11. Conclusions

Musk tibetene has a very low acute toxicity and is not irritant. Musk tibetene produces conjunctival irritation in the rabbit eye. Musk tibetene shows no sensitization reaction in humans. Musk tibetene is not photoirritating and has only very weak photosensitization potential in guinea-pigs. The no-observed-effect-level was 75 mg/kg b.w./d for male and female Sprague Dawley albino rat determined in a 90 d dermal toxicity study. There are no data on mutagenicity/genotoxicity, long-term-toxicity/carcinogenicity, teratogenicity, toxikokinetics and dermal absorption. Based on the data mentioned above no definite evaluation of musk tibetene can be made. However, in view of the similar chemical structure with musk xylene and the lacking toxicological data, it is concluded that a use of musk tibetene in cosmetic products cannot be accepted at present.

Classification: D

MUSK XYLENE

1. General

1.1 Primary name

Musk xylene

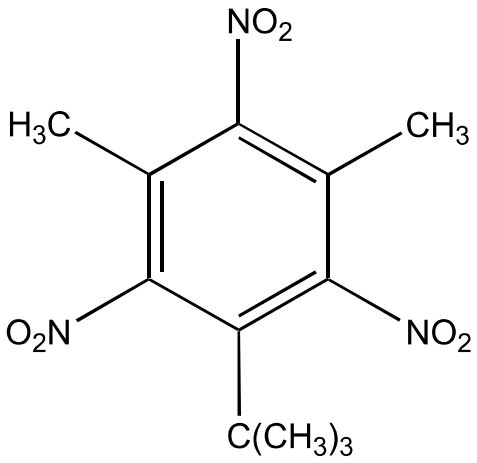
1.2 Chemical names

1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene
1-(1,1-dimethylethyl)-3,5-dimethyl-2,4,6-trinitrobenzene
5-tert-butyl-2,4,6-trinitroxylene
musk xylol

1.4 CAS no.

81-15-2
EINECS No.: 201-329-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{12}H_{15}N_3O_6$
Mol weight: 297.27

TOXICOLOGICAL CHARACTERISATION

3.1. Acute oral toxicity

LD_{50}, rat, oral: >10g/kg b.w.

Remarks: No original data available.

LD_{50}, mice, oral: >4g/kg b.w. (Maekawa 1990)

Remarks: 1/10 died (female given 4g/kg b.w.).

3.2 Acute dermal toxicity

LD_{50}, rabbit, dermal: >15 g/kg b.w.

3.4 Repeated dose oral toxicity

Musk xylene was fed ad lib. to groups of eight male and eight female SPF $B6C3F_1$ mice at concentrations of 0 (control), 0.3, 0.6, 1.25 or 5% in the diet for 14 d.

Results: All of the mice given ≥ 0.6 % musk xylene in the diet (approximately 0.9 g/kg b.w./d) died after 2-4 days of treatment, except for one female in the 0.6 % group. In contrast, all of the mice in the 0.3 % (approximately 0.45 g/kg b.w./d) and control groups survived to the end of the study. No toxic lesions specifically caused by musk xylene were noted in the brain or other organs.

3.7 Subchronic oral toxicity

Musk xylene was fed ad lib. to groups of ten male and ten female SPF $B6C3F_1$ mice at concentrations of 0 (control), 0.0375, 0.075, 0.15, 0.3 or 0.6 % in the diet for 17 wk.

Results: All of the mice given 0.6 % (approximately 0.9 g/kg b.w.) musk xylene in the diet, and eight males and all of the females given 0.3 % (approximately 0.45 g/kg b.w./d) died during the study. No significant differences in body weight or food intake or regarding organ weights were seen between the treated groups given ≤ 0.15 % (approximately 225 mg/kg b.w./d) and the control groups and although the absolute and relative liver weights were increased slightly in all treated groups except the 0.075 % males, the increases were not dose related. Histologically, enlargement and irregularity of liver cells were observed in male and female mice fed 0.15 % musk xylol.

3.8 Subchronic dermal toxicity

Daily doses of 7.5, 24, 75 or 240 mg musk xylene/kg b.w./d in phenylethyl alcohol were dermally applied to 15 male and 15 female Sprague Dawley Crl:CD^R(SD)BR albino rats for 90 d. The vehicle control group of 30 male and 30 female rats was treated with phenylethyl alcohol alone.

Results: The only effects of application were significant increases in relative and absolute liver weight at the higher doses, but these were not associated with histopathological changes. The no-effect-observed-levels were 75 mg/kg b.w./d for males and 24 mg/kg b.w./d for females.

4. Irritation & corrosivity

4.1. Irritation (skin)

Musk xylol applied full strength to intact or abraded rabbit skin for 24 hours under occlusion and was not irritating. Tested at 5 % in petrolatum, it produced a mild irritation after a 48 h closed-patch test on human subjects.

Remarks: No original data available.

5. Sensitization

A maximization test was carried out on 25 volunteers. The material was tested at a concentration of 5 % in petrolatum and produced no sensitization reactions.

Remarks: No original data available.

Photoirritation and Photosensitization: Dunkin-Hartley albino female guinea-pigs were used in groups of four for preliminary photoirritation studies, and in groups of 12 for photoallergy tests. Musk xylene (0.1 ml) was applied to clipped and shaved interscapular skin and the guinea-pigs irradiated with 100 kJ m^{-2} UV. The procedure was repeated 24 h later. Ten to 14 d after induction the guinea-pigs were challenged using clipped and shaved lumbar skin. Test and control animals were treated identically. Three concentrations (0.1, 1 and 10 %) of the test substance were applied (10 µl on 14 mm diameter skin). 30 min later the animals were irradiated with 100 kJ m^{-2} UV. After irradiation the test substance was applied to fresh skin sites to check contact sensitivity. Results: Musk xylene was not irritant or photoirritant under the conditions studied. There was no contact sensitivity reaction. Photoallergic reaction was elicited in one out of 12 guinea-pigs at 10 % challenge concentration.

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

Single topical application of ^{14}C-musk xylene (0.5 mg/kg b.w.) in a mixture of phenylethyl alcohol and ethanol (1mg/ml) to 21 adult male rats (16 CD Sprague-Dawley, 5 Long-Evans), application rate 0.01 mg/cm^2. Results: About 8 % of the applied dose was absorbed during 6 h; after this time the remaining dose was washed off. Approximately 14 % of the dose remained on the skin after washing which continued to be absorbed, a total of about 20 % of the dose being absorbed during 48 h with 2 % remaining on the skin. All the absorbed radioactivity was excreted during 5 d. Radioactivity was detected in nearly all the tissues of animals killed up to 24 hours. Concentrations were highest at 8 h and then declined. Highest concentrations were present in liver, fat, pancreas, kidneys and gastro-intestinal tract. One metabolite, a glucuronic acid conjugate of hydroxymethyl musk xylene, was the main metabolite in the bile (>50 %).

Single topical application of ^{14}C-musk xylene (mixed with nonradioactive material to a specific activity of 50.4 µCi/mg) in a mixture of phenylethyl alcohol and ethanol (1mg/ml) to two human volunteers (1mg per subject), application rate was 0.01 mg/cm^2. Results: ^{14}C-musk

xylene was very poorly absorbed in man; 90 % of the applied dose was recovered from the site of application after 6 h. After 120 h a mean of 0.26 % of the dose had been excreted in the urine and faeces.

Single oral dose of 70 mg/kg b.w. ^{3}H-musk xylene in 0.5 ml olive oil to three male Wistar rats, oral doses of 200 mg musk xylene/kg b.w. given consecutively for 2 wk to six male Wistar rats. Results: Urinary and fecal excretion accounted for 10 and 75 % of the single dose (70 mg/kg b.w.), respectively, on day 7 after application. Total residue of radioactivity in tissues on day 7 was less than 2.0 % of the administered dose. The highest concentration was found in adipose tissue and the second was in liver. The major route of excretion for musk xylene was the faeces via bile. The reduction of the 2-nitro group to the amino group was a key step in metabolism.

Male Wistar rats were injected i.p. for five consecutive days with either 0 (control), 50, 100 or 200 mg musk xylene in corn oil/kg b.w. and were starved 24 h prior to killing. The livers were homogenized and P-450 and cytochrom b_5 levels were determined. Results: Musk xylene increased both the total P-450 content and the cytochrome b_5 content about 1.4 and 1.5-fold, respectively. Musk xylene induced P-450IA2 strongly and preferentially and the ratio of P450IA2/P-450IA1 was about 12 at the lowest dose tested.

Male Wistar rats were injected i.p. for five consecutive days with either 0 (control), 50, 100 or 200 mg musk xylene in corn oil/kg b.w. and were starved 24 h prior to killing. The livers were homogenized and the enzyme activities for Phase I and Phase II drug-metabolizing enzymes were determined using kinetic and immunochemical methods. Results: Musk xylene induces both Phase I cytochrome P450 mixed-function oxidase (CYP1A2 specific) and Phase II metabolizing enzyme systems (DT-diaphorase, GST Ya subunit and UDPGT).

8. Mutagenicity

Musk xylene was tested for mutagenic activity in the *in vitro* Salmonella/rat liver microsome plate incorporation assay (Ames test). Results: The compound does not induce mutations in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 100 and TA 98 at concentrations up to 200 µg/plate, in the presence and in the absence of a rat liver homogenate fraction.

Musk xylene was assayed for mutagenicity in the *S. typhimurium* strains TA 100 and TA 98 with and without a rat-liver S-9 activation system at concentrations of 50, 100, 250 and 500 µg/plate. Results: Musk xylene was not found to be mutagenic.

Musk xylene was tested in the chromosome aberration assay using Chinese hamster ovary cells. The assay was conducted both in the absence of an Aroclor-induced S-9 activation system at dose levels of 2.5, 5, 10, 20 and 40 µg/ml and in the presence of an Aroclor-induced S-9 activation system at dose levels of 1.9, 3.8, 7.5, 15 and 30 µg/ml. Results: No increase in chromosome aberrations was observed in either the non activated or S-9 activated test system. It is concluded that musk xylene is negative in the CHO cytogenetics assay.

Musk xylene was tested in the L5178Y TK+/-Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor induced rat liver S-9. The non-activated cultures selected for cloning were treated with doses of 400 to 20 µg/ml and exhibited Total Growths from 8 % to 78 %. The S-9 activated cultures selected for cloning were treated with doses of 125 to

10 µg/ml which produced from 9 % to 113 % Total Growth. Results: None of the non-activated and activated cultures that were cloned exhibited a mutant frequency which was at least twice the mean mutant frequency of the solvent controls. A dose-dependent response was not noted in the treated cultures.

Musk xylene was tested in the Unscheduled DNA Sythesis Test using primary cultures of rat hepatocytes. Musk xylene was tested at nine dose levels ranging from 150 to 0.5 µg/ml and was fully evaluated at five dose levels of 30, 15, 10, 5.0 and 1.0 µg/ml. Results: Musk xylene did not cause a significant increase in the mean number of net nuclear grain counts, at any dose level.

9. Carcinogenicity

Long-term toxicity/carcinogenicity study: Musk xylene was fed ad lib. to groups of 50 male and 50 female SPF B6C3F$_1$ mice at concentrations of 0 (control), 0.075 or 0.15 % in the diet for 80 wk, that was 170 and 91 mg/kg b.w./d for males and 192 and 101 mg/kg b.w./d for females given 0.15 or 0.075 %, respectively. Results: The overall tumour incidences in all treated groups of both sexes were significantly higher than those in the corresponding controls. Malignant and benign liver cell tumours were clearly increased (adenomas: males 9/19/20, females 1/14/13; carcinomas: males 2/8/13). In males the incidence of Harderian gland tumours was also significantly greater in both treated groups than in controls (2/9/10).

10. Special investigations

Concentration in human tissue: 22 human milk samples from 15 women and 13 human fat samples (surgery samples) were analysed for their musk xylene content. Results: The residues ranged from 0.02 to 0.18 mg/kg fat in the human milk and from 0.02 to 0.22 mg/kg in human fat samples. In addition, 37, 314 and 391 human milk samples were analysed for their musk xylene content. Results: In 30 (81 %), 264 (84 %) and 391 (100 %) samples, respectively, musk xylene was found. The highest content was 1.17, 0.33 and 1.22 mg musk xylene/kg fat, respectively. Mean content of the investigation with 391 samples was 0.1 mg musk xylene/kg fat in human milk.

11. Conclusions

Musk xylene has a very low acute toxicity. It is mild irritating under occlusion on humans, it is not irritating on rabbit skin. Musk xylene is not sensitizing on humans and has only very weak photoallergic potential in the guinea-pig. It has no contact sensitivity potential. In a 17 wk feeding study in mice there was a no-observed-effect-level of 0.075 % determined, approximately 110 mg/kg b.w./d. The no-observed-effect-level determined in a 90 d-dermal toxicity study on Sprague-Dawley albino rats was 75 mg/kg b.w./d for males and 24 mg/kg b.w./d for females.

It was demonstrated that musk xylene was carcinogenic in B6C3F$_1$ mice when given at dose levels of 0.075 % or 0.15 % in the diet for 80 wk. Musk xylene has no genotoxic potential *in vitro*. Musk xylene applied once dermally to the skin of male rats (application time 6 h) has been absorbed to an extent of 20 % of the applied dose during 48 hours. 85 % of an oral dose

was excreted within 7 d. The major route of excretion was the faeces via bile. Musk xylene is a P450AIA2 inducer in male Wistar rats.

There is evidence that musk xylene is a non-genotoxic carcinogen in mice. For final risk assessment further studies are needed, e.g. a carcinogenicity study in rats and/or mechanistic studies in mice. In addition to the carcinogenicity in one species musk xylene is readily absorbed through the skin and present in human milk and human fat samples. Therefore, it is concluded that musk xylene should not be used in cosmetic products.

Classification: D

USE OF BORIC ACID AND BORATES IN COSMETICS

The SCC have been asked to give an opinion on the analytical problems relating to the use of boric acid and borates in cosmetic products and also specifically on the oral hygiene use in view of recent data on reproductive toxicity.

These 2 aspects are considered separately below.

ANALYTICAL QUESTIONS

It is reasonable to include salts of boric acid in the Annex III entry namely, boric acid, borates and tetraborates.

Regarding analytical methods it is possible to determine free boric acid, total boron levels, and hence to calculate levels of both boric acid and borates.

The amount of each present will be pH dependent. The distribution as a function of pH together with an analytical strategy is shown in Fig 1.

The extent of absorption through undamaged skin will also be pH dependent since it is the free acid that is absorbed, and gives rise to concern in this regard. In all formulations a warning not to use on damaged skin is appropriate.

Boric acid in talc is not absorbed through healthy skin due to the formation of calcium metaborate. Formulations containing borates (i.e. under alkaline or neutral conditions) would be expected to be poorly absorbed. Acidic formulations contain predominantly free boric acid and are known to be well absorbed through the skin. The use of boric acid, borates and tetraborates should thus be limited to products that are neutral or slightly alkaline, except in the case of talc.

In order to have confidence in the lack of significant absorption through normal skin it may be necessary to specify a limit on the free boric acid content of neutral and alkaline formulations of borates and tetraborates in cosmetic products for skin application. In order for such a value to be identified more detailed information on the extent of skin absorption from such neutral formulations would be needed.

A tentative Annex III listing is attached.

BORIC ACID AND BORATES IN COSMETIC PRODUCTS

SUMMARY

Possible boron species distribution in aqueous solution as a function of pH

	Boric acid	Borates	Metaborate
Acid pH (below pH 5.0)	Boric acid	Boric acid	Boric acid
Neutral pH	Mixture of boric acid, borates and metaborate at different relative concentration levels. (boric acid and relative concentration: about 40 %)		
Alkaline pH (above pH 9.0)	Metaborate	Some borates Metaborate (mainly)	Some borates Metaborate (mainly)

Tentative analysis methodologies on solutions containing boron compounds

Formulations at acid pH:	1 ° Free boric acid determination. 2 ° To assess the possible presence of borate salts.
Formulations at neutral pH:	1 ° Free boric acid determination. 2 ° Total boron determination (boric acid + borates). 3 ° To quantify separately boric acid and borates (expressed as boric acid equivalent) in order to know the contribution of each one.
Formulations at alkaline pH:	1 ° Boron determination (borates and metaborates), expressed as boric acid equivalent.

Dermal absorption studies

Formulation at acid pH:	Mainly due to boric acid existence.
Formulation at neutral pH or slight alkaline pH:	Some studies on the possible transformation from borates to boric acid should be carried out. Depending of the extension level of such transformation, a concentration limit will be indicated for boric acid, borates or a global figure for both.

ANNEX III

First Part

		Restrictions			
Order	Substance	Field of application and or use	Maximum concentration in finishing product	Other limitations	Use conditions and Precautions
a	b	c	d	e	f
1a	Boric acid, Borates and Tetraborates	a) Talcum powder b) Products for oral hygiene c) Other products (except bath products and products for waving hair)	a) 5 % (expressed as boric acid) b) 0.5 % (expressed as boric acid) c) 3 % (expressed as boric acid)	a) - Do not use for care children - Not to be used on damaged skin b) and c) When boric acid solutions are used, the pH value must be neutral or slightly alkaline	a) - Do not use for care children under three years of age - Not to be used on damaged skin c) Not to be used on damaged skin for products to be applied to the skin if the free-soluble-borate/boric acid concentration, expressed as boric acid exceeds x % (depending on percutaneous absorption)
1b	Tetraborates	a) Bath products b) Products for waving hair	a) 18 % b) 18 %	a) and b) The solution pH must be neutral or slightly alkaline	a) Do not use for bathing children under three years of age b) Rinse thoroughly

USE OF BORIC ACID IN ORAL HYGIENE PRODUCTS

Recent data on the effects of boric acid on the reproductive system (testicular toxicity and developmental toxicity) have prompted a request to review these data, and to consider the adequacy of the safety margins arising from the oral hygiene use. Since boric acid in talc is not absorbed through normal skin, such use does not give rise to concern.

EFFECTS OF BORON (AS BORIC ACID OR BORAX) ON THE REPRODUCTIVE SYSTEM

Effects on male fertility

There are a number of reports in the literature of adverse effects of boron on male fertility.

Limited details are available on studies in Eastern Europe (Russia) showing reduced testicular weight and sperm count in rats exposed to drinking water containing 6 ppm boron for 6 months, the NOAEL being 0.3 ppm in the drinking water. Interest in such studies was prompted by claims of a high incidence of male infertility in certain parts of the USSR where boron levels in drinking water were 0.4 - 1.2 mg/l but few details are available. However, attempts to confirm the Russian studies in rats failed, with no effects being seen in the gonads of animals given 0.3, 1 and 6 ppm boron (as borax) in drinking water for up to 90 days.

Short-term (14 day) exposure of rats to high levels of boron (1 gram/kg as boric acid)

resulted in marked testicular toxicity (atrophy, severe degenerative changes).

An earlier extensive series of studies on the effect of boron (given as either boric acid or borax) to rats and dogs, involving both 90 day and 2 year repeated dose studies, and reproductive studies, has clearly indicated effect on male fertility. In the 90 day study in rats boron levels in the diet of 1750 and 5210 ppm (as boric acid or borate) produced signs of general toxicity (reduced weight gain, skin lesions) as well as degenerative changes in the testis. The NOAEL was 525 ppm boron. In 2 year studies the NOAEL was 325 ppm boron in the diet with testicular damage at 1170 ppm. In a reproductive toxicity study no effects were seen at 117 and 350 ppm on fertility, lactation, litter size and development. Similar effects were seen in dogs. In a 2 year study no effects were seen on the testis at dietary levels of 58-350 ppm boron but marked toxicity was seen at 1750 ppm boron. In neither the rat nor the dog studies were dose levels given on a mg/kg body weight basis. However, the NOAEL in the dog (350 ppm boron in diet) is equivalent to a dose level of the order of 10 mg boron/kg body weight/day. Similarly the NOAEL in the chronic study in the rat, 325 ppm boron in the diet, is equivalent to a dose level of the order of 20 mg boron per kg body weight per day.

The same group of workers more recently have published a subchronic study in rats specifically to investigate testicular damage. Animals were fed 500, 1000 and 2000 ppm boron (as borax) in the diet for 30 and 60 days. No significant adverse effects were seen at 500 ppm. At 1000 ppm and above dose related effects on the testis were observed (reduced weight, degenerative changes). Infertility was shown to be persistent for at least 8 months indicating prolonged germ cell depletion. The no effect level was 500 ppm in the diet. No data were provided on the daily dose in mg/kg body weight but this is estimated to be of the order of 30 mg boron/kg/day.

Recently full details have been published of a reproductive toxicity study in mice using a continuous breeding protocol. Male and female mice were exposed to boric acid in the diet for 27 weeks at levels of 1000, 4000 and 9000 ppm, stated to be equivalent to 160, 636 and 1262 mg/kg boric acid body weight/day. Marked effects on fertility were seen at 4000 ppm (reduced to 5 % control value in later stages of study) and 9000 ppm (sterility at all time points). No significant effects on fertility were seen at the lowest dose. The only effect reported in this group was a slight reduction in sperm motility, but this did not affect fertility, a significant reduction in sperm motility. A crossover mating trial of the controls and 4000 ppm group confirmed that reduced fertility was solely due to affects in the males. The NOAEL in this study was 160 mg boric acid/kg for affects in the males; this was equivalent to 27 mg boron/kg body weight/day.

Studies to investigate the mechanism of action of boron as a testicular toxin have been reported using short-term exposure (up to 4 weeks) to high levels of boron (9000 ppm). Under these conditions the first effect seen was a reduction in basal serum testosterone levels from day 4 with an inhibition of spermiation from day 7. Widespread exfoliation of apparently viable germ cells and pachytene cell death appeared during the 2nd week. Extreme epithelial disorganisation and germ cell loss was noted after 28 days. There was no evidence of any accumulation of boron in the testis.

Summary; effects on male fertility

Ingestion of boron, either as boric acid or borax has produced severe testicular toxicity in both rats and dogs. The NOAEL in the rat after sub-chronic (90 day) dietary exposure was 500 ppm boron, estimated to be equivalent to a dose of the order of 30 mg boron/kg body weight per day. In chronic studies in the rat the NOAEL was 325 ppm boron in the diet equivalent to a dose of the order of 20 mg boron/kg body weight per day. In 2 year studies in the dog the NOAEL was 350 ppm boron in the diet, equivalent to a dose of the order of 10 mg boron/kg body weight per day.

DEVELOPMENTAL (TERATOGENICITY) STUDIES

The teratogenic potential of boric acid has recently been investigated in rats and mice.

Pregnant rats were given boric acid in the diet at 1000, 2000 and 4000 ppm throughout gestation and also at 8000 ppm on day 6-15 of gestation. These levels were estimated to be equivalent to 78, 163, 330 and 539 mg/kg boric acid body weight per day. Animals were sacrificed and the uteri and contents examined on day 20. A significant reduction in maternal weight gain was seen at 330 mg/kg and above, with histological evidence of nephrotoxicity and hepatoxicity in maternal animals at 163 mg boric acid/kg/day and above. Regarding effects on the developing offspring, the percentage of resorption and fetal deaths was increased at 539 mg/kg. No significant effects were seen on litter size or viability at the other dose levels. An increase in gross malformations was seen at 330 mg/kg and above, including abnormalities of the eye, CNS and cardiovascular system. An increase in skeletal malformations was seen at 163 mg/kg. Total body weight was significantly reduced at all dose levels and in addition there was an increase in the number of litters with one or more affected implants (non live implants plus fetal abnormalities) at the lowest dose level. The NOAEL in this study for effects on maternal animals was 78 mg boric acid/kg body weight per day but a NOAEL was not

identified for effects on the offspring since some adverse effects were seen at the lowest dose level investigated namely 78 mg boric acid/kg/day or 14 mg boron/kg/day.

Summary of Developmental Studies

In rats given boric acid in the diet on day 6-15 of gestation the NOAEL for effects on the maternal animals was 78 mg boric acid/kg body weight (equivalent to 14 mg boron). This dose level however produced slight adverse effects on the developing offspring (limited to reduced fetal weight). A NOAEL was not identified for adverse effects on the developing fetus in the rat. In studies in mice the NOAEL for effects on the developing offspring was 248 mg boric acid/kg body weight. This is equivalent to 43 mg boron/kg body weight. This dose produced slight effects on the maternal animals.

Thus 14 mg boron/kg was a marginal effect level in the rat.

ESTIMATION OF EXPOSURE AND SAFETY MARGINS

Exposure from oral hygiene use

Boric acid is permitted for use in oral hygiene products at concentrations up to 0.5 %.

Based on data recently provided by COLIPA on usage, the following exposures for an average and an extensive user are anticipated.

Product	Typical quality per application	Frequeny per day	Exposure per day normal	extensive
toothpaste	1.4 g	1-2	1.4 g	2.8 g
mouthwash	10 g	1-5	10 g	50 g

Assuming 1 gram of mouthwash (10 %) and 0.24 g (17 %) of a toothpaste, is swallowed total ingestion of an extensive user is to 5.48 grams of product. Assuming that all products used contain the maximum amount of boric acid (0.5 %) this is equivalent to 27.4 mg boric acid or 4.66 mg boron.

$$\text{Thus total daily dose} = \frac{4.66 \text{ mg boron/kg}}{60} = 0.078 \text{ mg boron/kg}$$

SAFETY MARGINS

Based on male fertility studies, and taking the NOAEL in the 2 year study in dogs is the critical effect, namely 10 mg boron per kg per day.

$$SM = \frac{10}{0.078} = 128$$

Based on developmental effects

Assuming a NOAEL of 1.4 mg boron per kg for developmental effects (since 14 mg/kg was a marginal effect level).

$$SM = \frac{1.4}{0.078} = 18$$

It is suggested that this is unacceptable.

However, if the maximum permitted concentration was reduced to 0.1 %, the SM would then be 90. This is acceptable [the similar metabolic profile of boric acid and borates across species would support the acceptance of a SM somewhat below 100].

It is thus recommended that the maximum in use concentration for oral hygiene products be reduced to 0.1 %.

THE USE OF GLYCERYL MONOTHIOGLYCOLATE

Introduction

Currently, thioglycollic acid esters may be used at up to 8 %, for general use and 11 % for professional use. There is a requirement that products containing the esters should be labelled with the caution:

"May cause skin sensitization in the event of skin contact; Wear suitable gloves." (Annex 111, Part 1; OJ C322/46).

Glyceryl monothioglycolate, used in 'acid' permanent waving products, does occasionally sensitise consumers, but it is more usually considered as an occupational hazard for the hairdresser. Glyceryl monothioglycolate can persist in permanent-waved hair for up to 3 months.

The compound is unstable at room temperature, either in water or petrolatum, and has a half life of about 1 year. Prepared dilutions for patch testing (allergy testing) should be refrigerated. The recommended patch test dilution is 1 %.

The Problem of Contact Sensitivity

The frequency of hypersensitivity (allergy of Type 1V type) to glyceryl monothioglycolate in the *general* population, *unselected* clients of hairdressers (end users) or hairdressers themselves, is not known.

However, there is considerable information on the incidence of hypersensitivity to the compound in hairdressers presenting for investigation of a dermatitis (eczema) from which they have been suffering. From within this context of the evaluation of individuals *with* eczematous skin conditions, the following published data can be extracted:

The German Contact Dermatitis Research Group has reported a rate of sensitisation to glyceryl monothioglycolate at 38 % in 87 hairdressers with dermatitis. lt should be noted that the principal author of this paper also provided the data for Dortmund in the multicentre study discussed later.

The Italian Contact Dermatitis Research Group has published their results on a panel of 302 hairdressers with contact dermatitis collected from 9 Italian centres. 24 % reacted to p-phenylenediamine and 11 % to glyceryl monothioglycolate.

To obtain data on the frequency of sensitization among European hairdressers, the patch test results from 9 centres were reviewed. 8 allergens recommended by the European Environmental and Contact Dermatitis Research Group (EECDRG) in the hairdressing series of contact allergens and p-phenylenediamine (PPD) in the standard European series were used to patch test 809 hairdressers and 104 clients for evaluation of suspected allergic contact dermatitis (i.e., presenting for investigation of an eczematous skin and, therefore, a highly selected group) (Table 1).

In this pan-European study, among hairdressers with suspected contact dermatitis the mean frequencies of sensitization ranked as follows:

glyceryl monothioglycolate	19 %
p-phenylenediamine	15 %
ammonium persulphate	8 %
ammonium thioglycolate	4 %

The frequency of sensitization showed marked regional variations but account must be taken of the bias in the selection of individuals for testing. Thus, in Dortmund, the German centre, 28/55 (50.9 %) of hairdressers tested were allergic to glyceryl monothioglycolate but most of these hairdressers were tested *"as part of an expert opinion in claims for legal compensation".* In a series of 416 hairdressers presenting for investigation of a hand eczema at the Institute of Dermatology, London 78/416 (18.80 %) were found to be allergic to the compound.

Glyceryl monothioglycolate is a major contact sensitiser for hairdressers in Europe. Sensitization is at least as frequent as to p-phenylenediamine.

Glyeryl monothioglycolate poses a higher risk of sensitization to hairdressers than the alkaline ammonium thioglycolate used since the 1940s. The lower figures for sensitization to glyceryl monothioglycolate in some centres may be explained by lower usage in salons *or* by more careful handling. In Denmark, most hairdressers wear gloves when dyeing and permanent waving. In Germany, most hairdressers protect their hands only against hair dyes. There is still a strong prejudice against the use of gloves in this occupation. This attitude was confirmed in Italy: only 12,5 % of 240 hairdressers wore gloves for permanent waving, whereas 51 % wore them for hair dyeing.

In a series of 261 hairdressers' clients with a dermatitis *suspected* of being related to use of hair dyes or permanent wave solutions in Italy, only 49 were patch tested. Within this subgroup of 49 individuals, allergy to glyceryl monothioglycolate occurred in 3 % but 7 % were allergic to p-phenylenediamine and allergy to nickel sulphate was shown in 26.5 %.

Data from the pan-European study on 104 hairdressers' clients investigated for dermatitis (and all of whom were patch tested) shows a rate of hypersensitivity of 5.8 % for glyceryl monothioglycolate but 19.2 % for p-phenylenediamine.

Summary

Glyceryl monothioglycolate is an important occupational allergen. Better occupational hygiene precautions should help to reduce the incidence of hypersensitivity to the compound in hairdressers.

Hairdressers need to be instructed to handle this type of permanent wave with greater care and follow the directions already in legal force. Direct skin contact should be avoided. Gloves and improved handling technique may lead to a decrease in the frequency of sensitization.

For the hairdressers' clients there is no data to suggest that there is an unacceptable risk of the acquisition of hypersensitivity.

Table 1: Positive patch tests in hairdressers tested with the hairdressers' series and PPD

Material (pet.)	Barcelona *n = 36*		Belfast *n = 28*		Gentofte 1985-90 *n = 47*		Leuven 1987-91 *n = 49*		Dortmund 1990-91 *n = 55*	
	34 F	2 M			42 F	5 M			52 F	3 M
1. o-nitro-p-phenylenediamine 1 % (ONPPD)	5	13.9 %	1	3.6 %	0	0 %	3	6.1 %	6	10.9 %
2. resorcinol 2 %	1	2.8 %		NT	0	0 %	0	0 %	1	1.8 %
3. p-toluene-diamine sulphate (PTD) 1 %	8	22.2 %		NT	1	2.1 %	8	16.3 %	9	16.4 %
4. glyceryl monothioglycolate (GTM) 1 %	8	22.2 %	9	32.1 %	4	8.5 %	8	16.3 %	28	50.9 %
5. ammonium thioglycolate (ATM) 2.5 %	1	2.8 %	1	3.6 %	0	0 %	5	10.2 %	3	5.5 %
6. ammonium persulphate (APS) 2.5 %	2	5.6 %	2	7.1 %	2	4.3 %	9	18.4 %	11	20.0 %
7. p-aminodiphenylamine hydrochloride (PADH) 0.25 %	3	8.3 %		NT	0	0 %	4	8.4 %	4	7.3 %
8. pyrogallol 1 %	0	0 %		NT	0	0 %	0	0 %	1	1.8 %
9. p-phenylenediamine (base) (PPD) 1 %	9	25.0 %	2	7.1 %	2	4.3 %	20	40.8 %	13	23.6 %
% positive per patient	37/36 = 1.0		13/28 = 0.5		9/47 = 0.2		57/49 = 1.2		76/55 = 1.4	

Continuation of Table 1: Positive patch tests in hairdressers tested with the hairdressers´series and PPD

Material (pet.)	London *n = 416*		Bordeaux *n = 11*		High Wycombe 1985-9/91 *n = 84*		Oulu *n = 83*		**Total (19 centres)** *n = 809*		Range
	376 F	40 M	11 F		80 F	4 M	82 F	1 M			
1. o-nitro-p-phenylenediamine 1 % (ONPPD)	16	3.8 %		NT	1	1.2 %	1	1.2 %	33/798	4.13	0-13.9 %
2. resorcinol 2 %		NT		NT	0	0 %	0	0 %	2/354	0.56 %	0-2.8 %
3. p-toluene-diamine sulphate (PTD) 1 %	29	7.0 %	0	0 %	3	3.6 %	1	1.2 %	59/781	7.55 %	0-22.2 %
4. glyceryl monothioglycolate (GTM) 1 %	78	18.8 %	0	0 %	14	16.7 %	2	2.4 %	151/809	18.66 %	0-50.9 %
5. ammonium thioglycolate (ATM) 2.5 %	19	4.6 %	1	9.1 %	1	1.2 %	0	0 %	31/809	3.83 %	0-10.2 %
6. ammonium persulphate (APS) 2.5 %	35	8.4 %	2	18.2 %	2	2.4 %	1	1.2 %	66/809	8.15 %	1.2-20.0 %
7. p-aminodiphenylamine hydrochloride (PADH) 0.25 %		NT	0	0 %	1	1.2 %	1	1.2 %	13/365	3.56 %	0-8.3 %
8. pyrogallol 1 %	3	0.7 %	0	0 %	1	1.2 %	1	1.2 %	6/781	0.76 %	0-1.8 %
9. p-phenylenediamine (base) (PPD) 1 %	64	15.4 %	5	45.5 %	5	5.9 %	0	0 %	120/809	14.83 %	0-45.5 %
% positive per patient	244/416 = 0.6		8/11 = 0.7		28/84 = 0.3		7/83 = 0.1				

OPINIONS ADOPTED DURING THE 56TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 24 June 1994

P 70: *BENZETHONIUM CHLORIDE*

1. General

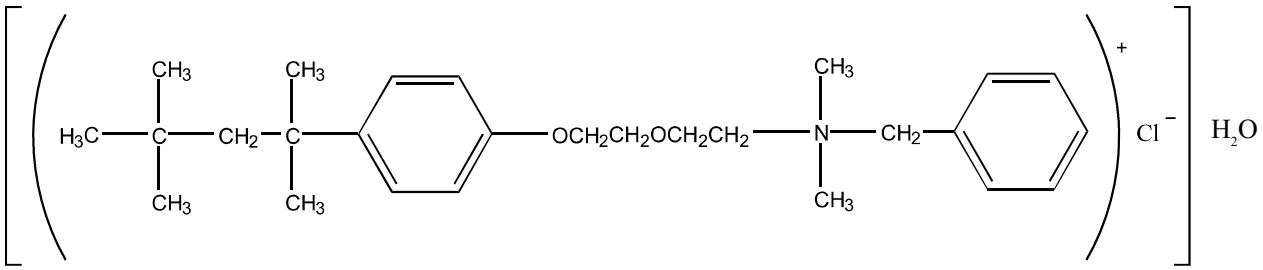
1.1 Primary name

Benzethonium chloride

1.2 Chemical names

4'- (1,1,3,3 - tetramethylbutyl) phenoxy-ethoxyethylene-dimethyl-benzyl-ammonium chloride
Hyamine 1622
benzethonium chloride
phemerol chloride

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{27}H_{42}NO_2.Cl$
Mol weight: 447

1.9 Solubility

Soluble in water, alcohols and other organic solvents.

2. Function and uses

Used in cosmetics at levels of 0.1 %.

TOXICOLOGICAL CHARAKTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} values for the rat are: oral 420 mg/kg, i.p. 33 mg/kg, and i.v. 19 mg/kg. Intranasal administration of 0.06 ml of a solution of 0.25 % or more was lethal to rats.

3.4 Repeated dose oral toxicity

In a 28-day feeding study, rats received diets with 0, 20, 100, 500 or 2500 ppm, providing intake levels of 0, 1.7, 8, 40 or 200 mg/kg b.w./day. The changes in the top-dose group included growth retardation, caecum enlargement, signs of liver damage and decreased serum levels of inorganic phosphorus in males. The latter finding was the only effect considered treatment-related in males fed 500 ppm. The diet with 100 ppm (8mg/kg b.w./day) was a clear no-effect level.

A supplementary 28-day study in rats with the same feeding levels was conducted to verify and extend certain findings in the previous study. The results confirmed most of the changes seen at the top-dose, including caecal enlargement, The latter finding was not accompanied by histopathological changes. Decreased levels of serum-P seen at the two higher levels in the previous study did not occur in the present study. Therefore, 500 ppm (or c. 40 mg/kg b.w.) was the NEL in the supplementary study.

3.5 Repeated dose dermal toxicity

Upon subacute, dermal application of 2 ml 0.1 % solution to the skin of rabbits daily, 5 days/week for 4 weeks no systemic effects were observed (summary report).

3.8 Subchronic dermal toxicity

Subchronic (13-wk) dermal studies in rats and mice are being conducted by the NTP.

3.10 Chronic toxicity

In a one year study, groups of 3 dogs were fed 0, 5, 100 and 500 ppm in the diet. No changes were observed in growth rate, haematology or in gross- or microscopic pathology. A two year study has been conducted with groups of 5 rats/sex, fed diets containing 0, 50, 200, 1000, 2500 and 5000 ppm. The top dose induced mortality. With 2500 and 5000 ppm testicular atrophy and caecal enlargement occurred. With 1000 ppm there was only caecal enlargement.

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin irritation in rabbits did not occur when 2 ml of a 0.1 % dilution were applied daily 5 days a week for 4 weeks. In humans, 0.1 ml of a 5 % aqueous solution applied under patches for 48 hours, was irritating.

4.2 Irritation (mucous membranes)

Very slight irritation to the eye of rabbits was produced at concentrations as low as 0.01 and 0.03 %.

5. Sensitization

A sensitization test in humans with 0.12 % in formulations applied to the skin under closed patches was negative.

6. Teratogenicity

An oral teratogenicity study in rabbits with 1, 3 and 10 mg/kg/day revealed signs of maternal toxicity with 3 and 10 mg, increased mortality of mothers and pups with 10 mg, and an increased incidence of supernumerary ribs with 3 and 10 mg. The latter finding was attributed to stress.

In a second teratogenicity study in rabbits with oral dosing of 1.125, 3.558 and 35.576 mg/kg/day, the high dose induced maternal and foetal mortality. A dose-related increase in foetal resorptions occurred in all treatment groups although the change was statistically significant only in the high-dose group. The mid-dose was not clearly without effect.

In a teratogenicity study in rats with oral dosing of 1.125, 3.558 and 35.576 mg/kg/day the high-dose group showed decreased maternal body weight and an increased number of smaller pups. An increased incidence of skeletal variants (ossification effects) occurred in all treated groups. Skeletal malformation was increased in the high-dose group. Slight hydrocephalus was seen in one pup of the mid-dose group and in 5 pups (in 2 litters) of the high-dose group.

A second oral rat teratogenicity study with 0.059, 1.125, 3.558 and 35.576 mg/kg showed lower maternal body weights, increased variation of skeletal ossification and increased incidence of skeletal malformations (wavy ribs) in the top-dose group only. The latter finding was considered to be within the limits for historical controls. Fertility and reproductive performance were examined in rats treated orally with 1.125, 3.558 and 35.576 mg/kg/day prior to and during mating and during the gestation and lactation period. The high-dose produced growth depression, increased irritability, respiratory signs in the parents and decreased viability, and body weight of pups at birth. Fertility and general reproductive performance were not affected. Peri- and postnatal effects were examined in rats dosed orally with 1.125, 3.558 and 35.576 mg/kg/day from day 15 of gestation through day 20 of lactation. A slight decrease in foetal viability occurred in all dose groups and in postnatal survival in the mid- and top-dose group.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption was examined by applying 1.0 ml of a 10 % aqueous solution of the ^{14}C-labelled compound under occluded patches to the skin of two rabbits on 4 consecutive days. One rabbit had the skin abraded. Blood samples taken on each day, showed an average concentration of 0.2 ppm, which corresponds to 0.003 % of the amount applied. No mention is made of analyses in urine, faeces or carcasses and it is not possible to make any assessment of the total amount absorbed.

The percutaneous absorption of a 0.5 % aqueous emulsion has also been investigated in human volunteers by measuring the rate of deposition in the stratum corneum and circulation of the permeability constant. In the first study using 6 volunteers and a surface recovery method (at approximately hourly intervals from 0-6 hours) rapid transfer to the stratum corneum was

noted (9.12 µg/cm^2/hr) with 4.56 µg/cm in the stratum corneum in 30 minutes. Percutaneous penetration rate was calculated as 51 µg/cm^2/hr. In a second experiment the amount present in the stratum corneum was determined after 30 minutes using an abrasion technique to remove surface layers. The results indicated a similar permeability constant, namely about 50 µg/cm^2/hr. The value of this method for measuring skin absorption is not completely clear, but the data do suggest appreciable absorption through the skin can occur with a 0.5 % formulation.

Data from *in vitro* studies using an aqueous emulsion of 0.5 % compound and excised abdominal skin did not, however, indicate any significant absorption. The concentration of benzethonium chloride in the receptor fluid remained below the detection limit during the 72 hour exposure.

Total exposure, assuming use in all types of cosmetics up to a maximum concentration of 0.1 % will be 0.46 mg P70/kg/day. Since this level is in the same order as the low NOEL obtained in teratogenicity studies in rats and rabbits (c.1 mg/kg b.w./day) virtual absence of absorption through the skin is essential for P70. However, contradictory results were obtained in various percutaneous absorption studies conducted with P70 and there were uncertainties about the reliability of the methods used. Therefore industry should provide information which conclusively remove the present doubts with respect to the rate of percutaneous absorption.

Maternal and foetal absorption of the ^{14}C-labelled compound was examined in pregnant rats treated orally with 1.125 and 3.558 mg/kg/day on days 6 through 15 of gestation. Average blood levels in the two groups were 1.5 and 0.97 ng/g respectively. In urine, the maximum levels were 52 and 149 ng/ml after a single oral dose. Virtually all radioactivity was recovered in the maternal faeces and carcass. Results of foetal analyses varied between not-detectable and 6.8 ng/g foetus.

Several subcutaneous injection studies have been conducted in rats and mice. In one study in rats, a dose-related increase in the incidence of granulomatous reactions (mainly fibrosarcomas) occurred at the injection site.

Concentrations as low as 0.002 % inhibited the mortility of the isolated ileum of rats and rabbits. Blood pressure measurements in the dog indicated nearly complete blockage of sympathetic ganglions at an i.v. dose of 2 mg/kg.

8. Mutagenicity

Mutagenicity studies using the Ames test have given negative results using up to 100 nmoles/plate, and up to 7500 µg Hyamine 1622/plate. It was stated that in an *in vitro* assay with CHO cells no evidence was found of sister chromatid exchange or chromosome aberrations, but a report is not available.

11. Conclusions

In summary bezethonium chloride has moderate acute toxicity by the oral route and high toxicity following parental exposure. It produces slight eye irritation at very low concentrations (i.e. 0.01 %) and significant skin irritation at 5 %. The No effect level in a 28 day repeated dose oral study was 8 mg/kg/day with only marginal effects at 40 µg/kg; these

were not seen in a second study. Chronic studies in rodents indicate a no effect level of around 200 ppm on the diet with only caecal enlargement at 1000 ppm but testicular effect at 2500 ppm. There was no evidence of mutagenicity using the Salmonella assay and the compound was reported to be negative in a metaphase analysis for clastogenicity in CHO cells. The data from teratogenicity studies in rats and rabbits indicate that both maternal toxicity and adverse effects on the developing fetus are seen at 3 mg/kg with malformations at 30 mg/kg. The no effect level was around 1 mg/kg. Data from studies in humans suggest significant absorption through the skin using a 0.5 % formulation.

Industry should be asked to provide the following information:

- eye irritation study with the 'in use' concentration (0.1%)
- sensitization test according to present requirements,
- the results of the 'NTP' subchronic dermal studies in rats and mice.

It was noted that industry proposed to limit the use of this preservative to areas where there is limited skin contact namely deodorants, hair care products and aftershaves. However, even with these limitations the requested data should be provided to enable meaningful safety assessment.

Classification: D

P 91: *3-IODO-2-PROPYNYL BUTYL CARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butyl carbamate

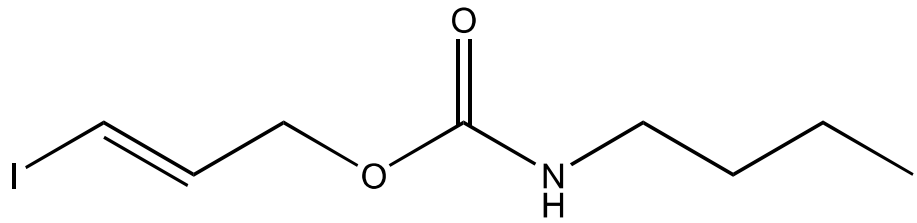
1.2 Chemical names

iodo propynyl butyl carbamate
3-iodo-2-propynyl butyl carbamate

1.4 CAS no.

55406-53-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}NO_2I$
Mol weight: 281

1.9 Solubility

It has low solubility in water (156 ppm at 20°C) and is soluble in organic solvents.

2. Function and uses

It is proposed for use as a preservative in all types of cosmetic products at up to 0.1 %

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal signs of toxicity were seen at 500 mg/kg or below. In a percutaneous toxicity study in rabbits

a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing resulted in no deaths. The only signs of toxicity seen were slight irritant effects at the site of application.

3.7 Subchronic oral toxicity

In a sub-chronic study rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg. Histological examination showed hepatocyte enlargement at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The no-effect level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritancy study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe oedema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours. Severe effects were however noted in an eye irritation study in rabbits. The substance (0.1g) produced moderate to severe hyperaemia, chemosis and discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. If the compound was washed out of the eye 20-30 seconds post instillation only transient irritant effects were seen.

4.2 Irritation (mucous membranes)

The eye irritancy of a 0.5 % solution of IPBC in corn oil has been tested in rabbits and also the effects of 0.5 % IPBC in a baby shampoo. Groups of 6 animals were used in each case. No signs of any irritant effects were seen with the corn oil formulation. In the case of the baby shampoo, signs of slight irritant effects were seen for about 24-48 hours, but similar effects were seen in the ‘control’ baby shampoo that did not contain IPBC. Thus 0.5 % in corn oil or in a baby shampoo formulation produced no eye irritancy.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximization test. Induction concentration were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson Kligman tests have been carried out on formulations containing 0.05 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical) were used. In the second case the concentrations were 0.1 % and 0.5 % respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation.

There was no evidence of sensitization in either test. These studies suggest that the compound does not have any significant potential for sensitization. No data are available on sensitization in humans, or on the potential of this carbamate to cross react with dithiocarbamates used in the rubber industry.

6. Teratogenicity

Teratogenicity studies have been carried out in both rat and the mouse. In the study in rats compound was given on day 6-15 of gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The No Effect Level was 50 mg/kg. A similar dosing regime was used in the study in mice. No compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The No Effect Level was 125 mg/kg.

6.2 Two-generation reproduction toxicity

A two generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week premating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound-related effects were seen at any dose level on clinical chemistry or at necropsy. Slightly reduced weight gain was seen in the males at 750 ppm during the premating period in both the initial generation and the F1 generation. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index (= no. of pups alive at day 1/total number of pups) at 750 ppm in either generation, while a marginal effect was also noted at 300 ppm in the F1 generation. Postnatal growth of the offspring however was not affected and no effects were seen on the development of the offspring. The No Effect Level was 120 ppm test compound in the diet (roughly equivalent to a dose of 10 mg/kg b.w./day).

7. Toxicokinetics (incl. Percutaneous Absorption)

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C radio-labelled material. Following iv administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the level and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicate that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

8. Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies. Negative results were obtained in the Salmonella assay versus strains TA 1535, 1537, 1538, 98 and 100 but this study was limited by investigating only 3 concentrations (6.2 - 55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537, 98 and 100 and concentrations of 1-1000 µg/plate against TA 1535. In all cases the top concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 %-25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

The compound is a carbamate and studies have been carried out to investigate whether significant blood cholinesterase inhibition occurs in the rat following intravenous administration. The compound was given in PEG/400: water vehicle at 2-16 mg/kg and blood samples taken and analysed for erythrocyte cholinesterase activity at 15, 30, 60 minutes and 2 and 5 hours post dose. No effects on blood cholinesterase levels were observed.

Data on minimum inhibitory concentrations of 3-iodo-2-propynyl butyl carbamate demonstrated the efficacy of this compound at levels ≤ 0.1 %.

11. Conclusions

The substance has moderate acute toxicity by the oral route and low toxicity following dermal exposure. It is a mild to moderate skin irritant, but is a severe (corrosive) eye irritant; however concentrations of 0.5 % do not produce any eye irritation. Negative results were obtained in 3 Magnusson Kligman maximisation tests for skin sensitization. In a subchronic (90 day) oral study in the rat the No Effect Level was 50 mg/kg.

Mutagenic potential has been investigated in Salmonella assays for gene mutation, in a study to investigate Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro*, and in an *in vivo* micronucleus test. Negative results were consistently obtained. There was no evidence of teratogenic potential in studies in two species (rats and mice). In a 2 generation reproductive toxicity study in rats a reduction in life birth index was observed. The no-effect-level in this

study was 120 ppm in the diet (c. 10 mg/kg b.w./day). The compound is well absorbed orally but is rapidly metabolised and excreted.

Concern was raised about the safety margin of P 91 in relation to the relatively low No Effect Level obtained in the 2 generation reproductive toxicity study in rats. On the basis of the information provided on the efficacy of P 91, it was noted that the maximum permitted concentration of P 91 should be reduced to 0.1 % (instead of the 0.5 % originally requested). However, reduction of the concentration below 0.1 % is not feasible and hence the safety margin cannot be further increased this way.

Therefore Industry should be asked to indicate whether the use of P 91 could be restricted to a limited number of products, and to provide a realistic estimate of the total exposure to P 91.

At present the exposure is estimated as follows: No data are available on skin absorption and therefore 100 % absorption is assumed. The extreme worst-case scenario (assuming that all products contain this preservative at a maximum concentration of 0.1 % and all are used extensively) is an exposure to 27.6 mg P 91/ human/ day (from 27.6 grams product comprising 5.54 g oral hygiene and 22 g skin contact).

In addition, Industry should provide a clear proof for the absence of cross sensitivity, e.g. a patch test in humans known to be sensitive to thiuram/carbamate rubber accelerating chemicals.

In a literature search no evidence was found for cross-reactions of 3-iodo-2-propynyl butyl carbamate with dithiocarbamates used in the rubber industry. This information is, however, considered unsatisfactory proof for the absence of cross sensitivity.

Classification: B

S 46: *UROCANIC ACID*

1. General

This substance occurs naturally in the skin and cornea. It is produced by deamination of histidine, and is metabolised to formiminoglutamic acid, in which form it is excreted. In the skin, however, it is converted to the *cis*-isomer under the influence of UV radiation, in which form it is not metabolised. The amount found in the skin increases with increasing exposure to UV radiation. Its use as a sunscreen was proposed by Zenisek and Kral.

1.1 Primary name

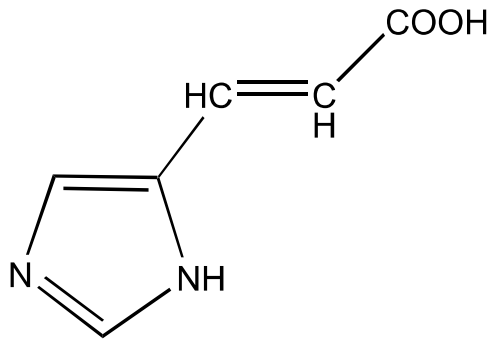
Urocanic acid

1.2 Chemical names

Urocanic acid

4-imidazole acrylic acid.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_6N_2O_2$

Mol weight: 138.2

1.9 Solubility

Poorly soluble in water; insoluble in alcohol, ether.

2. Function and uses

Authorised for use as a sunscreen at concentrations up to 2 %, expressed as acid.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The LD_{50} is reported to be greater than 3 mg/kg bw intraperitoneally. No further details are given.

3.2 Acute dermal toxicity

Phototoxicity.

Guinea pig. In a preliminary screen for the production of primary irritation, 4 animals were shaved and 0.1 ml of an emulsion (nature not specified) applied to (presumably) 5 areas of the skin. The concentrations of *trans*-urocanic acid used were (%): 0, 0.02, 0.2, 0.5, 1 & 2. No irritation was produced, and 2 % was taken to be the maximum non-irritating concentration (although no concentration high enough to produce irritation had been used).

In the main test, 3 groups of female animals were used: 10 test, 10 vehicle controls, and 5 positive controls. The skin of the dorsum was chemically depilated and then stripped with tape. Areas of 2 x 2 cm were delineated, and 0.1 ml of the test solution or of the vehicle were applied to each site. The positive control was an ethanolic solution of 0.01 % 8-methoxypsoralen. After this, one of the sites, and an area of the dorsum, were protected by aluminium foil, and the remainder of the dorsum irradiated for 3 hours with UVA at 1.12×10^8 ergs/cm^2. The spectral range of the light source was 320 to 400 nm, and the dose was monitored by a UV meter. Reading was at 24 and 48 hours, and 7 days. A Draize scoring system was used.

There was no primary irritation of the skin. There was no evidence of phototoxicity in any of the test animals, or in the vehicle control animals; nor was there any evidence of clinical abnormality or weight loss. The positive control animals showed effects on the skin in all animals at 24 hours, and at 4/5 animals at 48 hours and 7 days (mean scores, 2.2, 2.0 and 2.4 respectively).

4. Irritation & corrosivity

4.1 Irritation (skin)

An ointment containing an unspecified concentration of a.i. was applied daily to the abdominal skin of guinea pigs for 14 days. No irritation was produced.

A similar preparation was applied to the abdominal skin of 10 rabbits and 6 guinea pigs, daily for 21 days. No abnormality was produced.

4.2 Irritation (mucous membranes)

A solution of 10 % was made up in water with 1 % triethanolamine as a solubiliser. This was placed in (presumably) rabbit eye, without rinsing. No further details are given. No adverse effect was produced.

5. Sensitization

Photosensitization.

Guinea pig. Female animals of the Dunkin-Hartley strain were used. The a.i. used was trans-urocanic acid.

(i) A primary skin irritation screen was carried out. It is stated that 4 sites were prepared on the skin of 2 animals; however, 5 concentrations of a.i. were used in an emulsion at concentrations (%): 0.02, 0.2, 0.5, 1 and 2. Reading was at 1, 2 and 24 hours, No abnormality was seen at the sites of application or in clinical appearances. The maximum non-irritant concentration was taken to be 2 %.

(ii) Primary phototoxicity screen. Six animals had the same concentrations of an emulsion of a.i. applied to 2 sites on the dorsum. On the left side, the areas were exposed to 30 J/cm^2 of UVA (70 minutes). The right side was shielded. Reading was at 1, 2 and 24 hours. No abnormal local or clinical changes were seen. A concentration of 2 % was taken to be the maximum non-phototoxic concentration.

(iii) For the main test, 3 groups each of 10 female animals were used. Animals of the first group were treated with an emulsion of 2 % a.i.; of the second group with vehicle only, and of the third group with the positive control substance, 5 % 6-methylcoumarin. The sequence was as follows:

Day 1: a. Four injections of 0.1 ml emulsified FCA in the nuchal region;
b. Skin stripped with tape;
c. 0.1 ml of test (or control) sample applied;
d. UV irradiation at a dose of 10 J/cm^2 for about 24 minutes. The flux was subject to monitoring to ensure a correct dose. Reading was at 24 hours.

Days 2 to 5
Procedure of day 1 repeated, with reading at about 24 hours after the applications.

Day 19 Dorsum stripped in all animals, and 0.1 ml of the appropriate solution applied to both sides. The right side was shielded, and the left side irradiated with UV as before. Reading was at 24, 48 and 72 hours.

A numerical scoring system was used. There was no reaction in the irradiated or non-irradiated animals except for a slight reaction in one non-irradiated animal at 48 hours. The positive control animals subjected to irradiation were all positive; 7/10 had eschar formation at 72 hours. The mean scores at the readings were, respectively, 2.4, 1.9, 1.6. The non-irradiated animals also showed some reaction, with mean scores of 0.2, 0.3 and 0.4; 1 animal at 72 hours had eschar formation.

The test was regarded as negative.

7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption.

Human and hairless mouse skin *in vitro*.

A lotion of unspecified composition was used; it contained 0.2 % of a.i. Mouse skins were divided into two parts, and the experiments carried out in duplicate; the human skins, obtained in a frozen state, were divided into either 3 or 6 replicates, according to size. The skins were mounted in a Franz chamber of area 1 cm^2; the receptor fluid was distilled water and the epidermal surface was exposed to air. The integrity of each skin specimen was first assured by studying the permeation of ^3H_2O; less than 2 microlitres/cm^2/30 minutes was acceptable. The receptor fluid was supposed to be sampled at 2, 4 , 8 and 24 hours; the graphical results suggest that the sampling intervals were 1, 3, 6 and 16 hours.

Lotion was applied at the beginning of the experiment, and the amount applied determined by difference. At the end of the period of exposure, the surface of the skin was washed twice with isopropanol, and the skin separated into dermis and epidermis with a scalpel. Estimation of the a.i. was by HPLC.

In the first series of experiments, 6 mouse skins were used, each divided into 2 parts, and 3 specimens of human skin, the first divided into 6 replicates, and each of the others into 3 replicates. About 10 mg of lotion was applied to each skin. From the results, the following values may be derived.

(a) In the mouse skin, the mean net amount applied (amount applied less amount recovered in washings at the end of the experiment) may be calculated to be 13.577 (all figures are in ug/cm^2). The amounts found in the receptor fluid over the period of the experiment totalled 14.195, and the amounts in the epidermis + dermis + receptor fluid amounted to 31.083.
(b) In human skin, the mean net amount applied was 4.04, and the amounts in the receptor fluid 2.87; the total in epidermis + dermis + receptor fluid amounted to 22.22.

It is clear, therefore, that there was probably a substantial endogenous contribution to the amounts of a.i. found.

In a second set of experiments, 2 samples were obtained from each of 3 mouse skins, and half of them were treated and the other half not. The skin from 1 human donor was divided into 6 parts, and one half of these treated and one half not. Unfortunately, the net amounts applied are not given for this experiment.

(a) In the mouse skins to which a.i. had been applied, the (mean) amount found in the receptor fluid was 14.59, and in the epidermis + dermis + receptor fluid 24.24. In those skins to which no a.i. had been applied, the corresponding figures were 5.58 and 18.50.
(b) In the human skin, when a.i. had been applied, the figure for the amount in the receptor fluid was 3.13, and in the epidermis + dermis + receptor fluid was 11.14. When no application of a.i. had been made, the corresponding figures were 3.62 and 9.66.

The time course of the appearance of the a.i. in the receptor fluid is given in the form of graphs. From these, the following figures may be derived.

(1) *First experiment* (a.i. applied to all specimens):

(a) Mouse skins. A maximum of 0.65 ug/cm^2/hr was found at 3 hours. This fell to about 0.1 at 6 hours, and then rose slowly to 0.3 at 16 hours.
(b) Human skins: Maximum at 1 hour to 0.22, followed by a slow fall to near zero at 16 hours.

Second experiment (a.i. applied in half of the tests).

(a) Mouse: Following application of a.i., there was a peak of about 0.4 at 1 hour, followed by a fall to about 0.25, after which the level remained more or less constant up to 16 hours. This is somewhat different from the findings in the first experiment, but the number of experiments is smaller.

In the absence of any application, the initial rise to 0.35 is about the same, after which the level falls to near zero at 6 hours, and stays there.

(c) In human skin, the pattern is about the same whether or not an application has been made. There is a peak of about 0.45 at 1 hour, followed by a fall to near zero at 4 hours, which remains up to 16 hours. This is again somewhat different from the pattern in the first experiment.

It may be concluded that in mouse skin about half the a.i. found in the receptor fluid and in the skin is endogenous; in experiments with human skin, perhaps 75 % of the amount in the skin is endogenous, and all the amount found in the receptor fluid. From these experiments, it may perhaps be supposed that percutaneous absorption of the a.i. in man would be small. It should be noted that there seems to have been no attempt to estimate the isomers separately in these experiments.

Hairless mouse *in vitro*. Urocanic acid (chiral status not specified) was made up in an o/w emulsion at concentrations of a.i. of 2 %, 0.2 % and 0.02 %. Skins were mounted in Franz cells and treated with 2 mg/cm^2 of the formulations in duplicate. There were 2 untreated cells as controls. The receptor fluid was water; 2 ml of this was collected at 4 hours and replaced with fresh water; the receiving fluid was then collected in toto at 24 hours. At this time the exposed surface of the skin was wiped and the skin stripped 10 times; the strippings were analysed for a.i. in groups of 5. The skin was then homogenised and the content of a. i. determined. Analyses were by HPLC.

At 4 hours, the amounts in the reservoir (ug/ml) were: 0, 0.276, 0.21 , 0.188 (control, 2 %, 0.2 %, 0.02 %, respectively). At 24 hours, the corresponding values were 0.785, 1.154, 0.604 and 0.781. Strippings 1 to 5 yielded (ug/cm^2) 0.2718, 17.355, 1.550, 0.5805; strippings 6 to 10 yielded 0.6058, 1.9545, 0.9945 and 0.5755. In the skin homogenates, no a.i. was found; the authors term these samples “subepidermal murine skin”. In summary, it may perhaps be concluded that the amount in the reservoir with the 0.02 % concentration derives from endogenous sources; with higher concentrations, absorption may be proportional to concentration of a.i. The strippings seem to show that the amount in the stratum corneum of endogenous origin is about 0.44 ug /cm^2 the values increase with increasing concentration of a.i. The failure to find any a.i. in the skin following stripping is puzzling.

Human skin *in vitro*.

Full thickness human cadaveric abdominal skin was stripped of muscle and fat and mounted in a Franz cell of diameter 1 cm^2. The a.i. was prepared as 3 concentrations of the potassium salt in an o/w emulsion; the concentrations of a. i. were 2 %, 0.2 % and 0.02 %, probably expressed in terms of acid. The receptor fluid was water; the epidermal surface was exposed to the ambient environment in the laboratory, but kept moist by a wick. Each concentration was tested

in duplicate, and two untreated preparations were set up as controls. Sampling was at 4 hours and 24 hours. At the end of the experiment the epidermal surface was wiped. The skin was stripped with adhesive tape 20 times; the content of a.i. in the strips was estimated in groups of 5 strips. The epidermis was then separated from the dermis by immersion in sodium bromide solution at 40° for 90 minutes. The experiments were repeated twice, and the results pooled. Estimation of the a.i. was by HPLC.

Results: (a) No a.i. was found in the receptor fluid at any time (less than 0.1 ug/ml). There was no significant difference between the strippings in the treated and untreated groups. The mean figures were (ug/cm^2): 2 %, 7.51; 0.2 %, 6.07; 0.02 %, 4.52; control 4.67. On inspection of the individual figures there did not seem to be any definite gradient of concentrations with number of strippings.

In the dermis and epidermis, there was no detectable level of a.i. (less than 0.1 ug/cm^2).

In this investigation, there does not seem to have been any attempt to separate the *cis*- and trans-isomers. In contradistinction to a previous investigation there was no evidence of production of endogenous a.i.

Man. A study to determine the amount of deposition of a.i. in the stratum corneum was carried out in 30 healthy female volunteers. Groups of 15 subjects were randomly allotted to apply 1 gram of o/w emulsions of a.i. twice daily to the volar surface of both forearms for 16 weeks. The preparations applied by members of each group were identical except that one preparation contained 0.2 % of a. i. and the other 1 %. The upper arms of each subject served as controls. A template 3.61 cm^2 in area was used to delineate skin areas which were stripped 20 times, using adhesive tape, at the following time intervals: before treatment, and at weeks 1, 2, 4, 8, 12 and 16 after beginning treatment. For the first 2 samples, the amounts of a. i. in the strips were estimated in groups of 5 strips, but thereafter all the strips from a given area were analysed together. Estimation of a.i. was carried out in a blinded fashion using HPLC. It is mentioned that *trans*-urocanic acid was among the reagents used for the HPLC, but the chiral status of the a. i. used in the emulsion is not specified.

There was no significant difference in the amount of urocanic acid between test sites and control sites at any sampling, with the exception of 2 of the sampling times using the 1 % emulsion, and in these cases, the difference between the control and test areas showed a greater amount of urocanic acid in the control strippings than in the strippings from the sites of application. In general, with a few exceptions, the amount of urocanic acid was greater in the control than in the test strippings. The authors conclude that under the conditions of the experiment there was no deposition of a.i. in the stratum corneum.

Man. An investigation was carried out to determine the levels of urocanic acid in human skin.

(a) Samples of "stratum corneum/callus" (presumably thickened stratum corneum) were obtained from 10 volunteers and the amount of urocanic acid in each estimated by HPLC.

(b) Five cadaver skins were used. These were stripped 20 time using adhesive tape over an area of 3.24 cm^2. This procedure was carried out in triplicate in each skin. The strippings were extracted in groups of 5 successive strips, and the amounts of urocanic acid estimated by HPLC.

Results: The mean amount of urocanic acid in the samples of stratum corneum/callus was 0.263 % (w/w). The individual values were reasonably uniform (SD = 0.065). The mean amount of urocanic acid in the strippings was 6.06 ug/cm^2, but there was considerable variation in the amount from skin to skin. The range was 0.99 to 15.30; the SE is given as 2.75, which indicates a standard deviation of 6.15 and a coefficient of variation of 101.5 %.

8. Mutagenicity

A standard Ames test was carried out according to GLP; in addition, *E. coli* WP2 uvrA was also tested. The a.i. was provided as 2 powders: *cis*- and *trans*-urocanic acid. Equal quantities of these were mixed extemporaneously and the mixture dissolved in (probably) 0.5 N sodium hydroxide. Further 1 N alkali was added drop by drop until complete dissolution of the a.i. had occurred. The stock solution so produced (presumably consisting of the sodium salts of the isomers, together with some free alkali) was then further diluted with water. Following preliminary range finding experiments, the concentrations chosen for the tests were (ug/plate): 33 , 100 , 333, 1 000, 3 333, 10 000. There was no evidence of toxicity or precipitation at these concentrations. Suitable controls were used, and activation was by "Aroclor"-induced rat liver microsomal preparations. The criteria for a positive result included the finding of at least a three fold increase in revertants in a dose related manner for strains TA1535, 1537 and 1538; for TA98 and 100, and for E. coli WP2 uvrA a two fold increase was required. The study appears to have been a well conducted one. There was no evidence of any increase whatever in revertants in any experiment; the positive controls gave satisfactory responses.

In an appendix, a method for estimation of the a.i. by HPLC is given. The distinction between the *cis*- and *trans*-isomers is mentioned in the notations written on the records, but does not seem to have been taken into account in the accompanying text.

A test for chromosomal aberration *in vitro* was carried out in Chinese hamster lung cells, according to the Japanese guidelines for toxicity studies of drugs (1989). In the preparation of the stock solutions of the *cis*-and *trans*-isomers of the a.i. in physiological saline, it was noted that the *cis*-isomer was somewhat more soluble than the *trans*-isomer. The concentrations used for the test were 1.25, 2.5, 5 and 10 mmoles/l (approximately 173, 346, 691 and 1382 ug/ml). Careful initial cytotoxicity tests were carried out: these were negative up to 10 mmole with the *trans*-isomer, but positive at 5 and 10 mmole with the *cis*-isomer. This was probably due to the difference in solubility, noted above. Metabolic activation was carried out with S9 mix induced by phenobarbitone + 5,6-benzflavone. Suitable negative and positive controls were used. Incubation with the a.i. was for 2 days without activation, and with activation for 6 hours (in a 4 day culture of cells) followed by washing and a further 18 hours incubation with a.i. but without activation mix.

The experiments seem to have been properly carried out. There was no evidence of chromosomal aberration at any concentration of a.i. The positive controls gave marked aberrations.

Two tests were carried out in cultures of human fibroblasts.

(a) Earlier work had shown that a combination of UV irradiation + a.i. led to the formation of thymidine-acrylic acid (and perhaps cytidine acrylic acid) adducts in calf thymus; however, the

UV fluxes used in those experiments were much too high to allow of DNA repair afterwards. It had also been shown that irradiation of the a.i. before mixing with DNA had no effect: it was necessary to irradiate the a.i. in the presence of the DNA to bring about the formation of adducts. The present experiments were designed to use a flux which would permit survival of the cells and possible DNA repair. They were carried out in accordance with the Code of Federal Regulations (USA).

Calf thymus DNA was exposed to 2 mmolar a.i. (= 276 ug/ml). A mixture of equal parts of the *cis*- and *trans*-isomers was used. Irradiation was with UVB from "an FS20 sunlamp". This equipment produced a peak output at about 313 nm; the output was measured at the culture level to ensure that 100 kJ/m^2 was administered. Untreated DNA was used as a control. Following enzymatic digestion, the DNA was end-labelled with ^{32}P-ATP and the products run on TLC; the radioactive spots were identified by autoradiography and were eluted. The results are given as follows:

Treatment	Relative adduct labelling
a.i. + UVB	9.4×10^{-9}
a.i. alone	8.9×10^{-9}
neither a.i. or radiation	2.3×10^{-9}

If the a.i. had formed a specific adduct with DNA, the migration on the tlc plate would have been expected to be different; it was not. The author does not regard the approximate 4-fold increase in RAL following a.i. as significant; it was thought due to purification and handling of the material.

(b) Unscheduled DNA synthesis (UDS) was studied. The experiments were so arranged that the cultures were either protected from radiation or exposed to UVB at 500 J/m^2. The dose of radiation was measured by a meter at the level of the culture. If a.i. was to be incorporated in the culture, the radiation was first passed through an 0.5 cm layer of 2 % a.i., with a consequent increase of exposure time to ensure the dose of UVB was standard. After irradiation, the cultures were incubated with ^{3}H-thymidine for 4 hours. After preparation of slides and autoradiography, the nuclear grains in "up to 25" lightly labelled cells were counted.

In all, 5 experiments are reported.

(i) Cultures with and without 2 % a.i. were compared; unexpectedly, the number of grains was significantly reduced (17 %) in the preparations from cultures containing the a.i.
(ii) The experiment was repeated with a.i. at 1 % and 0.1 %, and no reduction in grains was found; yet a 10 % reduction was found at 0.01 %. The author notes that although the reductions found were significant, they were small in absolute terms.
(iii) A comparison was made between cultures containing a.i., one set being irradiated and one set not. A reduction of 18 % in grain count was found in the presence of the a.i. The filter interposed in the radiation path in this case was an 0.5 cm layer of a 2 % solution of the *trans*-isomer of the a.i. A reduction of 18 % in grain count was found.
(iv) A similar experiment to (iii) using *cis*-a.i. instead of *trans*-a.i. in the filter gave a fall of 33 %.
(v) If the irradiation was filtered through *trans*-a.i. and incubation carried out with and without 2% *trans*-a.i., there was an increase in UDS in the culture lacking a.i. This finding excluded the

possibility that it was photolysis of *trans*-a.i. which produced the suppression noted in the previous experiments.

The conclusion of the author was that adduct formation and UDS did not occur under the circumstances of the experiment.

10. Special investigations

Study of amounts of urocanic acid in skin.

Man. (a) The ratio of *cis/trans*-urocanic acid is greater in summer than in winter.

(b) The ratio of *cis/trans*-urocanic acid is greater in the forearm and cheek than in the skin of the back.
(c) UV irradiation of the skin of the back increased the *cis*/*trans*-urocanic acid ratio; this reverted to normal in 18 days, but the total level of urocanic acid remained elevated at that time.
(d) Both *cis*- and *trans*-urocanic acid, sodium salt, had about the same protective effect as each other against ultra-violet radiation.

The compound monosodium 4-(5)-imidazolylmethylidenemalonate, an analogue which is incapable of isomerisation, also had about the same protective effect.

Effects on immune function.

Immune function in the skin is known to be reduced by UV radiation, and it is suggested that urocanic acid may be a photoreceptor for this effect, the *cis*-form produced by the radiation then influencing the Langerhans cells.

(a) The *cis*-isomer of urocanic acid inhibits the delayed type hypersensitivity induced by experimental herpes simplex virus infection in the mouse.

(b) The contact hypersensitivity produced in hairless mice by oxazolone is suppressed.

(In this investigation, the tumour production induced by urocanic acid was also studied; this part of the investigation is summarised below).

(c) High levels of histidine (the precursor of urocanic acid) in the diet produced a much increased level of urocanic acid in the skin of mice. Following this, the reduction in contact sensitivity to DNCB in the skin following UVB irradiation was studied. It was found that the effect of the feeding with histidine was to cause much greater inhibition of contact sensitivity compared with controls.

(d) Ultraviolet radiation can produce activation of herpes virus infections. Urocanic acid is plausibly postulated to be the intermediate in this reaction.

(e) In the rat, heart transplants showed less rejection if the recipients were treated with injected urocanic acid daily for 7 days. In 40 % of the treated animals, rejection seemed to have been prevented permanently.

(f) Urocanic acid binds covalently to thymus DNA under the influence of ultraviolet radiation. These adducts have been identified.

(g) There is evidence that UVB irradiation at the relatively high level of 50 kJ/m^2 suppresses contact hypersensitivity of the skin at a distant non-irradiated site. Whether urocanic acid plays a part in this reaction is not known. It has been shown that the time course of this reaction is identical with that of local suppression.

(h) It has been reported that stripping of the skin, which removes most of the Langerhans cells, prevents the reduction of contact hypersensitivity induced by UV irradiation. Another investigation, however, contradicts this report.

(i) In cultures of human monocytes, which contained *Staphylococcus epidermidis* to promote IL-1 production, cis- but not trans-urocanic acid depressed its production, and the proportion of DR-positive monocytes. In cultured lymphocytes, the proportions of helper and suppressor T-cells was altered by cis- but not trans-urocanic acid.

(j) In a study of the nature of the chromophore responsible for the immunosuppression associated with ultraviolet irradiation, the authors suggested that the cyclobutylpyrimidine dimers, which are known to be produced under such conditions, are probable chromophores. They availed of the fact that in the South American opossum, Monodelphis domesticus, there is an enzyme in the skin, activated by visible light, which repairs DNA by breaking down the cyclobutylpyrimidine dimers and restoring the integrity of the DNA. By using this species, and studying the effect of ultraviolet and white light on the contact hypersensitivity induced by 1-fluoro-2,4-dinitrobenzene, they concluded that urocanic acid was an unlikely candidate for the chromophore, and that the dimer was a more probable one.

(k) In an investigation of the mechanism of the reduction of contact hypersensitivity by UVB irradiation, the authors point out that not all strains of mice are equally sensitive to this effect. They showed that in sensitive strains, compared with relatively insensitive ones, there was a greater reduction in the hypersensitivity to dinitrofluorobenzene brought about by injection of cis-urocanic acid. (By this term the authors mean trans-urocanic acid, irradiated with UVB; in their laboratory, this gives just over 50 % cis-urocanic acid in the racemic mixture). However, although cis-urocanic acid would induce this lack of sensitivity, the authors had earlier shown that tumour necrosis factor-alpha (TNF-alpha) had a similar effect. In the present investigation, the authors were able to show that TNF-alpha had a similar effect to cis-urocanic acid on the Ia antibody in the Langerhans cells, and also on the histological changes in these cells, and on the effect on contact hypersensitivity. Furthermore, they were able to show that prior injection of an anti-TNF-alpha preparation inhibited these effects. They therefore postulate: in sensitive strains of mice, UVB induces isomerisation of trans-urocanic acid; this in turn combines with a receptor, possibly in the Langerhans cell, but more likely in cells in the stratum spinosum, to produce TNF-alpha, which in turn is responsible for the changes in the Langerhans cells and the immunosuppression.

(l) Since it was recognised that immunosuppression was associated with ultraviolet radiation, and since the lymphocyte proliferation induced by phytohaemaglutinin (or concanavalin A) was inhibited by ultra-violet radiation, the authors tested, by two methods, the hypothesis that *cis*-urocanic acid might be the chromophore.

(i) Normal human lymphocytes from 6 healthy volunteers were cultured, and incubated for 4 days with either phytohaemagglutinin or concanavalin A; tritiated thymidine was added for

the last 6 hours of culture. In addition to control tests, *cis*-urocanic acid and *trans*-urocanic acid were added to the cultures in concentrations from 10^{-10} molar upwards. The results showed that *trans*-urocanic acid had no effect, but that *cis*-urocanic acid inhibited the incorporation of thymidine at concentrations of 10^{-2} molar (1.4 mg/ml) and above. It was noted that normal human skin contains about 0.4 % of *trans*-urocanic acid (wet weight) (this may be calculated to be roughly 2.9×10^{-2} molar or 4 mg/g); ultra-violet radiation *in vitro* converts about half the amount of *trans*-urocanic acid exposed to irradiation to the *cis*-isomer).

(ii) Six human volunteers were subjected to prick tests 4 months apart. Seven antigens were given (tetanus toxoid, diphtheria, tuberculin, etc: this was a ready-made preparation, "Multi-test Merieux"). A cream containing 5 % of finely divided powdered crystals of *cis*-urocanic acid or a dummy cream was applied to either forearm in a double-blind manner; the first application was 3 hours before the first prick test, and was repeated 3 times a day for 2 days. A second prick test was applied 4 weeks later; each subject served as his own control. The application had no effect on the delayed hypersensitivity.

(m) Phototoxicity is associated with PUVA treatment. A new bifunctional psoralen, which does not have this effect, is 4,4'5-trimethylazapsoralen (TMAP). This compound was investigated in mice.

It was known from earlier investigations that TMAP with low dosage UVA had induced such changes as reduced numbers of Langerhans cells and Thy-I^+ cells in BALB/c mice. In the present work, SPF female C3H/HeN(MTV^-) mice were used. The radiation used was 320-400 nm, controlled with a spectroradiometer.

TMAP in 70 % alcohol was applied to the shaved dorsal skin 3 times a week; 45-60 minutes after each application, 10 kJ/m^2 of ultra-violet radiation was applied to the dorsal skin. This schedule was continued for 4 weeks. The ears were shielded from irradiation. Controls were non-irradiated animals; in addition, some animals received drugs alone; others alcohol applications alone; others alcohol + ultra-violet radiation; others ultra-violet radiation alone. In yet another set of animals, 8-methoxypsoralen (8-mop) replaced the TMAP.

(i) Skin in the irradiated area was removed and examined for immune cells by staining and counting the numbers of dendritic cells.
(ii) Dorsal skin of irradiated mice was treated with dinitrofluorobenzene (DNFB); 6 days later a challenge with DNFB was made on each ear. These mice were then killed, and single cell suspensions were made from the spleen, which were injected intravenously into normal syngeneic mice. The recipient mice were then sensitised by DNFB and challenged 6 days later, as above.
(iii) "Twenty-four hours after the last treatment" i.e., probably after the last ultra-violet irradiation, the dorsal skin of the animals was painted with DNFB; 18 hours later, a single cell suspension was prepared from inguinal, axillary and subscapular lymph nodes. This suspension was injected into each hind foot pad of syngeneic mice. These latter mice were then challenged 8 days later with DNFB on the ears.

The results may be summarised as follows.

All animals treated with 8-mop + ultra-violet radiation showed severe phototoxicity; this was absent in those animals treated with TMAP + UVA, UVA alone, or drugs alone.

The number of immune cells in the skin was reduced by ultra-violet radiation alone and by alcohol + ultra-violet radiation; the addition of 8-mop or TMAP reduced the number of cells still further. The reduction in $ATPase^+$ cells and Ia^+ cells was significantly greater in the skin from animals treated with 8-mop + ultraviolet radiation, compared with that from animals treated with TMAP + ultra-violet radiation; the number of $Thy\text{-}1^+$ cells was reduced to the same extent in both groups.

Contact hypersensitivity. No change was found in skin from animals treated with drugs alone; despite changes in numbers of immune cells, ultra-violet radiation alone had no effect; but the addition of TMAP or 8-mop to the ultraviolet radiation produced marked decrease in contact hypersensitivity.

(iv) Transfer of reduction of contact hypersensitivity responses. Those animals receiving suspensions of spleen cells taken from animals treated with either 8-mop or TMAP followed by ultraviolet radiation showed reduced hypersensitivity. Thus it was concluded that lymphoid suppressor cells were present in the spleen following such treatment.

Cell suspensions from lymph nodes. Contact hypersensitivity was produced when DNFB challenge was administered, 6 days later, to recipient mice. This hypersensitivity was much reduced if the donor mice had previously been treated with 8-mop or TMAP + ultra-violet radiation. Thus, antigen presenting cells are functionally altered by such treatment.

(v) It is possible that the 50 % reduction of immune cells produced by ultra-violet radiation might be insufficient to cause decreased overall immune function; or, morphological changes may not correlate with impaired function. Doses of ultra-violet radiation alone and of TMAP + ultra-violet radiation were chosen so as to give about the same degree of reduction (about 50 %) of the numbers of cutaneous immune cells.

The ability of cell suspensions from lymphatic glands after such treatment to induce hypersensitivity was not affected by ultra-violet radiation alone, but was much reduced by ultra-violet radiation + TMAP. Thus there is a qualitative difference between the effects on hypersensitivity produced by ultra-violet radiation and that produced by ultra-violet radiation + TMAP.

(n) It is known that contact hypersensitivity (CHS) is depressed by psoralen + UVA treatment. This rather resembles the effect of UVB by itself, which is also known to be associated with systemic immunosuppression. In the present investigation, both monofunctional and bifunctional psoralens were investigated.

The animals used were C3H/HeNCr(MTV^-) and BALB/c AnNCr mice. UVB and UVA were produced from tubes which had outputs of 270-390 nm and 320-400 nm (wavelengths checked by spectroradiometer). The outputs at 20 cm were 4.1 and 22 J/m^2/second repectively.

(i) A keratinocyte cell culture line was used. It was exposed to UVB without psoralen, or to UVA with appropriate doses of the psoralen under test; after 12 hours, supernatant was taken for use in testing.

(ii) C3H mice were injected with 15 ug of supernatant protein; after 5 days, the mice were immunised with allogeneic BALB/c mouse splenic cells; after 6 days, the animals were challenged with the same cells by injection into each hind footpad. Suitable negative and

positive controls were used. This procedure demonstrated that delayed type hypersensitivity was suppressed by supernatant protein from cultures that had been exposed to UVB and also when the cultures had been irradiated with 200 to 500 J/m^2 of UVA + 400 ng/ml of 8-methoxypsoralen. Higher doses of UVA were cytotoxic.

(iii) The cultures were irradiated with UVA at 500 J/m^2; 8-methoxypsoralen was added in concentrations from 0 to 1000 ng/ml. Concentrations greater than about 200 ng/ml gave rise to a supernatant which reduced contact hypersensitivity.

(iv) The irradiation of the cultures with UVA was maintained constant at 500 J/m^2, and equimolar doses (1.85 nmoles/ml) of the following compounds added to the incubation: trimethylazapsoralen; 8-methoxypsoralen; 5-methoxypsoralen; angelicin; 4,4',6'-trimethylangelicin. All the agents had much the same effect in producing a supernatant which would inhibit delayed type hypersensitivity.

(v) Inhibition of contact hypersensitivity. Mice which had been injected with supernatant protein as above were tested for inhibition of contact hypersensitivity. After 5 days, dinitrofluorobenzene (DNFB) was applied to the abdominal skin. After 6 more days, a DNFB challenge was applied to each ear. Suitable positive and negative controls were used. Exposure of cultured keratinocytes to 200 J/m^2 of UVA alone caused the release of a factor into the supernatant which reduced contact hypersensitivity induced by DNFB. A dose of 50 J/m^2 was subthreshold, but at this dose the addition of 200 ng/ml of 8-methoxypsoralen caused release of a factor which suppressed contact hypersensitivity to DNFB.

The authors conclude that the dose relationships support the hypothesis that different mechanisms are involved in the suppression of contact hypersensitivity and delayed type hypersensitivity under the conditions of these experiments. The type of psoralen used does not seem to make much difference.

(o) In a similar investigation, the effect of UVA on immunosuppression was investigated. Mice of the C3H/HeN(MTV$^-$) and BALB/c strains were used. UVA was produced at wavelengths from 320 to 400 nm, measured with a spectroradiometer. An established mouse keratinocyte culture was used. The C3H mice were shaved on the back and subjected to 10 kJ/m^2 of UVA 3 times a week for 4 weeks. Some animals had 122 ug of 8-methoxypsoralen in 300 ul of alcohol applied ot the area 45 minutes beforehand (about 400 ug/ml). After 24 hours the animals were killed and the epidermal sheets stained for immune cells, which were counted.

Contact hypersensitivity (CHS) was induced by shielding the ears of the irradiated animals from the UVA. Twentyfour hours after the last treatment, the skin in the treated area was painted with dinitrofluorobenzene (DNFB). Both ears were challenged with DNFB 6 days later.

Delayed type hypersensitivity was induced by first immunising the animals with BALB/c spleen cells, 24 hours after the last ultra-violet irradiation. After 6 days, the same cells were injected into each footpad.

Induction of immunosuppressant material. A culture of keratinocytes was exposed to UVA, with or without the addition of 8-methoxypsoralen, followed by incubation for 12 hours. This

was given IV to C3H mice. Contact or delayed hypersensitivity was induced after 5 days, as described above.

The results were as follows: UVA irradiation with or without alcohol pretreatment gave a 50 % reduction in immune cells in the exposed skin; there was also altered morphology. These changes were more marked if topical 8-methoxypsoralen were used 45 minutes before the ultraviolet irradiation.

Contact hypersensitivity was not impaired after UVA treatment or 8-methoxypsoralen treatment individually. Thus the changes in the cutaneous immune cells (above) did not affect the response. However, the simultaneous use of 8-methoxypsoralen with ultra-violet radiation did reduce the responses markedly.

Delayed hypersensitivity responses behaved in the same way as the cutaneous hypersensitivity responses (above).

To produce immunosuppressant protein from cultured keratinocytes a dose of 100 J/m^2 UVA had to be applied to the culture; this produced a protein which suppressed contact hypersensitivity, but not delayed type hypersensitivity. However, the addition of a psoralen (probably 8-methoxypsoralen) to the culture as well as irradiation produced factors which suppressed delayed type hypersensitivity as well.

(p) In view of the known immunosuppressive effect of ultra-violet radiation below 340 nm, and the animal evidence that ultra-violet radiation at 340 to 440 nm may enhance immunity, in animal experiments, the authors decided to investigate human volunteers. The radiation was provided by a commercial sunbed device, which emitted radiation very carefully filtered to remove radiation below 340 nm, and also to remove radiation from 440 to 800 nm, and infrared radiation from 800 to 3000 nm. The radiation produced was checked by metering. The doses used were: 1,130,000 J/m^2 of UV-A1 and 1,290,000 J/m^2 of UV-A1-light (the distinction between these categories is not further commented upon). The respective values in W/m^2 were 750 and 860. In all, 14 irradiations were carried out.

Twenty-seven healthy volunteers were recruited; the test groups comprised 7 females and 6 males, and the non-irradiated control group 7 females and 7 males. Subjects giving a marked erythematous reaction to a test exposure were excluded. The whole body was irradiated for 50 minutes at each session. The experiments were commenced in November, to minimise any effects of natural insulation.

Tests for immunity were as follows:

(i) A “Merieux multitest” applied to the left forearm.

(ii) Counting of lymphocytes: total lymphocytes, and lymphocytes in the following categories: pan-T, T-helper (T4), T-suppressor (T8); and the T4/T8 ratio.

The left forearm is said to have been protected from radiation. The timetable of the investigation was as follows:

Before commencement: lymphocyte counts, multitest application.

Day 2: reading and scoring of multitest.

Day 5: begin phototherapy.
Day 23: end phototherapy: 14 irradiations in all, weekends excluded.
Day 33: lymphocytes counted, multitest applied.
Day 35: multitest read and scored.
Day 57: lymphocytes counted, multitest applied.
Day 59: multitest read and scored.

The results are considered for day 35 and day 59. On day 35, the reaction to the multitest was significantly reduced compared with the control on the "irradiated left forearm". The protocol, however, calls for the left forearm to be protected from radiation. No other differences were found. On day 57, no differences between the control and irradiated groups could be found. The authors conclude that they had failed to show any effect of exposure to these wavelengths on the immune status of the subjects. They review 2 other studies in which such differences were found, but the irradiation used in those investigations was not identical with that used in the present investigation.

(q) Ultra-violet radiation is known to stimulate cultured human keratinocytes to generate products which block spleen cell proliferation in the mixed lymphocyte reaction to antigenic stimulation. Cytokines are also produced which cause immunosupression in the intact animal. Human fibroblasts in culture which carry the chloramphenicol acetyltransferase gene (under the control of the HIV long terminal repeat promoter) are caused to express the gene by exposure to ultra-violet radiation.

The present investigation examines whether *cis*-urocanic acid produces these effects.

Human keratinocytes in culture were exposed to 200 J/m^2 of UVB or exposed for 1 hour to *cis*- or *trans*-urocanic acid. The cells were then cultured for 18 to 24 hours and the supernatant removed. Twenty ug of protein from the supernatant was injected iv into the tail veins of 2 or 3 C3H/HeN mice. After 5 days, subcutaneous injections of spleen cells from BALB/c mice were given to these animals. Seven days later, spleen cells were taken and mixed with gamma-irradiated BALB/c stimulator cells. The spleen cells were cultured for 4 days, and for the last 18 hours, ^{3}H-thymidine was added. The incorporation of the thymidine into the DNA was measured.

Human fibroblasts in culture were transfected by using a plasmid containing pHIVcatSVneo (the chloramphenicol acetyltransferase gene and the long terminal repeat chain of the HIV virus). The fibroblasts were incubated for 18 hours with *cis*- or trans-urocanic acid or (as positive control) exposed to 5J/m^2 of UVC (about 254 nm). Expression of the cat gene was measured by exposure to labelled chloramphenicol, followed by ethyl acetate extraction and TLC.

Results. The factors released by keratinocytes subjected to ultra-violet radiation significantly suppressed the ability of the C3H mouse spleen cells to proliferate. No effect of *trans*- or *cis*-urocanic acid at 10 ug/ml was found.

Ultra-violet radiation powerfully stimulated the expression of the cat gene by the transfected fibroblasts, but *cis*-urocanic acid gave the following results: 0.01 % (100 ug/ml) no effect; 0.1 % (1 mg/ml) a non-significant increase in cat activity of about 12 %; 1 % (10 mg/ml) a significant increase of about 28 %. The last concentration was highly cytotoxic.

In this investigation, urocanic acid does not seem to have had the same effects as ultra-violet radiation on the tests used; however, it should be noted that UVC was used in the fibroblast experiment.

(r) Mouse. This communication gives a short account of an investigation into a hypothesis that DNA damage initiates the immunological changes which follow ultra-violet radiation to the skin.

The excision repair of DNA damage in the mouse skin following ultra-violet radiation can be accelerated by the application of T4N5 liposomes (containing T4 endonuclease V) to the skin after exposure. In these experiments, the liposomal preparation was applied to mice immediately after ultra-violet radiation. The effect sought was prevention of suppression of delayed type hypersensitivity to *Candida albicans*. The hypersensitivity was unaffected by ultra-violet irradiation if the liposomes were applied; and inactivation of the T4N5 by heat treatment removed its ability to prevent the delayed type hypersensitivity associated with ultra-violet radiation. The authors therefore suggest that it is DNA which is the primary photoreceptor, and not urocanic acid.

In an abstract which seems to reproduce the same data as those summarised in reference, the authors again suggest that DNA is the primary photoreceptor in the skin for the suppression of immunity by ultra-violet radiation.

(s) Since it is known that exposure to UVB (280 to 320 nm) causes a dose related suppression of systemic cell-mediated immunity, it has been postulated (by the authors and others) that the *trans-cis* isomerism of urocanic acid in the skin in response to ultra-violet radiation is the photoreceptor for this effect. Since the absorption spectrum of urocanic acid lies partly in the UVA, the authors investigated the possibility that UVA might also cause immunosuppression.

Shaved mice were exposed to banks of fluorescent tubes consisting of either BlackLightBlue (Sylvania), Blue (F40B, Philips) or PUVA (Sylvania). Following irradiation, skin was removed from the treatment site and a non-irradiated site, extracted, and analysed for urocanic acid content by HPLC. A dose dependent isomerisation of urocanic acid was found at the irradiated site with all three tubes. Their efficacy in this regard, in descending order, was: PUVA, BLB, Blue. No further details are given.

(t) In experiments in female mice of the strain C3HBu/Kam(H-2^K), it was found that migration of dendritic cells to draining lymph nodes was produced by UVB. It is probable that this effect plays an essential part in the inhibition of contact hypersensitivity in the skin under these circumstances. This effect was enhanced if the skin was first sensitised with fluorescein isothiocyanate. The mediator of this response was possibly tumour necrosis factor-alpha. Neither the *cis*- nor the *trans*-isomers of urocanic acid had any effect on dendritic cell numbers in the skin, whether there had been previous sensitization or not. The authors conclude that the immunosuppressant action of urocanic acid acts by a different mechanism to that described in this work, and may not play a part in the suppression of hypersensitivity induced by UVB.

(u) In an important review article on urocanic acid and immunosuppression, Norval et al make the following points relevant to the present summary. Firstly, urocanic acid is the major absorber of ultraviolet radiation in the skin, and it may be the chief naturally occurring photoprotective agent in man. Secondly, it is formed by the deamination of histidine, and the

ratio of urocanic acid to histamine in skin may be important. Thirdly, it seems to be the chemical mediator of the transient alteration in immune surveillance following ultraviolet radiation. Fourthly, while the equilibrium ratio of *cis*/*trans* urocanic acid *in vitro* is 74 %, it is about 40 % in the superficial layers of human skin, following 32 mJ/cm^2 (= 2 MED) of ultraviolet radiation, falling to 15 % in the deeper layers. Fifthly, the absorption spectrum of urocanic acid is the only one which corresponds to those wavelengths which produce immunosuppression.

(v) In another article from the same laboratory, the authors studied the supression of the delayed type hypersensitivity response (DTH) to *Herpes simplex* virus (HSV) in a mouse model. The isomers of urocanic acid, and various analogues, were tested. It was known from previous work that prior painting of the skin with urocanic acid suppressed the subsequent DTH reaction to HSV. The results showed that the *cis*-isomer of urocanic acid was much more powerful than the *trans*-isomer in suppressing the DTH. However, several analogues were also nearly as powerful. For instance, the *cis*- and *trans*-isomers of 2-pyrrole-acetic acid (which lacks the N_3) were so; replacement of the N function in the latter compound with S (2-thiophene-acrylic acid) also yielded a potent inhibitor, and so on. Hydrogenation of the side chain (dihydrourocanic acid) also gave a compound which was potent; and histamine itself was not very much less potent than urocanic acid.

It may be wondered whether the activity of such a variety of analogues does not shed some doubt on the specifity of urocanic acid in suppressing DTH *in vivo*.

(w) Pane (1992) draws attention to the formation of a cyclobutane dimer of urocanic acid in the guinea pig skin *in vitro*, following irradiation. Its significance is uncertain, but it may be relevant to the matters discussed in this section.

(x) An investigation of the possible effects of urocanic acid on the reaction of human skin to DNCB was carried out. A group of 40 healthy subjects was recruited (32 female and 8 male), and tested in four groups each of 10 subjects. Members of a group were asked to apply a preparation of *cis*-urocanic acid to the lower half of the body, amounting to about half the surface area of the skin. The amount applied may be calculated to be about 0.8 mg/cm^2. The concentrations of urocanic acid applied by members of each group were, respectively, 0, 0.02 %, 0.2 % and 2.0 %. These applications were made daily for 17 days; the applications for the last 3 days were supervised by nurses. On day 18 a challenge dose of 40 ug of urocanic acid was applied (the author states that he recognises this to be a low dose, but says it might serve to "...maximise the chance that a subtle difference might be detected.") These applications were made to treated skin.

After a rest period of 21 or more days, subjects were challenged with four doses of DNCB applied to the inner surface of untreated skin of the upper arm; the inner aspect was chosen as an area with little exposure to sunlight. The doses of DNCB (ug) were 0, 3.125, 6.25 and 12.5. The reactions produced were graded clinically on a scale from 0 to 3, and the area of induration and the skin thickness were also measured. Spontaneous reactions to DNCB were commonly found after 10 to 20 days; there was no significant difference between the groups, despite different pretreatment with urocanic acid. Thus, there was no evidence that sensitivity to DNCB was affected by urocanic acid. In addition, a "subset of 20 patients" (how selected is not stated) was subjected to extensive haematological investigation, including determination

of lymphocytes, T cell count, B cell count, T helper cell count, T suppressor cell count, and the response of lymphocytes to various mitogens. Skin biopsies for counting Langerhans cells were taken before treatment with urocanic acid began, and daily during the first 14 days of treatment. It is not clear whether these biopsies were taken from the „subset of 20" or from all subjects. The results of the haematological investigations showed no significant difference whether or not urocanic acid had been used; there was also no definite trend with increasing concentration of urocanic acid in the treated groups. The Langerhans cell counts showed that skin treated with placebo had a significantly lower count than control untreated skin; this difference disappeared when untreated sites were compared with sites which had been treated with placebo at the end of the experiment. The finding in the early part of the experiment appears to be an aberration. It is difficult to understand the author's interpretations of the haematological findings. His tables 10 and 11 are reproduced and included for the reader's inspection (see next pages). Overall, urocanic acid appears to have had little if any effect on immune function or the response to DNCB, under the circumstances of the experiment.

(y) In a further experiment an attempt is made to see whether the elicitation of skin reactions to DNCB could be affected by prior topical urocanic acid. From internal evidence, it seems likely that the 20 subjects were the same as that "subset of 20" investigated in the previous study, but this is not stated. All had been sensitised to DNCB; the concentrations of DNCB used were lower than those used in the earlier work. From the individual records provided it is possible to determine that six subjects were tested with the dose of DNCB that had previously elicited a reaction; four subjects were tested with half of the dose which had previously elicited a positive reaction, and 10 subjects were tested with 10 % of the dose of DNCB that had previously elicited a positive reaction. The DNCB was applied after the application of 4 different concentrations of *cis*-urocanic acid to four different skin sites, on the previous day. Reading was carried out 2 to 4 days later. All subjects reacted to DNCB; although the intensity of the reactions varied with the subjects and the dose of DNCB, there was no suggestion that prior application of *cis*-urocanic acid had any effect.

TABLE 10

VARIANCES IN LABORATORY TESTS BY UCA TREATMENT GROUPS ON DAY 14

TEST	Urocanic Acid Concentration				
	0 %	0.02 %	0.2 %	2 %	Variance P
Langerhans Cells Mean Number	21.6 *	16.0	15.2	18.2	
Change in LC #	5.2	0.0	-1.2	-0.8	0.16
WBC $10^3/mm^3$	7.02	6.69	6.44	6.94	0.87
Lymphocytes mm^3	2320	2059	2355	2002	0.58
T Lymphocytes %	78	80	78	77	0.74
B Lymphocytes %	10	11	11	12	0.85
Helper Cells %	42	43	50	41	0.19
T Suppr Cells %	29	29	22	27	0.36
Helper/Suppr Ratio	1.4	1.6	2.4	1.6	0.10
Lymphocyte Prolif Control	2030	1107	1840	1274	0.63
Lymphocyte Prolif OKT3	67671	94311	85587	98517	0.76
Lymphocyte Prolif Pokeweed	58122	61525	44051	52891	0.72
Lymphocyte Prolif PHA	206808	207886	272293	210560	0.56
Lymphocyte Prolif (ConA)	283926	330701	336740	289837	0.91

* Langerhans cell count at contralateral control (no treatment) site is 16.4.
(From Dahl M.V. (41). To accompany UCA summary).

TABLE 11

SIGNIFICANT CHANGES OF PRE-AND POST-UCA TREATMENT LABORATORY VALUE

The Langerhans cell count in skin treated with placebo cream was higher than in skin not treated at all (contralteral arm).

Pretreatment values (Day 0) were compared to posttreatment values (Day 14). No significant differences were seen except:

Treated with placebo cream

Langerhans cells count fell* $p < 0.01$

Treated with UCA 0.02 % cream

Lymphocyte count fell** $p < 0.02$
Lymphocyte proliferation to media fell $p < 0.04$

Treated with UVA 0.2 **%** cream

No significant changes

Treated with UCA 2 % cream

Lymphocyte count fell *** $p < 0.04$
T helper cell count rose *** $p < 0.01$

* The mean Langerhans counts in untreated sites were the same as the post treatment placebo treated skin. The high mean count in the pretreatment placebo-treated skin appears to be an aberrant finding.

** Mean lymphocyte counts rose in placebo-treated subjects, but fell in all UCA-treated groups. The significance (if any) is unclear.

*** T helper cell % for placebo also rose ($p < 0.20$).

(From Dahl M.V. (41). To accompany UCA summary.)

Tests for effect on tumour production.

Hairless mouse. This is part of the paper describing the suppression of the contact hypersensitivity to oxazolone in the hairless mouse (mentioned above). It is recognised that UV irradiation induces immunosuppression, and that *trans*-urocanic acid is a strong candidate for the cutaneous chromophore involved. The *trans*-isomer is isomerised by UV irradiation.

In the investigation, groups of 15 mice were subjected to irradiation of 2.7×10^{-4} w/cm^2 of UVB (280-315 nm) and 5.2×10^{-3} w/cm^2 of UVA (315-400 nm). One group had applications of an o/w emulsion containing 0.2 % *trans*-urocanic acid; a second group had emulsion only, and a third group had the emulsion with a.i. but no irradiation. The sequence of the experiment was as follows:

Day 1: 100 ul of the emulsion (with or without the urocanic acid) was applied to the dorsal skin. Thirty minutes later, irradiation was carried out. A third group had the application of emulsion containing urocanic acid, but no irradiation. These applications were continued (probably daily) for 10 weeks.

Days 70 to 229 monitoring for tumour production. Day 230: Application of the tumour promoter croton oil, 100 µl of a 0.1 % solution, to dorsal skin daily for 4 weeks, to reveal latent tumours. Days 258 to day 314: observation, and final classification of tumours produced.

The results show that tumours were not produced in the absence of UV irradiation. Before and after the application of croton oil, the numbers of tumour bearing mice were much the same whether or not urocanic acid had been applied. However, there was a highly significant increase in the numbers of tumours per animal in those given urocanic acid even before the applications of croton oil (1.94 times as many) and the effect was even more marked after the croton oil (3.6 times as many). In addition, the animals treated with urocanic acid showed, as well as an increase in the number of tumours, a decidedly higher incidence of malignant tumours among them, compared with the animals irradiated but treated with the emulsion without the urocanic acid.

This paper also describes the study of immunosuppression by urocanic acid, which is briefly summarised above.

Since ultra-violet radiation is known to produce melanoma in certain strains of mice, and since this is associated with, and may be due to immunosuppression, an investigation was carried out in which immunosuppression due to ultra-violet radiation was investigated, and also the effect of this on the transfer of melanomatous tumours between syngeneic mice. The protocol was a very elaborate one, but may perhaps be briefly summarised as follows:

SPF C3H/HeN/Cr-(MTV$^-$) mice were used.

(a) Mice were treated with an amount of ultraviolet radiation known to be sufficient to reduce the numbers of Langerhans cells and impair contact hypersensitivity. The radiation used was 5 W/cm^2 over the wavelength 280-320 nm (measured by a spectroradiometer). Because of screening by the cage the dose-rate recived by the mice was 3 J/m^2 /second. The mice were irradiated for 27 minutes (4.8 kJ/ m^2) twice weekly for 4 weeks. The mice in this part of the experiment were not shaved, so that only the tails and ears were exposed to the full dose of radiation; one ear of each animal was protected from irradiation. Twenty-four hours after the

last irradiation, K1735 melanoma cells were injected into the pinna, but in some animals, apparently, the injection was delayed until 8 days after the last irradiation. The melanoma cells had been induced in the same strain of mouse by UV irradiation, and maintained in tissue culture. If the tumour cells were injected within 24 hours of the last irradiation, the percentage tumour incidence was increased over the first 3 to 4 weeks; however, the total increase in tumours was the same in both groups after 5 weeks. A control group also gave the same percentage increase in tumour growth; it is not clear from the test what the treatment protocol for this group was: probably irradiation was omitted. If the growth of the melanoma cells had been accentuated by an effect of the ultraviolet radiation on skin immunity, it would have been expected that the dendritic cells in those areas of skin would have been affected. While the numbers of $ATPase^{+}$, Ia^{k+} and $Thyl.2^{+}$ cells in the pinna were reduced, the time course of these reductions was not related to that of melanoma cell growth. In addition, a test for reduction of contact hypersensitivity in the unexposed pinna, using dinitrofluorobenzene induction on the irradiated pinna, was carried out. The degree of contact hypersensitivity was the same in the treated mice and in the control animals. Thus suppression of contact hypersensitivity did not seem to play a part in the growth of the melanoma cells.

(b) In this part of the experiment, mice were shaved on the ventral skin, and this area was exposed to 400 J/cm^2; the head and ears were protected. Control animals were identically treated without irradiation. This treatment was carried out on 4 consecutive days. Some of the animals were sacrificed and the exposed skin was removed for enumeration of dendritic cells; other animals were sensitised on the exposed skin with fluorescein isothiocyanate; and others were injected with melanoma cells in the irradiated area. In this part of the experiment, first, immune cells were reduced in the irradiated area; secondly, contact hypersensitivity induced with fluorescein isothiocyanate was considerably reduced compared with control animals; thirdly, however, the growth of the melanoma cells was unimpaired, whether measured by incidence or time of appearance of tumours.

(c) To test the effect of ultra-violet radiation on immunity to melanoma cells, fragments of such tumours were injected into one pinna and allowed to grow for 3 weeks; they were then removed by excising the ear. The mice used were treated with ultra-violet radiation (probably on the treated pinna); a control group was (probably) not irradiated, and it may be inferred that some mice were not treated with melanoma cells to induce immunity. Three weeks after removal of the pinna, mice were challenged with melanoma cells injected into the opposite pinna. There was no difference between the groups immunised with melanoma cells, although both showed less growth than controls not pretreated with melanoma cells. The results suggest that irradiation had no effect on immunity to melanoma. Overall, the authors feel that the enhancement of tumour growth by ultra-violet radiation is not due to immunological causes.

Hairless mouse. This investigation was designed to repeat “the photocarcinogenesis portion of a study in which the conclusion was reached that ultraviolet photoproducts of urocanic acid augmented ultraviolet photocarcinogenesis”. The protocol of this experiment was, however, somewhat different from that of the earlier study.

The study was carried out according to GLP guidelines of the USFDA. Five groups, each of 20 female albino hairless mice of the strain Crl SKH1 (hr/hr) BR were used (groups a, b, c, d, e). Daily applications of 100 µl of an o/w emulsion of urocanic acid were made 5 days a week, for

10 consecutive weeks. The concentrations of a. i. used were: 0, 0.2, 2 and 20 mg/ml. Animals of group e had the same applications as those of group d, but these animals were not irradiated. Animals of groups a to d were irradiated 5 days a week, shortly after the applications of urocanic acid, as follows. In the first week, a minimal inflammatory dose was administered daily from an SSR source (W/cm^2 UVA 2.7×10^{-3}; UVB 5.4×10^{-5}). This was increased by 20 % in the second week, and similarly in subsequent weeks, so that by the tenth week the dose of ultra-violet radiation was 2.8 times greater than at first. From weeks 33 to 36 inclusive, each mouse had applied 100 µl of an acetone solution of 12-*O*-tetradecanoyl-phorbol-13-acetate 3 days a week, initially at 32 µg/ml, and increasing in subsequent weeks to 64, 128 and 256 µg/ml. Mice were examined daily; tumours were looked for and recorded weekly. Any mice dying or sacrificed before the end of the experiment were subject to full macroscopic necropsy. At the end of the experiment all remaining animals were sacrificed and subjected to necropsy. Tumours were classified macroscopically at week 45. They were allotted to the groups: papillomas, squamous cell carcinomas, or tumours other than these. Representative tumours were sectioned and examined histologically by an independent pathologist. There was a good correlation between the clinical diagnoses and the histological ones.

Among the groups treated with ultra-violet radiation, there were no significant differences; the incidence of tumours, the intervals before their appearance, and so on, were the same. There was only one tumour bearing animal in group e (in which the animals were not irradiated). There was a suggestion from the histological evidence that the high dose of urocanic acid might have some protective effect, in that the percentage of carcinomas showing deep penetration was less in these groups than in the others.

The study was carefully carried out to a good protocol, and fully reported; it showed no evidence of a photocarcinogenic activity of urocanic acid.

In a paper by Forbes, from the same institute as that which carried out the negative carcinogenic study, there is a detailed examination of the discrepancies between the report of positive carcinogenicity by Reeves *et al* and the negative report by Sambuco *et al*. The paper may fairly be summarised as follows:

(a) The incidence of tumours in the study by Reeves *et al* did not differ significantly between the two treatments (ultraviolet radiation only and ultraviolet radiation + urocanic acid). Incidence is defined as the percentage of mice bearing at least 1 tumour greater than 1 mm in diameter.

On the other hand, the tumour yield (defined as the number of tumours per mouse) was much higher in the group treated with urocanic acid and ultraviolet radiation, compared with the group treated with ultraviolet radiation only. (The figures: respectively, urocanic acid only, ultraviolet radiation only, and urocanic acid + ultraviolet radiation: all tumours, 0, 51, 141; papilloma, 0, 82.4, 61; carcinoma in situ, 0, 15.7, 30.5; squamous cell carcinoma, 0, 2, 8.5).

(b) The author states that Reeve et al determine tumour yield by the total number of tumours/number of affected animals. Most workers prefer to record the total number of tumours/all surviving animals. The former method gives higher values than the latter, since in the latter the denominator must be higher (unless, indeed, all animals bear tumours). He suggests that the figures for tumours in the animals treated with ultraviolet radiation + urocanic

acid in the Reeves study are what would be expected historically with ultraviolet radiation with or without treatment with urocanic acid, and considers the control figures an aberration. "Panel 8", from Forbes is included for inspection.

(c) The author claims several advantages for the study by Sambuco et al: it used SPF mice, individual housing, and 3 concentrations of urocanic acid (instead of one). In addition, he claims that the published figures for ultraviolet radiation in the report by Reeves et al are less than the amount of ultraviolet radiation actually used, on the basis of correspondence with the latter authors.

(d) The negative findings in the study by Sambuco et al correspond to historical controls. Forbes concludes his analysis with a theoretical justification for preferring the methods employed by Sambuco et al to analyse tumour production.

On the whole, the protocol of the experiments of Sambuco et al seems superior to that of the experiments of Reeves et al; but I do not think that the analysis of Forbes satisfactorily accounts for the differences between the groups with and without urocanic acid in the latter study.

11. Conclusions

Acute toxicity was low.

Dermal absorption studies suggest a low absorption, but studies are complicated by the physiological presence and synthesis of urocanic acid in the skin. Tests for absorption in skin of hairless mouse *in vitro* in some experiments, showed greater amounts in skin + receptor fluid than had been applied; in other experiments there was perhaps a small degree of absorption. In cadaveric human skin *in vitro* there did not seem to be any absorption. Application to the skin of volunteers did not lead to any increase in the amount of urocanic acid found in strippings, when compared with controls. Percutaneous absorption seems slight, but there is considerable variation.

Dermal irritation was not produced by an application containing 2 % of urocanic acid, but this is the use concentration. In other animals tests, no irritation was produced, but the concentrations used are not stated.

Up to 10 % of urocanic acid is reported to be non-irritant in the eye.

Tests for phototoxicity and photosensitization in guinea pigs were negative.

Tests for mutagenic activity in *S. typhimurium* and in *E. coli* were negative, as were tests for chromosomal aberration *in vitro* in Chinese hamster lung cells. Unscheduled DNA synthesis in human fibroblasts *in vitro* was not induced.

A test for unscheduled DNA synthesis in human fibroblasts *in vitro* following exposure to UVB at 500 J/m^2 was negative.

Urocanic acid forms an adduct with calf thymus DNA *in vitro*; this was not increased by exposure to UVB at 100 kJ/m^2.

One test for photocarcinogenicity in mice was negative. In a different set of experiments in mice an increase in tumours was found, but these findings have been criticised as being analysed by methods which would exaggerate the apparent incidence of tumours.

Most of those reported experiments, which were designed to study the effects of urocanic acid on the reduction of immunity by exposure of the skin to ultraviolet radiation, showed that urocanic acid enhanced the effects of ultraviolet radiation on reducing immunity.

The evidence is strong that this compound, when applied to the skin, enhances the effect of ultraviolet radiation in reducing immunity. Under these circumstances, the subgroup feels that it cannot conclude that the compound is suitable for use in cosmetics.

Classification: D

OPINIONS ADOPTED DURING THE 57TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 21 October 1994

S 27: *PROPENOIC ACID 3-(4-METHOXYPHENYL)-3-METHYLBUTYL ESTER*

1. General

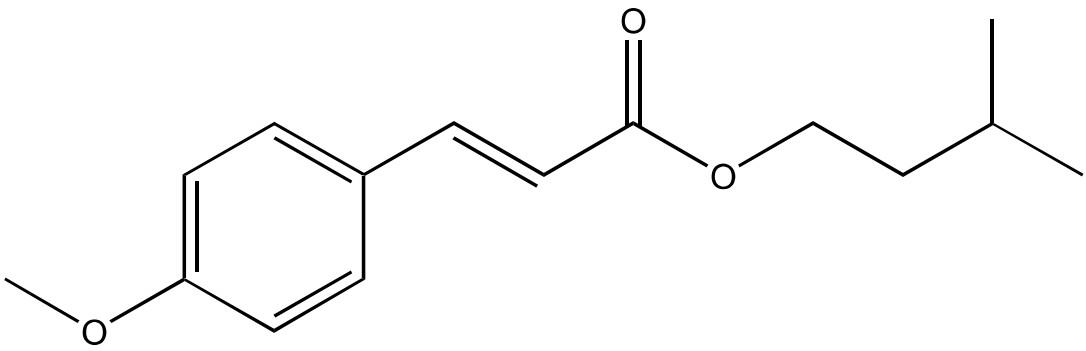
1.1 Primary name

Propenoic acid 3-(4-methoxyphenyl)-3-methylbutyl ester

1.2 Chemical names

Propenoic acid 3-(4-methoxyphenyl)-3-methylbutyl ester, mixed isomers
Isopentyl-4-methoxycinnamate

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{20}O_3$
Mol weight: 248.4

1.7 Purity, composition and substance codes

Clear yellowish liquid, not less than 98 % pure; absorption maximum 308 nm.

1.8 Physical properties

Subst. code: $C_{15}H_{20}O_3$
Appearance: Clear yellowish liquid.

1.9 Solubility

Soluble in oils, ethanol, isopropanol. Immiscible with water.

2. Function and uses

Proposed for use as a sunscreen in concentrations up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. Oral. Values of 9.6 to 9.9 g/kg b.w. were found. No details are given.

3.2 Acute dermal toxicity

Acute dermal toxicity testing was carried out according to OECD guidelines. No abnormalities were found up to 20 g/kg b.w.

3.4 Repeated dose oral toxicity

Rat: A 3 week oral toxicity study was carried out as a range finding study. Four groups, each of 5 m and 5 f animals, were used. The doses were 0.3, 0.9 and 2.7 ml/kg b.w./day suspended in 0.8 % hydroxypropylmethylcellulose and given by gavage (in mass units, 312, 935 and 2805 mg/kg b.w./day). There were no deaths. There was decreased weight gain in both sexes at the high dose. All animals were subjected to necropsy. At the top dose, the absolute and relative weights of the spleen and thymus were significantly decreased in both sexes. In males, the weights of the gonads were significantly reduced at the top dose. At 2.7 ml/kg b.w./day in males, and at 2.7 and 0.9 ml/kg b.w./day in females, the weights of the liver were increased significantly, and those of the spleen and thymus reduced.

Relative organ weights: In the text of the report the relative weights of spleen, thymus and gonads in males are stated to be decreased significantly at the top dose, and of the spleen and thymus in females. In the tables of the report, increased relative liver weights are seen at all dose levels, most pronounced in males at 2.7 ml/kg b.w. and in females at 2.7 and 0.9 ml/kg b.w. The NOAEL may be 0.3 ml/kg b.w./day.

3.5 Repeated dose dermal toxicity

Guinea pig: Twelve animals were used. The material applied is not specified: it may have been undiluted a.i. It was rubbed into the clipped skin of the flank for 30 seconds daily for 5 days. The test is stated to have been negative; no details are given.

Man: Thirty subjects were tested by applying undiluted a.i. to the skin of the back or of the inside of the forearm, followed (probably) by occlusion for 24 hours. No irritation is said to have been produced. No details are given.

Man: Tests were carried out on 65 m and 45 f patients hospitalised for various skin diseases. Three concentrations of a.i. in soft paraffin were tested: 1%, 5% and 10 %. They were applied to disease free areas of skin of the back by means of a Finn chamber. Contact time was 24 hours; reading was at 24 and 48 hours. In 15 subjects, the test was repeated one or more times. No adverse reaction was found in any test.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Following a preliminary study, the doses chosen were 0, 20, 200 and 2000 mg/kg b.w./day, administered daily by gavage 7 days a week for 13 weeks. Four groups of animals were used, each containing 15 m and 15 f. All animals were subjected to necropsy after sacrifice, and animals dying during the trial were subjected to necropsy as soon as possible after death. A wide range of tissues was fixed, and all from the control and top dose groups were subjected to histological examination. There were four deaths during the experiment: 1 control, 2 at 20 mg/kg b.w./day, and 1 at 200 mg/kg b.w./day. Weight gain was reduced in all animals at the top dose. Haematological changes were found, which were rather variable; in summary, it may be stated that the haemoglobin and MCHC values were increased at the top dose in both male and female animals at the end of the first and third months. There were many changes in the values obtained by clinical chemical analysis. The main ones, which may be significant, were: at 1 month, AP and GOT were increased at the top dose in both sexes, and cholesterol was reduced. The same finding was made at 3 months, and in addition the GPT was raised in female animals at that time. There were no urinary abnormalities.

Organ weights: (a) Absolute weights. At the top dose, both sexes showed increase in the weight of the liver; in females, the weight of the spleen was reduced, and in males the weight of the testis was reduced. (b) Relative weights. At the top dose, the weights of the liver and kidneys were increased. In males, the weight of the heart was increased and that of the spleen and adrenals slightly reduced. In females, the weight of the spleen was reduced.

The histological findings at the top dose showed patchy areas of increase in size of hepatocytes with clear cytoplasm and large nuclei. There was also increased iron-containing pigment in the spleen of both sexes and in the Kupfer cells of the liver in females. These changes were not seen at the lower dose levels. In sum the findings indicate that at the top dose there are effects on the liver, and possibly increased breakdown of red cells. The NOAEL is 200 mg/kg b.w./day. This appears to have been a well conducted study carried out according to OECD guidelines.

4. Irritation & corrosivity

4.2 Irritation (mucous membranes)

Chick: Applications of 0.2 ml of dilutions of a.i. in olive oil were made to the chorioallantoic membrane. The text gives data for tests in 1 egg only for each of the concentrations 1%, 10 % and control. The results were negative. This test is not yet officially recognized for this purpose.

Rabbit: Eight animals were subjected to a Draize test. A 50 % solution in olive oil was instilled into the conjunctival sac. In 4 animals rinsing was carried out. The result was reported as negative. No details are given.

5. Sensitization

Guinea pig: Twelve animals were used. The concentration used is not stated: it may have been undiluted a.i. It was rubbed into the flank skin for 30 seconds daily, 5 days a week, for 3 weeks.

After a 5 day rest, the a.i. was applied to the skin of the opposite flank daily for 3 days. The test is reported as negative. No details are given.

Man: Ten subjects had undiluted a.i. applied twice weekly to the same site for 7 applications. After 12 days a challenge application with undiluted a.i. was made. No abnormality was found. No details are given.

6. Teratogenicity

Fertile hen's eggs: Groups of 20 eggs were tested. The dose applied was contained in 0.1 ml of olive oil. The amounts applied were 0, 0.25, 0.625, 2.5 and 6.25 µl a.i. per egg. Injections were given into the white of the egg on day 1 of incubation in one series and on day 5 in another. The LD_{50} of injections on day 1 was 5.8 µl, and on day 5, 1.15 µl (approximately 120 and 25 ppm respectively). Deaths of embryos during the incubation were dose related. Following hatching, the chicks were anaesthetised and bled. The only abnormality found was a statistically significant reduction of blood glucose at 0.25 and 6.25 µl, but its biological significance is doubtful. [This test is not regarded as adequate for an evaluation of teratogenic or embryotoxic effects. In addition, injections are usually made into the yolk sac, or sometimes into the air space, and not into the white of the egg, as here.]

Rat: A study of the teratogenic and embryotoxic properties of the a.i. was carried out according to GLP. The a.i. was dissolved in 3 ml of olive oil and given daily by gavage in doses of 0, 0.25, 0.75 and 2.25 ml/kg b.w./day, from days 6 to 15 (inclusive) after mating. A positive control was used: tretinoin, similarly administered, at a dose of 15 mg/kg b.w./day. At day 20 the animals were killed by ether anaesthesia and subjected to post mortem examination. The foetuses were weighed, and about half of them were subjected to visceral examination and the remainder to skeletal examination.

The chief findings in the dams during the experiment were: a loss of weight in the high dose animals; an increase in water consumption in the high dose animals throughout the experiment, and in the low and intermediate dose animals in the second half of the experiment; a decrease in food intake in the intermediate dose animals in the first half of the experiment, and in the high dose animals throughout the experiment; and a dose related increase in hair loss in all dosed groups and in the positive control animals.

At necropsy, the weight of the adrenal glands was increased in the high dose animals; the weight of the liver was increased in the low dose animals, but this was not thought to be of biological significance.

The effect on fetuses were as follows. There was a dose related increase in intra-uterine mortality. There was a fall in foetal weights in the high dose animals and in the positive control animals. This was a well conducted study, and the a.i. does not show any teratogenic activity; the NOAEL was 780 mg/kg b.w./day. The positive control animals showed numerous foetal abnormalities.

7. Toxicokinetics (incl. Percutaneous Absorption)

Rat: Five experiments in all are reported; they are designated by the author by the letters A, B, C, D and E.

Experiment A: A 10 % formulation of ^{14}C a.i. in a w/o emulsion was applied (weight of formulation applied 210 mg) to the clipped skin of 3 m and 3 f rats for 24 hours, covering an area of 2.5 x 3.5 cm (this area was the same for all the subsequent experiments). A non-occlusive dome was applied over the area. A large number of organs was examined after sacrifice, but the account is confusing. The authors seem to suggest that absorption may be determined by summing the radioactivity in carcass + urine + faeces; this amounts to 11.24 %. Although there seems to have been some radioactivity in the various organs examined, the data given do not permit of any calculation of the amounts.

Experiment B: The same formulation was used in 1 female animal (weight of formulation applied 230 mg). The area was covered with an occlusive polyethylene sheet for 3 days. The total amount of radioactivity over the period in urine + faeces was 15.8 %. The carcass value was 0.7%, so that the total absorbed over the period is the taken to be 16.5 %.

Experiment C: A 10 % o/w formulation was used (weight of formulation applied 220 mg). One female animal was tested. A non-occlusive dome was sutured to the skin under anaesthesia, and the preparation allowed to remain in contact for 7 days. The total of the percentages of radioactivity for urine + faeces over the period was 64.8 %.

Experiment D. This was the same as C except that a 10 % w/o formulation was used (weight of formulation applied 180 mg). The total of radioactivity for urine + faeces over the period was 70.5 %.

Experiment E: One animal was used. A 10 % o/w emulsion was used (weight of formulation applied 200 mg) and the area of application covered with a non-occlusive dressing. After 6 hours, the area of application was washed and the dressing reapplied, and allowed to remain in place for 7 days. The amount found in the urine + faeces over the period was 3.18 % of the amount applied.

The report is difficult to interpret. It may be concluded that over a period of 6 hours, about 3 % of a.i. is absorbed from an application area of 8.75 cm^2, using a 10 % formulation; over 7 days about 70 % is absorbed.

Man: After 30 minutes exposure to formulations containing 10 % a.i., the skin was repeatedly stripped at the site of application. It is stated that OECD guidelines were followed. The formulations were w/o emulsions, one of which contained 13.5 % of liquid paraffin; the other contained 10.5 % of liquid paraffin + 3 % „Eusolex 8020“ (the sunscreen 4-isopropyl-dibenzoylmethane). The a.i. was labelled with ^{14}C. About 3 mg of each formulation was applied without conclusion to two different areas of the forearm, each measuring 2 cm^2. The period of exposure was 30 minutes. The subjects were 2 males and 4 females. Using the first formulation, the amount in the first two strips were 42.27 % and 13.28% respectively. (The area of application is not stated to have been washed before stripping). The authors suggest this may be ignored as being present only in the most superficial layers of the skin. The remaining strips yielded 42.21 % of the applied radioactivity. The amounts found in the strippings with the second formulation were not significantly different. There was a significantly higher amount of radioactivity in the strippings from the females than from the males. There was slight or definite erythema for up to 24 hours in 4 of the subjects treated with the first formulation. The results are difficult to interpret; if the results in all the strippings are taken into account, the

formulae developed by Rougier *et al.* suggest an absorption of 60 to 70 % over 4 days, or 60 to 70 mg/kg b.w.

8. Mutagenicity

Ames test. A standard Ames test was carried out, using a.i. dissolved in DMSO, up to 10 mg/plate. No evidence of mutagenic activity was found. With strains TA 1538 and TA 98, the level of revertants was some 3 to 5 times higher after activation, both with the vehicle control and the a.i. This may be related to the fact that the investigator used phenobarbitone + 5.6-benzoflavone as an inducting agent, instead of the customary Aroclor.

A second test using strains TA 98, TA 100, TA 1535 and TA 1537 was carried out. In this case precipitation was noted at levels greater than 5 mg. There was no evidence of mutagenic activity.

Mouse. Micronucleus test. The dose levels were 750, 1500 and 3000 mg/kg b.w., dissolved in olive oil and given as a single intraperitoneal injection. All animals showed toxic effects, most marked at the top dose. There was no evidence of abnormal micronucleus formation.

Human lymphocytes **in vitro**. The test was carried out according to GLP standards. Human lymphocytes were cultured and exposed to concentrations of a.i. in DMSO determined by preliminary toxicity testing, as follows: without activation 0, 10, 30, 100 µg/ml; with activation 0, 30, 100 and 300 µg/ml. The top doses gave 55 % to 70 % toxicity. Positive controls were cyclophosphamide and mitomycin C. Tests were carried out in duplicate. The cells were exposed to a.i. for 24 hours; they were then washed and cultured for a further 24 hours. At least 100 metaphases from each culture were counted.

There was a slight tendency to an increase in the number of gaps with increasing dose of a.i., but the authors report the test as negative, by comparing the values with those of the historic controls.

Test for photomutagenic activity.

A test was carried out, according to GLP, using two strains of *S. typhimurium*: TA 1537 and TA 102.

The tests were carried out in the same manner as the conventional Ames test. The positive control for TA 1537 was chlorpromazine, and for TA 102, 8-methoxypsoralen. The active ingredient and 8-methoxypsoralen were dissolved in DMSO; there was a tendency for the development of precipitates of the active ingredient at 5000 µg/plate. Chlorpromazine was dissolved in water. Each experiment was carried out twice. Metabolic activation was not used. The sensitivity of the strains to mutagenic effects was confirmed, before each set of experiments, by using plates containing 9-aminoacridine for TA 1537 and cumenehydroperoxide for TA 102, in each case without ultraviolet radiation. A xenon arc was employed to produce the ultraviolet radiation, and the intensity of the radiation was measured at the level of the plates. The values were (experiment 1): UVA/UVB 6.9/0.48, 13.8/0.96, 20.7/1.44, and 41.4/2.88 mJ/cm^2. There were trifling differences between these values and those measured in the second experiment. The doses of active ingredient used were 8, 40, 200, 1000 and 5000 µg/plate.

With TA 1537, some toxicity was seen at 40 µg/plate and above; precipitation was noticed at 5000 µg/plate. In the absence of ultraviolet radiation, there was no increase in the number of revertants in any plate. In the presence of ultraviolet radiation, there was an increase in the number of revertants with chlorpromazine, but none with the active ingredient. With TA 102, toxicity was not found, but precipitation occurred at 5000 ug/plate. In the absence of ultraviolet radiation there was no increase of revertants; when ultraviolet radiation was used, there was an increase in revertants with 8-methoxypsoralen, but not with the active ingredient. There was no evidence of photomutagenesis.

10. Special investigations

Tests for capacity to produce phototoxicity and photoallergy.

Guinea pig. Fifty animals were used in a maximisation procedure, according to the method of Guillot et al. GLP guidelines were followed. From preliminary experiments, it was decided to use a 50 % solution of a.i. in ethanol/DEP 1:4 as a nonirritant concentration for the tests. Irradiation was delivered from two lamps, which produced wavelengths from 285 to 400 nm. The two control groups (Ia and Ib) consisted each of 3 m and 3 f animals, and were treated identically with the respective test groups except that they were not irradiated. The two test groups (IIa and IIb) each contained 10 m and 10 f animals. Animals of group IIa had applications of the solution containing a.i.; those of group IIb had vehicle only. Both of these groups were irradiated.

(a) Phototoxicity. A single application of 0.5 ml of the solution of a.i. (test animals) or of vehicle (control animals) on a piece of gauze 2 cm x 2 cm was made to the depilated skin of the back. After 90 minutes, this was removed, and, in animals of groups IIa and IIb, immediately followed by irradiation. This consisted of exposure to both lamps for 5 minutes, followed by a 90 minute exposure to the lamp producing the longer wavelengths. The total irradiation was 12.5 J/cm^2, and amounted to a minimal erythema dose. The site was inspected after 24 hours. Any reaction was compared with that produced in the area surrounding the patch, which had also been exposed to a m.e.d.

(b) Photoallergy: Four days after the first test, using the same animals, intradermal injections of Freund's complete adjuvant (diluted with saline 50/50) were made at each corner of the site previously tested. The patches and irradiation were repeated. Further applications of patches and irradiation were made on days 7 and 9. A rest period of 14 days ensued. On day 23, a new site on the back was depilated and patches applied as before. The irradiation on this occasion, however, was from the lamp producing the longer wavelengths only, for 90 minutes. Tests on other Guinea pigs had shown that this irradiation did not of itself produce any skin reaction. Readings were made at 6, 24 and 48 hours.

Result: There was no evidence of any phototoxic or photoallergic reaction in any animal. There were no formal positive controls, but in an appendix the findings of a series of experiments using the same protocol are given. In these a wide range of chemicals capable of producing phototoxic and photoallergic reactions was tested (e.g. 8-mop, 5-mop, angelica extract; and promethazine, 3, 5, 4-tribromosalicyclamide, etc.). These gave the expected positive results.

Man: Ten subjects had undiluted a.i. applied by means of an occluded patch for 24 hours. The area was then exposed to UV irradiation of an intensity slightly below the m.e.d. No abnormality was seen. No details are given.

11. Conclusions

Acute and subchronic toxicity are low. Tests for irritation of mucous membranes and skin were negative. Tests for sensitization were unsatisfactory, but the results of the tests for photoallergenicity permit the deduction that sensitization is unlikely; the substance is a very rare allergen and photoallergen in clinical practice. Tests for teratogenicity were negative. There was no evidence of mutagenic or photomutagenic activity in tests with *S. typhimurium*. The tests for percutaneous absorption are difficult to interpret.

Tests for chromosomal aberration *in vitro* under the influence of ultraviolet radiation should be carried out, and studies of percutaneous absorption giving clearcut results.

Classification: C

OPINIONS ADOPTED DURING THE 58TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 3 February 1995

COAL TAR

1. General

1.1 Primary name

Coal Tar

1.2 Chemical names

Tar, coal (chemic, IUPAC)
Coal tar (CA index name) (INCI/CI name)
Goudron de houille (Fr.)
Pix ex carbone (EP name)

1.3 Trade names and abbreviations

Pix carbonis[1] (Brit. Pharmacopeia): product of destructive distillation of bituminous coal at approximately 1 000°C

Coal tar solution[2] (USP XIX): alcoholic solution of coal tar (20% m/v)

Pix lithantracis[3] (Pharm. Belge) - product of the dry distillation of coal

Crude coal tar[4]

Coke oven tar, Coking tar, Supertar, Estar, Lavatar, Pixalbol, Zetar, Tarcrome 180, KC 261, Carbo-cort, Polytar bath, etc.

Coal oil = high temperature coal tar

Usage categories and concentrations
- Shampoos 2 %
- Hair lotions 1 %
- Soap 1 %

1.4. CAS no.

CAS No: 232 - 361 - 7
EINECS No: 8007 - 45 - 2

1.7 Purity, composition and substance codes

EINECS definition (UVCB index)[5]

[1] Synonym communicated by COLIPA (22/10/93)
[2] Synonym communicated by COLIPA (22/10/93)
[3] Synonym communicated by COLIPA (22/10/93)
[4] Synonym communicated by COLIPA (22/10/93)
[5] UVCB-Complex substances requiring definition

By-product of destructive distillation (carbonisation) of coal.

Almost black semi-solid. A complex combination of aromatic hydrocarbons, phenolic compounds, nitrogen bases and thiophene.

N.B.: Depending on distillation temperature, a distinction is made between:
low-temperature tars (distillation temperature < 700°C)
high-temperature tars (distillation temperature > 700°C)

7. Toxicokinetics (incl. Percutaneous Absorption)

In vivo cutaneous metabolism in animals

Test material: USP coal tar
Concentration and vehicle: 20 % (m/m) in ethanol + polysorbate 80 (= standard USP solution)
Species tested: 6 x 6 newborn rats + 6 pregnant rats

Method:

1 After one cutaneous application, *in vivo* determination of the Acryl Hydrocarbon Hydrooxylases (AHH) activity in the skin and liver of newborn rats, pregnant mothers and foetuses.
The newborn rats are treated 4 to 6 days after birth with one application of 100 µl USP solution of coal tar and sacrificed 24 hours later.
The pregnant female rats are shaved and treated on the 19th day of gestation with one application of 500 µl of USP coal tar solution and sacrificed 24 hours later. The foetuses are removed.

2 Effects of applying different constituents of coal tar.
The newborn rats are treated with application of benzene, naphthalene, acridine, anthracene and benzo-alpha-pyrene at a single dose of 100 µg/kg pc in acetone and sacrificed 24 hours later.
The controls are treated with 100 µl acetone applied in the same conditions.
In each test, the skin and liver of six animals are collected for one determination of the induction of AHH activity.

Results:

1 Newborn rats - increase in AHH activity by a factor of:
10 in the isolated epidermis
18 in the isolated corium
15 in the entire skin

N.B.: Large variations in cutaneous and liver AHH activity are observed in the controls, which are housed in cages adjacent to those of the animals treated with coal tar. These variations are attributed to coal tar fumes.

Pregnant rats - increase in AHH activity by a factor of:
3.8 in the entire skin of the mothers
4.8 in the mothers´ liver
2.0 in the entire skin of the foetuses
1.9 in the foetuses´ liver

Benzene and naphthalene do not affect AHH.

Acridine does not affect liver AHH but induces significant cutaneous AHH activity (factor of 2.2).

Anthracene produces a comparable induction of cutaneous and liver AHH (factor of 2.7).

Benzo-alpha-pyrene is the most powerful inductor with an increase by a factor of 8.8 in the skin and 7.7 in the liver.

Cutaneous metabolism in volunteers

Test material:	USP coal tar
Concentration and vehicle:	20 % (m/m) in ethanol + polysorbate 80 (= standard USP solution)
Species tested:	6 volunteers suffering from psoriasis
	3 volunteers suffering from atopic dermatitis

Method:

Clinical study - Measurement of induction of AHH in human skin *in vivo* and *in vitro*

1 100 µl of standard USP solution (= 20 mg coal tar) are applied to the lumbar region on a 1 cm-diameter area of skin which is not clinically altered. For control purposes, an equivalent surface situated at least 10 cm from the initial surface is treated with the vehicle only.

AHH activity is measured in homogenates of entire skin prepared from biopsies taken 24 hours after contact.

2 In parallel, surgically-obtained human skin samples, less subcutaneous fat and homogenised, are incubated over 24 hours in a culture medium with or without 0.05 ml of standard USP solution (= 50 mg coal tar).

Results:

1 *In vivo*, application of the standard USP solution provokes an increase in cutaneous AHH activity by a factor ranging from 3.3 to 5.4 in the nine patients treated.

2 Similarly, an *in vitro* increase in AHH activity is observed in skin homogenates cultivated in the presence of coal tar. The activity induced depends on incubation time, the concentration of tissue proteins and concentration in substrate.

Toxicokinetics in volunteers

Test material:	Coal tar, pharmaceutical quality
Concentration and vehicle:	10 % (m/m) in a therapeutic zinc oxide ointment
Species tested:	9 volunteers in good health

Method:

1 Determination of percutaneous absorption speed of polycyclic aromatic hydrocarbons (PAHs), through measurement (luminescence) of surface elimination. The ointment is 1

applied to four volunteers at a dose of 2.5 mg/cm^2 (forehead, shoulder, forearm, palm of hands, thigh and ankle) during 45 minutes.

After standard rinsing, surface elimination is measured using a fibre optic luminoscope during 55 hours.

A negative control is effected by applying the vehicle on its own.

Results:

Measurement of luminescence directly after rinsing the skin indicates that approximately 0.04 % of the applied dose remains on the skin after rinsing.

The absorption speed constant (Ka) ranges from 0.036/h to 0.135/h with an average of 0.066/h. (On the basis of this constant, the author estimates that 20 to 56 % of the PAH dose is absorbed after six hours.) (?)

$\frac{dX}{dt} = -KaX$, where X is the luminescence signal.

2 Measurement of urinary excretion of the 1-OH pyrene metabolite via HPLC. A dose of 2.5 mg/cm^2 of the ointment corresponding to 10 n mole pyrene/cm^2 or 2 µg pyrene/cm^2 is applied under occlusion to 400 cm^2 skin (five different sites) in eight volunteers and rinsed six hours after application.

All the urines are collected from 24 hours before application to 72 hours after application and are analysed via HPLC to determine the presence of the 1-OH pyrene metabolite in free and conjugated form.

Results:

The quantities of 1-OH pyrene excreted in urine vary from 7.7 n moles to 14.6 n moles (average: 11.6 n moles or 0.29 % of the applied dose).

Bearing in mind that 46 % of the pyrene available in the system appears in the form of 1-OH pyrene and conjugated derivatives, that 90 % of the latter are excreted in the urine and that urinary excretion of 1-OH pyrene returns to the normal base level during the measurement period, the author estimates that 0.3 to 1.4 % of the pyrene present in the ointment is absorbed with an absorption speed of between 5 and 23 p moles/cm^2/hour.

N.B.: Cutaneous absorption of the pyrene is comparable to that of the other PAHs.

8. Mutagenicity

Genetic Mutations in Bacteria (1)

Test material:	Therapeutic preparations of coal tar
Concentrations and vehicles:	1. ZETAR emulsion: 30 % (m/v) CCT in polysorbates (bath products: 15 to 25 ml) 2. ESTAR gel: 0.6 % (m/m) “refined” coal tar in a hydro-alcoholic gel (ointment for local usage)

3. LAVATAR solution: 25.5 % (m/m) distillate of coal tar in colloidal + surfactant solution (bath product: 1 to 2 tablespoons)

4. USP solution: 20 % (m/v) in ethanol + polysorbate solution (used in shampoos and ointments)

Method:

Ames Salmonella/microsome test

The preparations are tested in DMSO at doses of 10 to 200 µg tar/plate vis-à-vis Salm. Typh. (LT_2), TA98, TA1538 and TA100 in the presence of 50 µl of an Sg mixture.

Benzo-alpha-pyrene mutagenicity is determined to control Sg activity and the sensitivity of the strains tested. The average of his revertants/µg tar is calculated in the rectilinear part of the dose/response curves.

Results:

All the preparations are mutagenic for the three strains tested in the presence of Sg at doses < 100 µg* tar per plate.

The TA98 strain is the most sensitive.

For the same concentrations in DMSO, the decreasing order of mutagenicity is as follows:
Zetar 7.0 revert/µg tar
Estar 3.8 revert/µg tar
Lavatar 2.0 revert/µg tar
CTS 1.4 revert/µg tar

*N.B.: The quantity of tar applied to the epicranium in a single application (10 ml) of a hair lotion containing a 1 % dose is of the order of 1000 times this dose.

Genetic Mutations in Bacteria (2)

Test material: Coal tar based shampoos
ZETAR emulsion
TERSA tar
PENTRAX
POLYTAR

Concentration and vehicle:5% (m/v) dry extract (hexane) in methanol or DMSO

Method:

Ames Salmonella test.

The shampoos are extracted using hexane to eliminate detergents, and the extracts are dry-concentrated.

5 µl of dry extract solution at 5 % (m/v) in methanol are tested on *Salmonella typhimurium* (LT_2) TA100 in the absence and presence of 500 µl of Sg mixture.

The results in terms of numbers of colonies of his revertants per plate are the average obtained for two plates.

Negative control: methanol

Positive control: benzo-alpha-pyrene in methanol: 1.0 µg/plate

The PAHs, including benzo-alpha-pyrene (B(a)P), are determined in the extracts via HPLC and GC/MS.

Results:

The extracts of the Zetar, Tersa and Pentrax shampoos are positive in the presence of the Sg fraction with 524, 348 and 329 revertants per plate respectively, this being greater than the number obtained for 1.0 µg of B(a)P (311 revertants per plate).

The physico-chemical analysis shows that the Polytar shampoo which was negative in the test conditions contains approximately 50 times less B(a)P than the Zetar shampoo.

N.B.: The author calculates that the quantity of B(a)P present on the epicranium (8 mg B(a)P) resulting from a single application of 5 ml Zetar shampoo is 470 000 times greater than the quantity found in one cigarette.

Genetic Mutations in Bacteria (3)

Test material: Coal tar (volatile part)

Method:

Taped plate assay.

Maron and Ames (Dislerath et al.)
The lower plate containing the material to be tested and the upper plate containing the microbe strain with or without the enzymatic fraction Sg are incubated for five hours at 37°C.
Benzo-alpha-pyrene and benzo-alpha-anthracene (non-volatile) are used as controls.

Results:

The volatile components of coal tar are mutagenic for *Salmonella Typhimurium* TA98 and TA100 in the presence of the Sg mixture.
The number of revertant colonies is a function of the quantity of coal tar tested (0 to 500 µg/plate).

N.B.: These volatile mutagens may present a genotoxic risk in addition to the risk caused by the presence of carcinogenic PAHs in coal tars. The mutagenic benzo-alpha-pyrene and benzo-alpha-anthracene, which however are non-volatile, are negative in this test.

Covalent binding in DNA *in vivo*

Test material:	Pharmaceutic preparation of coal tar Liquor Picis Carbonis
Concentration and vehicle:	20 % (m/v) in ethanol
Species tested:	Parkes male mice

Method:

Determination of DNA additions (covalent binding) formed *in vivo* in the skin *and* in the lungs of mice after topical application.

1 Determination of DNA additions in the skin of the mouse, 24 hours after one application to the shaved dorsal skin of 150 µl of ethanol solutions containing 6 mg and 30 mg CCT respectively per mouse.

   Negative control: ethanol.

2 Study of persistence of additions formed 1, 4, 7, 14 and 32 days after application of 30 mg coal tar in 150 µl ethanol.

3 Effect of repeated treatment on the formation of DNA additions in the skin and the lungs; two applications per week during five weeks of 6 mg CCT in 150 µl ethanol (4 % solution (m/v).

The isolated DNA on the basis of epidermis and lung homogenates taken after sacrifice is determined using the post-labelling technique at 32p.

N.B.: The same operations are conducted with creosote and bitumen solutions.

Results:

1 Detectable levels of additions are present in skin DNA 24 hours after one application of 6 mg coal tar (0.14 f mol additions/µg DNA), 5 µl of creosote (0.19 f mol additions/µg DNA) and 15 mg bitumen (0.09 f mol additions/µg DNA).

2 The maximum level of additions is observed 24 hours after application.

   Suppression of DNA damage takes place in two phases with a reduction of 1/2 to 1/3 of the initial damage after one week and persistence of 10 to 15 % of initial damage after 32 days.

3 An increase in the levels of additions to cutaneous DNA and to lung DNA observed during the five weeks of treatment. The level is lower for additions to lung DNA and a ceiling is reached after three weeks.

N.B.: (a) The appearance of DNA additions in the lungs indicates that carcinogenic components in coal tar are absorbed via the skin.

(b) Although the formation of additions to DNA in the tissues is a necessary but not sufficient condition for tumours, the author notes that there is evidence of an increase in the frequency of lung adenomas in mice treated by topical pathway with creosote, by comparison with the controls (IARC 1985).

(c) Although the possibilities of synergic metabolic activation or detoxification of PAHs is conceivable in complex mixtures, it clearly emerges from this test that the lower PAH levels in bitumen lead to lower levels of DNA binding than in the case of coal tar and creosote.

(d) Analysis of DNA obtained from samples of human skin in cultures treated with coal tar, creosote and bitumen shows formation of DNA additions similar to those observed in the skin of the mouse.

Formation of DNA Covalent bonds *in vivo*

Test material:	Coal tar, pharmaceutical quality: 20 % solution 20 % solution (m/v) in ethanol PAHs identified by GC
Species tested:	Parkes male mouse

Method:

The purpose of the study is to identify the PAHs responsible for formation of DNA covalent bonds in coal tar.

1 Identity and concentration of 19 principal coal tar PAHs are determined by GC. They are allocated to three groups depending on their carcinogenic potential:
A: the 19 identified PAHs
B: 7 PAHs presenting sufficient evidence of carcinogenicity in animals
C: 12 PAHs presenting limited or inadequate evidence of carcinogenicity in animals

2 The mice are treated by topical application of:

- 30 mg of coal tar solution
- synthetic mixtures of PAHs A, B and C present in 30 mg, of the coal tar solution
- 0.25 to 1 µmole in 200 ml acetone of:
B(a)A, B(b)F, B(j)F, B(k)F, B(ghi.)Perylene, B(a)P, cyclopental(c-d)pyrene, Indenol, (1,2,3-Cd)pyrene)
- acetone (control)

3 The DNA additions in the skin of the mice are analysed by 32p labelling and separated by TLC on cellulose and by HPLC.

Results:

The level of DNA additions formed by the group B PAHs is higher than that of the group C PAHs.

Benzo(a)pyrene plays a role in the formation of DNA additions in coal tar as well as the benzofluoranthenes (g, h and i) and benzo(g,h,i)perylene.

Benzo(a)anthracene, dibenz(a,h)anthracene and Indenol(1,2,3 cd)pyrene are not apparently involved in the formation of additions.

(Photo)mutagenicity: Suppression of DNA synthesis *in vivo*

Test material:	Crude coal tar (CCT): 60 % (m/v) in petroleum ether ESTAR Gel: 0.5 % pharmaceutical quality coal tar in a propylene glycol gel
Species tested:	Hairless mice
UV source:	UVA 360 nm

Method:

The preparations are applied to the hindquarters during two hours. After rinsing in soapy water, the treated sites are exposed to UVA during 45 minutes at a distance of 10 inches (360 nm, 6.7 joules/cm^2). An hour after irradiation, 25 µCi of H^3 thymidine are injected by intraperitoneal route; the animals are sacrificed one hour after the injection.

After sacrifice the DNA is extracted from the epidermic cells of the treated sites and (suppression of) DNA synthesis is measured by liquid scintillation counting.

The animals are also treated with UVA alone, with the vehicles alone (control) and with the two coal tar preparations without UVA.

Results:

Significant inhibition of DNA synthesis is observed in the epidermis of hairless mice treated with:

CCT (6 %) + UVA
ESTAR Gel (0.5 %) + UVA
CCT (6 %) only
ESTAR Gel (0.5 %) only

For the two preparations, the effect is greater with UVA than without UVA.

UVA radiation alone does affect DNA synthesis.

For ESTAR Gel + UVA (0.5 %), there is a dose-linked response and the critical quantity of UVA for inhibiting DNA synthesis lies between 0.9 and 1.6 joules/cm^2.

Urinary mutagenicity after percutaneous absorption

Test material:	Pharmaceutical quality crude coal tar
	Pix Carbonis
Species tested:	Clinical study
	3 patients suffering from psoriasis, non-smokers
	5 volunteers in good health, non-smokers, untreated (= controls)

Method: Ames Salmonella plate incorporation assay

Three patients receive a daily application of Pix Carbonis for three consecutive days followed by UV exposure (unspecified).

Their urines are collected from six hours after the first application to 36 to 48 hours after the final application and collected to obtain > 600 ml urine sample per patient.

The PAHs are determined by GC/MS in Pix Carbonis and in the urine samples.

Mutagenicity:

Pix Carbonis in solution in DMSO (1, 10, 100, 500 µg/100µl*)* is tested vis-à-vis Salm. Typh. TA98 and TA100 in the presence and absence of the Sg mixture.

The urine samples are suspended in DMSO after filtration, column purification and dry evaporation (0.4 ml DMSO for 100 ml urine) and tested vis-à-vis Salm. Typh. TA98 and TA100 in presence of the Sg mixture and in the presence and absence of ß-glucuronidase.

Results:

The crude coal tar is mutagenic for S. Typh. TA98 and TA 100 in the presence of Sg with doubling of the number of revertants for 10 µg of (CCT) per plate (TA98) and 16 µg of (CCT) per plate (TA100).

The total content of PAHs determined is approximately 3 %, with 0.2 % B(a)A; 2/3 of the PAHs consist of compounds with low molecular weight.

The urine samples are mutagenic for S. Typh. TA98 and TA100 in the presence of Sg. Mutagenicity is delectable 6 to 7 hours after first application and up to 40 hours after the final application.

The urinary PAH levels are high, mainly due to excretion of compounds with low molecular weight. B(a)A is present in small quantities and B(a)P is present in trace quantities [N.B.: the author notes the prevalence of faecal excretion for B(a)P in the animals].

There is a significant correlation between the PAH excretion values and urinary mutagenicity.

N.B.: the total PAH exposure dose to patients lies between 180 and 240 mg/day assuming an application of 2 g Pix Carbonis per 10 % of body surface.

Urinary Mutagenicity after percutaneous absorption

Test material:

1 Coal tar containing 23.2 mg pyrene per g (= 2.32 % m/m)
9.5 mg Benzol(a)Pyrene per g (= 0.95 % m/m)

2 Pix lithantracis dermata containing
16.7 mg pyrene/g (= 1.67 % m/m)
7.0 mg B(a)P/g (= 0.7 % m/m)

Species tested:

1. Male SPF wistar rats (3 x 4)

2. Clinical study
5 women patients suffering from contact dermatitis.
32 untreated volunteers (control).

Method: Ames Assay

1 Four groups of three rats are treated with 0.1 ml of a xylenol solution containing 0-2, 5-12.6 and 53 mg coal tar respectively. The solution is applied during 24 hours on 9 cm^2 of the shaved dorsal skin, 24-hour urines are collected during five days from time 0.

2 Five patients suffering from contact dermatitis are treated by daily application of 40 g ointment containing 10 % pix lithantracis dermata (= 4 g per application). The skin is cleaned with arachis oil before each new application.

A urine sample is taken after treatment; subsequently two samples per day are collected over three days.

Urinary metabolises

1-Hydroxypyrene and 3-hydroxy benzo(a)pyrene (3-OH.B(a)P) are determined by BPLC/fluorescence.

Mutagenicity

After extraction and concentration the 24 h (rat) urines or a volume corresponding to 1 m mole creatinine (man) are tested in DMSO vis-à-vis Salm. typh. TA98 in presence of Sg mixture and ß-glucuronidase.

Results:

1 24-hour urine mutagenicity in the rat is a function of the quantity of tar applied and the quantities of 1-OH P and 3-OH-B(a)P excreted, with a maximum of revertants between 24 and 48 hours.

2 Both in rat and man, there is a significant correlation between urinary excretion of the two metabolites with a higher level of 1-OH pyrene (a factor of 19 in the rat and 2 500 in man).

In the patients tested, the concentration of 1-OH pyrene increased approximately 100-fold after beginning of treatment and concentrations of 3-OH-B(a)P do not exceed 0.40 µ mole/mole of creatinine.

N.B.: The mutagenic potential of human urinary extracts could not be determined because of their toxicity for Salm. typh.

9. Carcinogenicity

IARC Classification

Carcinogenicity in animals: sufficient evidence

Carcinogenicity in man: sufficient evidence

Carcinogenicity *in vivo*: initiation of tumours

Test material:	Industrial coal tar (NBS)
(m/v)	Therapeutic stock solution of coal tar: 20 % in ethanol
Species tested:	Charles River CD 1 mouse

Method:

Cutaneous application (skin painting) following the procedure described by Mahlum.

Promotion: Acet.Phorbol.

Total duration of test: 197 days.

Initiation: The industrial coal tar (NBS) and coal tar extracted from the 20 % therapeutic solution are diluted to 50 % (m/m) in methylene chloride.

25 µl of these solutions are applied to the shaved skin (hindquarters).

Promotion: Two weeks after initiation, 50 µl of a solution of pharbol myristate acetate at a dose of 100 µg/ml are applied on the pretreated sites, twice per week during six months.

Results:

The industrial tar and the pharmaceutical quality tar both initiate tumours and the results indicate with a 90 % tumour incidence (details not available).

N.B.: This tumour initiation test is part of a complex study including measurement of mutagenic activity vis-à-vis Salm. typh. TA98.

10. Special investigations

Phototoxicity in volunteers

Test materials:	4 crude coal tars
	2 partly-refined coal tars
	1 detergent, Liquor Carbonis (LCD)
	(= alcoholic solution at 2 % m/v)
Concentration and vehicle:	5 % (m/m) in a hydrophilic ointment
Species tested:	26 volunteers
UV source:	UVA 360 nm

Method:

The phototoxic potential for human skin was compared for coal tars from different sources.

50 mg test material are applied under occlusion for 2 hours on 1 $inch^2$ of the forearm. After acetone rinsing the site is exposed to UVA (0.83 J/cm^2) at a distance of 2 inches from the light source for 10 minutes.

Phototoxicity is measured at 24 hours and 48 hours via intensity of erythema and oedema on a 4-point scale (0 = normal skin; 1 = minimal erythema; 2 = erythema; 3 = severe erythema and oedema). Three specimens are taken for biopsies immediately after irradiation, after 24 hours and after 48 hours, from four subjects.

Results:

Phototoxic reaction occurs in two stages (clinical and histological): an immediate urticaria reaction preceded by severe burning and erythema and followed by delayed infiltration reaction (after 12 hours, with a maximum between 24 and 48 hours).

Pigmentation is observed 7 to 10 days after exposure.

Intention of the delayed reaction differs with the material tested, partly refined tars being significantly less phototoxic than crude coal tars, and the 2 % alcoholic solution (LCD) being the least active.

Phototoxicity on humans

Test materials:	Crude coal tar (CCT)
Concentration and vehicle:	5 % (m/m) in petroleum ether
Species tested:	32 adult volunteers (skin types I to III), without photosensitivity problems
UV source:	UVA (310-400 nm)

Method:

One crude coal tar sample is tested to determine the minimum UVA dose that provokes erythema with distinct boundaries 24 hours after exposure (MPD or minimal phototoxic dose) and the minimum UVA dose provoking a subjective heating sensation (MSD), as well as the variables affecting the MPD.

MPD: Application during 15, 30, 60, 90, 120 and 180 minutes.
After thorough rinsing in soapy water, UVA irradiation (1.5 to 23.6 J/cm^2) during 4 to 60 minutes.

MSD: Application during one hour.

After rinsing in soapy water, UVA irradiation (at time 0, after 2 hours, 4 hours, 6 hours, 24 hours and 30 hours) (1.5 to 32.25 J/cm^2) during 4 to 90 minutes.

Tests of different types of rinsing (water, soapy water, mineral oil, soapy water + mineral oil).

Results:

Phototoxicity seems to peak after three hours application to the skin with a rapid increase after 15 minutes of application.

MSD is inferior to MPD at all times tested.

There is a logarithmic dose-response ratio between duration of application to the skin and reduction of MPD and MSD.

The rinsing methods do not affect the results.

MSD and MPD persist for at least 30 hours after removal of the tar.

S.C.C. OPINION CONCERNING COAL TAR, GOUDRON DE HOUILLE ADOPTED BY THE PLENARY SESSION OF THE S.C.C. ON 3.2.1995

Introduction

The International Agency for Research on Cancer has devoted a monograph to coal tars and their derivatives (IARC monograph, Vol. 35, 83-159, 1985 + suppl. 6, 186, 1987) including:

- physical and chemical data
- production, use, exposure, analysis
- experimental toxicity data
- animal carcinogenicity studies
- epidemological data on carcinogenicity in man

In its final evaluation the IARC concludes that there is sufficient evidence that coal tars can cause cancer in man and in animals.

The carcinogenic potential is directly linked to the polynuclear aromatic hydrocarbons (PAH) contained in the tars.

Physico-chemical analysis shows that the pharmaceutical quality coal tars in which COLIPA is interested have the same PAH content as crude coal tar.

The use of pharmaceutical quality coal tars for topical use in dermatology prompted skin toxicity studies and carcinogenicity studies which are summarised in the IARC monograph (see extracts in Annex 1).

In view of IARC's in-depth evaluation and the large quantity of data concerning "substances" derived from coal tar, only studies that meet the following criteria have been taken into consideration in preparing the report:

- very recent studies
- studies most directly relevant to assessing the risk associated with cosmetic use
- studies suitable for confirming genotoxic potential
- studies which relate specifically to crude or pharmaceutical quality coal tars corresponding to CAS RN 8007-45-2, EINECS 232-361-7.

COAL TAR - Substances of interest for COLIPA 22/10/1993

	EINECS CAS RN	DEFINITION
COAL TAR - Pix carbonis - coal tar solution - Pix lithrantracis - Crude coal tar	232-361-7 8007-45-2	By product from the destructive distillation of coal. Almost black, semi-solid. A complex combination of aromatic hydrocarbons, phenolic compounds, nitrogen bases and thiophene (UVCB-EINECS)***
COAL TAR DISTILLATE -Heavy anthracene oil** 7th primary distillate (ICT 1992)* distillates upperboiling 220 - 450°C	266-027-7 65996-92-1	The distillate of coal tar having an approximate distillation range of 100°C to 450°C, composed primarily of 2 to 4 membered condensed ring aromatic hydrocarbons, phenolic compounds and aromatic nitrogen bases (UVCB-EINECS)***
PITCH COAL TAR - Pitch coal tar, high temperature, softening point 40 °C - 180°C (ITC 1992)* - Coal tar pitch volatiles - Pitch: residue from distillates - Pine tar extract (? Colipa ?)	266-028-2 65996-93-2	The residue from the distillation of high temperature coal tar. A black solid with an apparent softening point from 30°C to 180°C composed primarily of a complex mixture of 3 or more membered condensed ring aromatic hydrocarbons (UVCB-EINECS)***

* ITC = International tar conference (7-04-92)

** Anthracene oil - Annex II, n° 38, directive 76/768/EEC

*** UVCB-EINECS (volume VIII) = chemical substance definitions index

PLANT TAR - Substances of interest for COLIPA 22/10/1993

	EINECS CAS RN	DEFINITION
TAR OIL - Naphtalene oil	292-305-1 8002-29-7	The volatile oil obtained by the distillation of wood tar composed primarily of phenolic substances and hydrocarbones. Exact composition varies with production methods and wood source (EINECS)
BEACHWOOD TAR OIL - Creosote wood	8021-39-4	A complex combination of phenols obtained as a distillate from wood tar.
BIRCH WOOD OIL - Birch tar oil - White birch oil	8001-88-5	Extractives and their physically modified derivatives - Betula alba, Betula pendula and Betula pubescens, Betulacae
BIRCH BUD OIL - Betula alba oil	8027-43-8	Extractives and their physically modified derivatives - Betula alba, Betulacae
BIRCH EXTRACT - Betula alba ext.	84012-15-7	Extractives and their physically modified derivatives such as tinctures, concretes, absolutes, essential oils, oleoresins, terpenes, terpenes free fractions, distillates, residues, etc... obtained from Betula alba, Betulacae (EINECS)
PINE TAR	232-374-8 8011-48-1	A product obtained from the destructive distillation of the wood of Pinus, Pinacae
CADE OIL - Juniper tar oil - Harlem oil	8013-10-3	Extractives and their physically modified derivatives Juniperus oxycedrus, Cupressacae
TAR PINE sulfurized - Sulfur tar complex - Willow oleoresin	305-840-4 95046-52-9	
TAR SOFTWOOD - Cade tar et Birch tar (Colipa)	307-057-3 97489-17-3	A distillate from high temperature destructive distillation of wood. Composed primarily of aromatic hydrocarbons with lesser amounts of sterols, fatty acids and their derivatives boiling in the range of approximatively 150 °C to 450 °C (EINECS)

Assessment

In clinical studies, 5 % concentrations of coal tar in an ointment or petroleum ether are phototoxic for human skin, leading to immediate erythema and a delayed infiltration reaction which persists after rinsing.

A single cutaneous application of a standard coal tar solution USP (20 %) triggers an increase in Acryl Hydrocarbon Hydroxylase (AHH) activity in the skin and liver of newborn rats, pregnant mothers and foetuses, benzo-alpha-pyrene being the most potent inductor of AHH in the coal tar constituents.

AHH induction has been confirmed *in vivo* and *in vitro* in human skin.

Therapeutic preparations of coal tar are mutagenic in the Ames test in the presence of metabolic activation at doses of less than 100 µg/patch; likewise, the volatile components of coal tar are mutagenic for Salm. Typh. TA 98 and TA 100 in the presence of S_9, which points to an additional genotoxic risk over and above that due to carcinogenic PAHs present in the coal tar, mutagenic but weakly volatile B(a)P and B(a)P and B(a)A being negative in this test.

Covalent binding to the DNA of mouse skin and lungs *in vivo* has been observed after a single application of 6 mg phamaceutical quality coal tar.

Repeated application triggers an increase in additions during five weeks, levelling out after three weeks. Analysis of DNA obtained from samples of human skin cultures reveals the formation of DNA additions similar to those observed in the skin of the mouse.

In vivo, in the presence and absence of UVA radiation, significant inhibition of DNA synthesis in the epidermis of hairless mice treated with coal tar preparations has been observed, including a gel containing 0.5 % pharmaceutical quality coal tar, where a UVA dose-linked response is observed.

Polyaromatic hydrocarbons contained in the coal tar are absorbed through human skin, the level of the 1-hydroxypyrene urinary metabolite after a single application of 20 g of a shampoo containing 285 ppm pyrene being comparable to that observed in coke oven workers after seven days of exposure at work.

Percutaneous absorption in man has been confirmed in clinical studies where a significant correlation has been observed between urinary excretion of polyaromatic hydrocarbons and the mutagenic potential of the urines in the Ames test in the presence of metabolic activation.

In the rat, mutagenicity of 24-hour urine is a function of the quantity of tar applied and the quantities of 1-hydroxypyrene and 3-hydroxy-Benzo-α-pyrene excreted. In the rat and in man there is a significant correlation between urinary excretion of the two metabolites with a higher level of 1-OH pyrene in man.

Most of the topical carcinogenicity tests evaluated by the IARC were conducted on the mouse (a species sensitive to carcinogenic PAHs) in the 70s. All pharmaceutical preparations containing coal tar which have been subjected to long-term tests provoke skin tumours, including carcinoma, some of which have metastasised. In these studies and in very many studies of creosotes, pitches and various varieties of coal tar, polynuclear aromatic hydrocarbons, in particular Benzo-α-pyrene, play a key role in triggering tumours. B(α)P is an

indirect carcinogen, which is active after metabolic activation used as a positive control in mutagenicity/genotoxicity tests and in biological carcinogenicity tests; according to the IARC evaluation (No 32. p. 211-224), B(α)P is also embryotoxic and teratogenic in the mouse, the induction of Acryl Hydrocarbon Hydroxylase (AHH) which is also observed in skin metabolism studies of coal tar being an important contributory factor.

Industrial tar and a pharmaceutical quality tar have been shown to induce tumours in a six-month study of cutaneous application in the mouse (no details available).

Conclusions

The clinical studies referred to in this report confirm the phototoxic and genotoxic potential of coal tars. They show significant cutaneous absorption of the carcinogenic polyarematic nuclear hydrocarbons contained in the tars, which correlates with urinary mutagenicity.

Most of the materials tested were of pharmaceutical quality and are comparable with those used in cosmetic products.

Coal tars, irrespective of their quality, should not be used in cosmetic products.

Classification: D

DRAFT SCC OPINION CONCERNING: MUSK XYLENE COLIPA SUBMISSION III

Assessment of the Enzyme Inducing Characteristics of Musk Xylene in B6C3F l Mice.

In a pilot study, groups of 10 male Charles River B6C3F1 mice received ip injections of 50, 100 or 200 mg MX /kg bw for 7 days. In a feeding study, groups of 25 male animals were given 0,015; 0,045 and 0,15 % MX (app. 22, 66 and 220 mg/kg b.w.) with the diet for 4 weeks. A recovery-group had access to control diet for another 14 days. Labelling Index was estimated using BrdU, liver slices were prepared for histology and electronmicroscopy and P450 isoenzyme induction studies were performed.

In the pilot study 50 mg gave rise to mild centrolobular hepatocellular hypertrophy. Hydropic changes, scattered mitoses and. nuclear size varations were seen in the 100 mg group. In the high dose group these effects were more marked and smooth and rough endoplasmic reticulum increased. Mitochondrial fragments indicated toxic effects. The nuclei were normal with some margination of the chromatin. Dietary administration of the substance gave essentially the same histological and electronmicroscopical pictures. There was no increase in number or size of peroxisomes.

In the pilot study increase of liver weight (up to 132 % of normal) and protein content (up to 170 %) as well as the induction of the P450 isoenzymes CYP IA1 and IA2 was seen in a dose dependent manner up to 1320 % for both isoenzymes and to 583 % for CYP IA2 alone. In the dietary study no effects were seen on hepatic parameters including enzyme induction at the 0.015 % dose level. At the two upper dose levels the relative liver weight (114/117 % from control) and protein content (117/148 %) as well as the induction of both isoenzymes (CYP IA1/IA2 = 441/602 %, CYP IA2 = 278/293 %) were increased in a dose dependent manner. No differences from controls were seen in the recovery groups.

The subacute feeding study suggests a NOEL of about 20 mg/kg b.w. for the evaluated parameters in this strain of mice.

It seems unlikely that the positive results seen in the carcinogenicity study can be associated to the results presented in this paper.

PHENOLPHTHALEIN

1. General

The substance is listed in Appendix III part 2 of the Cosmetics Directive (provisional list).

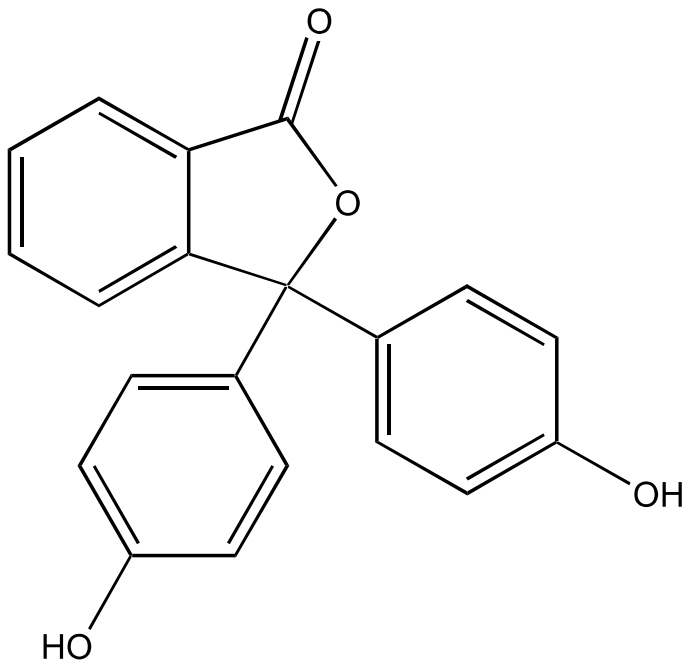
1.1 Primary name

Phenolphthalein

1.2 Chemical names

Phenolphthalein
3,3-bis-(4-hydroxyphenyl)-1-(3H)-isobenzofuranone

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{20}H_{14}O_4$
Mol weight: 318.33

1.8 Physical properties

Appearance: Phenolphthalein is a yellowish-white powder.

1.9 Solubility

Phenolphthalein is almost insoluble in water and readily soluble in alcohol and diethyl ether.

2. Function and uses

Industry are requesting approval for use at 0.037 % in 'motivational' toothpastes for children. It has apparently been used for this purpose within the EC since 1988. The estimated worst case ingestion by a child from such use is 0.86 mg.

Explanation of the term 'motivational':

Colipa indicates that "the product concerned makes use of the pH indicator property of Phenolphthalein. During brushing, and because of the slow downward drift of pH in the mouth, the colour of the foam changes from pink to colourless. This process takes an average time of about one and a half minutes, according to user studies. It is intended to encourage children, through its interesting visual action to continue brushing their teeth until the colour change is complete. Hence the term motivational toothpaste; children are motivated to brush their teeth for a reasonably adequate period".

The term "motivational" is indicated not to be used in the advertising, but to be an "incompany" description of the product concept.

The compound has been widely used since the early 1900s as a laxative and it is available in non-prescription medicines for this purpose. The recommended daily dose level is in the range 30-200 mg for adults.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat: According to data published by the Chemical-Biological Coordination Center from the U.S. National Research Council in 1953, the minimum oral LD is greater than 1000 mg/kg b.w.; the minimum intraperitoneal LD is greater than 500 mg/kg bw.

No more recent data are available.

3.7 Subchronic oral toxicity

No data are available from animal studies.

4. Irritation & corrosivity

4.2 Irritation (mucous membranes)

No data are available from animal studies.

A tolerance study involving 50 healthy volunteers (aged 10-18 years) who used toothpaste containing 0.0185 % Phenolphthalein for seven days revealed no difference compared to a matched group using a 'standard' toothpaste. Products were well tolerated with no induction compound related to mucosal irritation.

5. Sensitization

No data are available from animal studies demonstrating the absence of delayed sensitization.

6. Teratogenicity

No data are available from animal studies.

7. Toxicokinetics (incl. Percutaneous Absorption)

Mucous or percutaneous absorption:

No data are available from *in vitro* or animal studies.

8. Mutagenicity

A US publication from 1983: results obtained on 270 chemicals by 2 laboratories under contract to the US National Toxicology Programme (NTB) using strains TA1535, TA1537, TA98 (or TA97) and A100, were negative in both cases.

According to the opinion given by the SCC on 7.10.1992, a chromosomal aberration test was carried out in human lymphocytes in accordance with GLP standards. Suitable positive (CPH or MMC) and vehicle (DMSO) controls were used.

The assays were carried out with and without metabolic activation with S9 mix. Following a dose ranging study levels of active ingredient from 75 to 300 $\mu g\ ml^{-1}$ with S9 mix and from 12.5 to 150 $\mu g\ ml^{-1}$ without S9 mix.

Experiments were performed in duplicate; harvesting was carried out at 29 hours in both tests but an additional harvest time of 53 hours was included in the second test.

According to the conclusions of the Study Director, Phenolphthalein consistently induces structural aberration in this study in both presence and absence of S9 mix.

10. Special investigations

Adverse effects in humans from therapeutic use:

Phenolphthalein is a diphenylmethane stimulant laxative usually given in daily doses of 30-200 mg for short periods to adults. Doses of 270 mg or more should be avoided.

Laxative products include ‘chocolate’ squares and a number of cases of accidental ingestion of large single doses by children have occurred which give rise to particular concern with regard to the potential for severe diarrhoea and fluid depletion. A retrospective review of 204 cases reported to a Regional Poisons Information Centre in the USA has been published. Mean amounts ingested were about 300 mg. When symptoms occurred these were minor and did not persist for more than 24 hours. The authors concluded that children aged 5 years or under and who acutely ingest 1g or less of a Phenolphthalein-containing laxative product are at minimal, if any, risk of developing dehydration.

There are however occasional reports of marked adverse effects following therapeutic use of Phenolphthalein, specifically relating to allergic reactions. Various types of acute skin reaction

have been noted following oral ingestion of Phenolphthalein, in some cases followed by persistent pigmentation [Fixed drug eruptions and localised areas of pigmentation]. More rarely serious systemic effects have been reported that may be due to allergic effects. Fatalities have occurred in 2 children following the ingestion of amounts of the order of 1 gram; these may have been due to an anaphylactic type reaction and were associated with pulmonary and cerebral oedema. Hypotension, hypothermia, severe acidosis and pulmonary oedema occurred in an adult after ingestion of 2 grams of the compound in chocolate.

The FDA Advisory Committee on OTC laxatives and related compounds concluded in 1975 that Phenolphtalein was safe and effective in amount of 15-20 mg/day for children aged 2-5 at 30-60 mg/day for children 6 and over, when taken orally on laxative products for occasional use.

11. Conclusions

According to the given data, Phenolphthalein has low acute toxicity.

According to experimental results, it did not produce any mucosal irritation in adults when used in toothpaste in use conditions, at 0.018 %, that represents half of the approval concentration requested by Industry for children. This information therefore has no real interest.

Nevertheless, the extensive data existing on experience in use by humans allows to avoid a new evaluation of the mucous irritancy.

Ames tests investigated by 2 separate workers were negative but according to the results obtained in the *in vitro* chromosomal assay with human lymphocytes, Phenolphthalein appears clastogenic.

However the results do not enable to display a dose-effect relation possibly because of an interference of a toxicity at the higher doses (that is not in itself criticizable because the use of non-toxic doses may induce to the contrary false-negative diagnostic findings).

12. Safety evaluation

Indicative safety margins

Two approaches are under consideration:

- Based on the normal daily consumption corresponding to a 3 times application of toothpaste containing the maximum concentration of Phenolphthalein (0.037 %) and to a supposed 100 % absorption (1 g for children and 1.5 g for adult per application).

- Based on the acutely ingestion of a 75 g toothpaste tube containing the maximum requested concentration of Phenolphthalein (0.037 %) with a supposed 100 % absorption.

1. Adults

* Typical body weight: 60 kg

* Systemic exposure dose:

= in a normal use condition:

$$\frac{3 \times 1{,}500 \times 0.037}{100 \times 60} \times 100\ \% = 0.028 \text{ mg/kg b.w.}$$

= in a maximal risk condition:

$$\frac{75{,}000 \times 0.037}{100 \times 60} \times 100\ \% = 0.462 \text{ mg/kg b.w.}$$

* In laxative products, minimal adverse effects have been observed at mean amounts about 300 mg/day, i.e. 5 mg/kg b.w.

* Indicative safety margins are then:
= in a normal use condition, about 180
= in a maximal risk condition, about 10

2. Children aged 6 and over

* Typical body weight: 15 kg

* Systemic exposure dose:

= in a normal use condition:

$$\frac{3 \times 1{,}000 \times 0.037}{100 \times 15} \times 100\ \% = 0.074 \text{ mg/kg b.w.}$$

= in a maximal risk condition:

$$\frac{75{,}000 \times 0.037}{100 \times 15} \times 100\ \% = 1.850 \text{ mg/kg b.w.}$$

* According to the FDA Advisory Committee on OTC laxatives and related compounds, the safety dose of Phenolphthalein for occasional daily use by children aged 6 and over is 60 mg/day i.e. 4 mg/kg b.w.

* Indicative safety margins are then:
= in a normal use condition, about 50
= in a maximal risk condition, about 2.

Conclusion:

Since then, a first SCC opinion on Phenolphthalein was adopted in 1992 with classification B.

Concerning the term “motivational”, the explanation given by Colipa in submission II seems to be convincing.

Concerning the proposal to add Phenolphthalein in Appendix IV (submission II), there is already a precedent but in that case strong restrictions have to be introduced to avoid its employment as colourant.

Concerning the safety assessment,

- in spite of a low oral acute toxicity in rat, according to the clinical experience on human in laxative products, the safety margins appear low for a “side-effect” employement, especially for children who are the main consumers;

- the additional mutagenicity results given in Submission III conclude that Phenolphthalein reinst is clastogenic when treated for such effects *in vitro* to toxic concentrations with human peripheral blood lymphocytes; it is then strongly recommended to clarify this problem by undertaking complementary *in vivo* clastogenicity tests (mutaphase analysis and/or micronucleus).

Request data should be available within one year.

Classification: C is recommended.

P 91: *3-IODO-2-PROPYNYL BUTYL CARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butyl carbamate

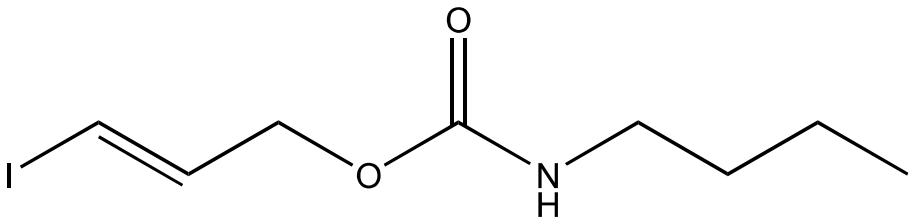
1.2 Chemical names

iodo propynyl butyl carbamate

1.4 CAS no.

55406-53-6

1.5 Structural formula



1.9 Solubility

It has low solubility in water (156 ppm at 20 °C) and is soluble in organic solvents.

2. Function and uses

It is proposed for use as a preservative in all types of cosmetic products at up to 0.1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal signs of toxicity were seen at 500 mg/kg or below. In a percutaneous toxicity study in rabbits a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing resulted in no deaths. The only signs of toxicity seen were slight irritant effects at the site of application.

3.7 Subchronic oral toxicity

In a sub-chronic study rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg. Histological examination showed hepatocyte enlargement at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The No Effect Level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritancy study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe oedema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours. Severe effects were however noted in an eye irritation study in rabbits. The substance (0.1 g) produced moderate to severe hyperaemia, chemosis and discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. If the compound was washed out of the eye 20-30 seconds post instillation only transient irritant effects were seen.

4.2. Irritation (mucous membranes)

The eye irritancy of a 0.5 % solution of IPBC in corn oil has been tested in rabbits and also the effects of 0.5 % IPBC in a baby shampoo. Groups of 6 animals were used in each case. No signs of any irritant effects were seen with the corn oil formulation. In the case of the baby shampoo, signs of slight irritant effects were seen for about 24-48 hours, but similar effects were seen in the 'control' baby shampoo that did not contain IPBC. Thus 0.5 % in corn oil or in a baby shampoo formulation produced no eye irritancy.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximization test. Induction concentrations were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson Kligman maximisation tests have been carried out on formulations containing 0.5 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical) were used. In the second case the concentrations were 0.1 % and 0.5 % respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation. There was no evidence of sensitization in either test. These studies suggest that the compound does not have any significant potential for sensitization. No data are available in sensitization in humans, or on the potential of this carbamate to cross react with dithiocarbamates used in the rubber industry.

6. Teratogenicity

Teratogenicity studies have been carried out in both rat and the mouse. In the study in rats compound was given on day 6-15 of gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The no-effect level was 50 mg/kg. A similar dosing regime was used in the study in mice. No compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The No Effect Level was 125 mg/kg.

6.2 Two-generation reproduction toxicity

A two-generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week premating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound-related effects were seen at any dose level on clinical chemistry or at necropsy. Slightly reduced weight gain was seen in the males at 750 ppm during the premating period in both the initial generation and the F1 generation. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index (= no. of pups alive at day 1/total number of pups) at 750 ppm in either generation, while a marginal effect was also noted at 300 ppm in the F1 generation. Postnatal growth of the offspring however was not affected and no effects were seen on the development of the offspring. The No Effect Level in this study was 120 ppm test compound in the diet (roughly equivalent to a dose of 10 mg/kg b.w./day).

7. Toxicokinetics (incl. Percutaneous Absorption)

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C radio-labelled material. Following iv administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the level and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicate that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

8. Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies. Negative results were obtained in the Salmonella assay versus strains TA 1535, 1537, 1538, 98 and 100 but this study was limited by investigating only 3 concentrations (6.2 - 55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537, 98 and 100 and concentrations of 1-1000 µg/plate against TA 1535. In all cases the top

concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 %-25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

The compound is a carbamate and studies have been carried out to investigate whether significant blood cholinesterase inhibition occurs in the rat following intravenous administration. The compound was given in PEG/400: water vehicle at 2-16 mg/kg and blood samples taken and analysed for erythrocyte cholinesterase activity at 15, 30, 60 minutes and 2 and 5 hours post dose. No effects on blood cholinesterase levels were observed.

Data on minimum inhibitory concentrations of 3-iodo-2- propynyl butyl carbamate demonstrated the efficacy of this compound at levels ≤ 0.1 %.

11. Conclusions

The substance has moderate acute toxicity by the oral route and low toxicity following dermal exposure. It is a mild to moderate skin irritant, but is a severe (corrosive) eye irritant; however concentrations of 0.5 % do not produce any eye irritation. Negative results were obtained in 3 Magnusson Kligman maximisation tests for skin sensitization. In a subchronic (90 day) oral study in the rat the No Effect Level was 50 mg/kg.

Mutagenic potential has been investigated in Salmonella assays for gene mutation, in a study to investigate Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro*, and in an *in vivo* micronucleus test. Negative results were consistently obtained. There was no evidence of teratogenic potential in studies in two species (rats and mice). In a 2 generation reproductive toxicity study in rats a reduction in life birth index was observed. The no-effect-level in this study was 120 ppm in the diet (c. 10 mg/kg b.w./day). The compound is well absorbed orally but is rapidly metabolised and excreted.

Concern was raised about the safety margin of P 91 in relation to the relatively low no-effect-level obtained in the two-generation reproductive toxicity study in rats. On the basis of the information provided on the efficacy of P 91, it was noted that the maximum permitted concentration of P 91 should be reduced to 0.1 % (instead of the 0.5 % originally requested). However, reduction of the concentration below 0.1 % is not feasible and hence the safety margin cannot be further increased this way.

Therefore industry should be asked to indicate whether the use of P 91 could be restricted to a limited number of products, and to provide a realistic estimate of the total exposure to P 91.[1]

A negative literature search was considered insufficient evidence for the absence of cross reactivity in humans with sulphur-carbamate sensitivity. Especially because of the general use of rubber gloves, Industry should provide a clear evidence for the absence of cross reactivity (e.g. patch tests in humans sensitive to thiuram-carbamate accelerating chemicals).

In a literature search no evidence was found for cross-reactions of 3-iodo-2-propynyl butyl carbamate with dithiocarbamates used in the rubber industry. This information is, however, considered unsatisfactory proof for the absence of cross sensitivity.

Meanwhile, information became available that the dossier with respect to P 91 is incomplete. It seems that e.g. evidence suggestive for sensitization and photosensitization in humans has not been submitted. All available information should be submitted, not only the information required as a minimum in the guidelines for the toxicity testing of cosmetic ingredients. A 'C'-classification for P 91 is maintained until the full dossier will be available.

Classification: C

[1] At present the exposure is estimated as follows: No data are available on skin absorption and therefore 100 % absorption is assumed. The extreme worst-case scenario (assuming that all products contain this preservative at a maximum concentration of 0.1 % and all are used extensively) is an exposure to 27.6 mg P 91/ human/ day (from 27.6 grams product comprising 5.54 g oral hygiene and 22 g skin contact).

OPINIONS ADOPTED DURING THE 60TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 23 June 1995

A 28: *3,4-DIAMINOBENZOIC ACID*

1. General

Summary of the opinion adopted by the plenary session of the SCC of 6 October 1992.

SPC/317/91.

3,4-diaminobenzoic acid has low acute toxicity by the oral route and studies suggest that dermal absorption from hair dye formulations is low. There was no evidence of skin or eye irritation following rapid (10 second) wash out with a 2.5 % solution. No data were available on animals not subject to rapid irrigation. There was no evidence of sensitization in a maximisation test in guinea pigs. In a 28 day oral study a no effect level of 50 mg/kg was reported. In a 90 day study effects on the thyroid and kidney were seen following histological examination at all three dose levels. These increased with increasing dose and were accompanied by other signs of dose-related toxicity at doses above 500 mg/kg. The histological findings were not reversed by a four week recovery period after dosing. Mutagenicity data was limited to negative results *in vitro* in gene mutation assays in *Salmonella* and *Eschericha coli* and *in vivo* in a micronucleus test. No adverse effects were reported in an oral teratogenicity study in rats up to 90 mg/kg.

Safety margins

Permanent hair dye use:

1g of 3,4-diaminobenzoic acid comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1.0 % 3,4-diaminobenzoic acid). With a maximal penetration of 1.152 %, this results in a dermal absorption of 11.52 mg per treatment, which is 0.192 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 300 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in rats in the 28-day study.

It should be noted that the NOAEL stems from a daily exposure for 28 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

However, further data on mutagenicity are required (an *in vitro* study to measure gene mutation and a study to measure chromosome aberrations by metaphase analysis, both in mammalian cells) to provide adequate reassurances in this regard.

Classification: B

CALCULATION OF SAFETY MARGIN

3,4-DIAMINOBENZOIC ACID

COLIPA NO. A 28

Based on a usage volume of 100 ml, containing at maximum 1.0%.

Maximum amount of ingredient applied	I(mg) = 1000
Typical body weight of human	60 kg
Maximum absorption through the skin	A(%) = 1.152
Dermal absorption per treatment	I(mg) x A(%) = 11.52 mg
Systemic exposure dose (SED)	SED = I(mg) x A(%)/ 60 kg b.w. = 0.192
No observed adverse effect level	NOAEL = 50 mg/kg

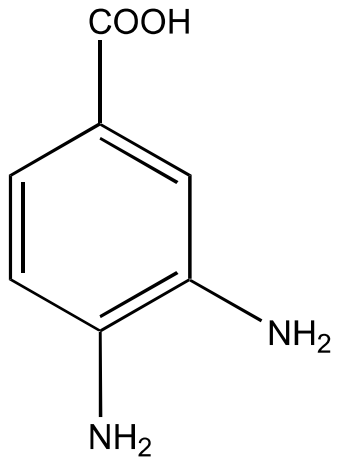
MARGIN OF SAFETY NOAEL/SED = 300

It should be noted that the NOAEL stems from a daily exposure for 28 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

1.1 Primary name

3,4-diaminobenzoic acid

1.5 Structural formula



8. Mutagenicity

Since then, only a chromosome aberration assay is provided. The evaluation of the study (Quality Assurance Declaration was included) is reported below.

Chromosome aberration assay in human peripheral blood lymphocytes in vitro.

3,4-diaminobenzoic acid was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with A 28, dissolved in DMSO, at dose levels of 100, 300 and 1000 µg/ml -S9-mix and 100, 1000 and 3000 µg/ml +S9-mix. Mitomycin and cyclophosphamide served as positive controls. 200 metaphases per culture were analyzed for chromosome aberrations.

3,4-diaminobenzoic acid did not induce chromosome aberrations in the presence as well as in the absence of S9 mix.

11. Conclusions

The information provided by the chromosome aberration assay is considered adequate.

Classification: A

A 39: *NORANTIPYRINE*

1. General

1.1 Primary name

Norantipyrine

1.2 Chemical names

1-phenyl-3-methyl-5-pyrazolone
2-pyrazolin-5-one, 3-methyl-1-phenyl
3-methyl-1-pheny-l-2-pyrazolin-5-one
3-methyl-1-phenyl-5-pyrazolone
1-phenyl-3-methylpyrazol-5-one
MCI 186

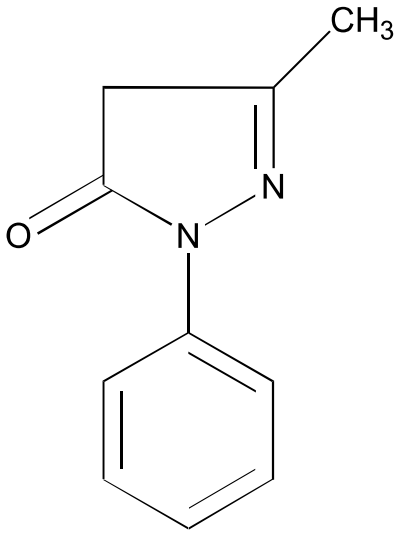
1.3 Trade names and abbreviations

Colipa No.: A 39

1.4 CAS no.

89-25-8

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_{10} H_{10} N_2 O
Mol weight: 174

1.7 Purity, composition and substance codes

sA: 1-phenyl-3-methyl-5-pyrazolone (purity: 99 %)

1.8 Physical properties

Subst. code: sA
Appearance: white to creamy powder, almost odourless
Melting point: 174°C.
Density: temp.: 20°C.
Vapour Press.: temp.: 20°C.

2. Function and uses

1-phenyl-3-methyl-5-pyrazolone is included in oxidative hair dye formulations at a maximum concentration of 0.5 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 0.25 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	oral	rat (f)	>2000	mg/kg b.w.		
sA	oral	rat (m)	>2000	mg/kg b.w.		
sA	oral	rat	3500	mg/kg b.w.		

Oral studies

1-phenyl-3-methyl-5-pyrazolone dissolved in 1,2-propanediol was administered once via stomach tube to Sprague-Dawley rats (5/sex). Rats received a dose of 2000 mg/kg b.w.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

No deaths occurred during the observation period. The test substance caused reduced activity. The test substance is slightly toxic.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	i.p.	rat	>2000	mg/kg b.w.		

Intraperitoneal study

1-phenyl-3-methyl-5-pyrazolone dissolved in distilled water was administered intraperitoneally to albino mice (5/sex). Mice received a dose of 2000 mg/kg b.w.

No deaths occurred during the study. The test substance is slightly toxic.

3.4 Repeated dose oral toxicity

Route: oral	Exposure: 28 days	DWE : 200 mg/kg b.w.
Species: rat	Recov.p. :	LED :1000 mg/kg b.w.
Subst.: sA		

Norantipyrine was administered, by gavage, once daily to 4 groups Sprague-Dawley rats (10/sex) for 28 days. The test substance was administered at dosage levels of 40, 200 or 1000 mg/kg b.w. The control group received the vehicle (0.5 % carboxymethylcellulose) only. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination was performed. Blood samples were withdrawn from all animals of each test group for haematological and clinical chemistry investigations, on days 0 and 28. Urine samples were taken from all animals, in week 4. Organ weights (c. 6) were measured and macroscopy and histopathology (c. 35 organs/tissues) was performed, on all animals.

1 animal died during the study (after blood sampling). In several high dose animals, hypersalivation, decreased motor activities and lacrimation were observed during the treatment period. Increased triglyceride was observed in the high dose male rats. A significantly increased spleen weight (abs., rel.) was observed in the high dose animals. In the high dose males, a significant increase in relative kidney and liver weights was observed. The spleen was coloured black in almost all high dose animals and was also enlarged in half of the high dose animals. Haemosiderin-laden macrophages were observed in all high dose animals.

The dose level without adverse effects was 200 mg/kg b.w.

3.10 Chronic toxicity

Route : oral	Exposure : 2 year	DWE : mg/kg b.w.
Species: mouse, rat	Carc.study: yes	LED : mg/kg b.w.
Subst. : sA		

Norantipyrine was administered to 2 groups of B6C3F1 mice (50/sex) and a control group (20/sex) and 2 groups of Fischer rats (50/sex) and a control group (20/sex). The test substance was daily fed at dose levels of 7500 and 15000 ppm, equivalent to 1071 and 2142 mg/kg b.w., during 102 weeks to mice and 2500 and 5000 ppm, equivalent to 125 and 250 mg/kg b.w., during 103 weeks to rats. An additional recovery period of 2 weeks followed.

All animals were observed daily for mortality and clinical signs. Food consumption data were collected monthly from 20 % of the animals in each group. Bodyweights were recorded monthly. Macroscopy and histopathology (c. 30 organs/tissues) was performed on all animals.

Rats:

At the end of the study, the survival rate for males was 65 %, 59 %, 74 % and for females 55%, 88 % and 88 % for the 0, 2500 and 5000 ppm group, respectively.

The test substance did not induce any toxicologic or neoplastic lesion in the rats. The dose level without adverse effects was 5000 ppm, equivalent to 250 mg/kg b.w.

Mice:

At the end of the study, the survival rate for males was 86 %, 80 %, 80 % and for females 68 %, 76 % and 90 % for the 0, 7500 and 15000 ppm group, respectively.

The test substance did not induce any toxicologic or neoplastic lesion in the mice. The dose level without adverse effects was 15000 ppm, equivalent to 2142 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin	Exposure: 4 hr	Pr.Irr.Index: 0.4
Species: rabbit	Dose: 0.5 ml	Effect: not irrit.
Subst.: sA	Concentr: 1 %	

Of a 1 % solution of the test substance, 0.5 ml was applied semi-occlusively to the right, clipped back of 3 male NZW rabbits for 4 hours. The substance residues were washed off.

Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours after patch removal.

Erythema was observed in all animals at 1 hr post application. The Draize score was 0.4 (not irritating).

4.2 Irritation (mucous membranes)

Route: eye	Exposure:	Pr.Irr.Index: 0.0
Species: rabbit	Dose: 0.1 ml	Effect: not irrit.
Subst.: sA	Concentr: 1 %	

Of a 1 % solution of the test substance, 0.1 ml was instilled into the conjunctival sac of the left eye of 3 male NZW rabbits. The untreated right eyes served as controls.

The eyes were examined 1, 24, 48, 72 hours after application.

No ocular reactions were observed. The Draize score was 0.0 (not irritating).

5. Sensitization

Subst. : sA	Conc.induc.: 0.5 %	Result:
Species: guinea pig	Conc.chall. : 0.5 %	
Method : Magnusson Kligman		

Twenty albino Hartley guinea pigs (10/sex) were used in this skin sensitization study.

The induction phase consisted of 10 topical applications of the test substance (0.5 %) in water, using occlusive patches and 2 intradermal injections of FCA. On days 1 and 10 the guinea pigs received an intradermal injection of 0.1 ml of FCA diluted to 50 % in sterile isotonic saline. The test substance is applied 3 times per week, with a 2 days interval, for 3 weeks and once at the start of the 4th week. The sites were rinsed after a one-hour contact.

Day 24-35: rest period.

On day 36, the challenge phase started; the left shoulder was treated with 0.5 ml of the test substance (0.5 %) in a 1 hour closed patch test.

Observations were made 1, 6, 24 and 48 hours after the end of the challenge exposure.

No skin reactions were observed in any of the test animals.

Remark: This test is inadequately performed, because no irritation was observed, neither irritation was induced by SDS-pretreatment. The induction concentrations appears to be too low. So, data on the preliminary test are needed.

6. Teratogenicity

Route: oral	Admin.Days: 615	DWE: mg/kg b.w.
Species: rat	LED: mg/kg b.w.	
Subst.: sA		

Norantipyrine was administered, by gavage, to 4 groups of c. 22 pregnant Sprague-Dawley rats (Crl CD (SD) BR). The test substance, dissolved in 0.5 % hydrogel carboxymethylcellulose, was daily administered at dosage levels of 40, 200 or 1000 mg/kg b.w. The control group received the vehicle (aqua dest.) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 6, 9, 12, 15 and 20. Food consumption was measured for the day intervals 0-6, 5-9, 9-12, 12-15 and 15-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Half of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and the remaining foetuses were evaluated for visceral imperfections (organic defects).

In the high dose group, all the treated animals showed orange coloured bedding from day 7-16 and females showed a significantly decreased food consumption from day 6-20. No irreversible structural changes were found.

The dose level without maternal and without embryo/foetotoxicity was 1000 mg/kg b.w.

Remark: In contrast to the requirements of OECD 414, no maternal toxicity was observed at the highest dose level tested.

8.1. Mutagenicity (Bact., Non mammalian eukaryotic, *in vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	*Salm typh*	TA98	frameshift mut	30-2000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	basepair subst.	30-2000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1535	basepair subst.	30-2000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1537	frameshift mut	30-2000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1538	frameshift mut	30-2000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA98	frameshift mut	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*Salm typh*	TA100	basepair subst.	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*Salm typh*	TA1535	basepair subst.	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*Salm typh*	TA1537	frameshift mut	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*Salm typh*	TA1538	frameshift mut	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*E.coli*	WP2 uvr	A reverse mut	0.3-10000 µg/pl	-	-	r,m,h	AR
*sA	*Salm typh*	TA98	frameshift mut	0-10000 µg/pl	-	-	r,h	AR
*sA	*Salm typh*	TA100	basepair subst.	0-10000 µg/pl	-	-	r,h	AR
*sA	*Salm typh*	TA1535	basepair subst.	0-10000 µg/pl	-	-	r,h	AR
*sA	*Salm typh*	TA1537	frameshift mut	0-10000 µg/pl	-	-	r,h	AR
*sA	CHO		chrom aber	0.1-2.0 mg/ml	-	-	r	AR
*sA	mouse lymph.	L5178Y	TK+/-	56.25-900 µg/ml		+	r	AR

8.2 Mutagenicity (*in vivo* mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	Swiss	micronuclei	400, 500 and 600 mg/kg b.w.	-

8.3. Mutagenicity tests (text)

Salmonella assays
5 Strains of *Salmonella typhimurium* were exposed to Norantipyrine dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose level tested was 30-2000 µg/plate. The negative control was DMSO; the positive control substance was 2-aminoanthracene.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

5 strains of *Salmonella typhimurium* and *E.coli* were exposed to Norantipyrine dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose level tested was 0.3-10000 µg/pl. The negative control was DMSO; the positive control substances were 2-aminofluorene, 2-aminoanthracene, sodium azide and 9-aminoacridine.

There was no mutagenic effect found in the 5 strains of *Salmonella typhimurium* and *E.coli*, neither in the absence nor in the presence of S9 mix.

4 strains of *Salmonella typhimurium* were exposed to Norantipyrine dissolved in DMSO, in the presence and absence of rat and hamster liver S9 mix. The dose level tested was 0-10000 µg/pl. The negative control was DMSO; the positive control substances were sodium azide, aminoacridine and 4-nitro-o-phenylendiamine without S9, 2-aminoanthracene with S9.

No mutagenic effect was found in the 4 strains, neither in the absence nor in the presence of S9 mix.

Chromosome aberration assay
Norantipyrine was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in DMSO, at dose levels of 0.1, 0.2, 0.4 and 0.8 mg/ml -S9mix and 0.5, 1 and 2 mg/ml +S9mix. Methylmetanesulphonate and cyclophosphamide served as positive controls. 100 metaphases per culture were analyzed for chromosome aberrations.

Norantipyrine did not induce an increase in chromosome aberrations.

Mouse lymphoma assay
Norantipyrine was tested for forward mutations at the $TK^{+/-}$-locus in mouse lymphoma cells. Cells were treated with sA, dissolved in DMSO, in the absence of S9 at 56.25, 112.5, 225, 450 and 900 µg/ml and in the presence of S9 at 100, 300, 500, 700 and 900 µg/ml. 3-Methylcholanthrene, methyl methanesulphonate and ethyl methanesulphonate served as positive controls.

Norantipyrine has no genotoxic activity at the $TK^{+/-}$-locus of L5178Y mouse lymphoma cells in the absence of S9 mix but is positive (mutation frequency is significantly increased; dose-related) in the presence of S9 mix.

Micronucleus assay
Norantipyrine was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of Swiss mice. The test substance, dissolved in DMSO, was administered

twice with a 24 hour interval, by gavage, to the animals (6/sex) at concentrations of 400, 500 and 600 mg/kg b.w. Mitomycin C was the positive control. Samples were taken 6 hours after the second administration. In each group 2000 polychromatic erythrocytes of 5 males and 5 females were analyzed for micronuclei.

Norantipyrine did not induce higher frequencies of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse.

10. Special investigations

Skin absorption of Norantipyrine

The method used is: *in vitro*, diffusion cell (Franz cell) using human breast epidermis.

0.5 % Norantipyrine* was applied 8 times, in absence and in presence of hair (adding 10 ml of finely cut bleached hair), using human breast epidermis, for 30 minutes. Then the skin was washed and dried.

The formulation was left for 30 minutes and was then rinsed-off using 10 ml distilled water. The contact area was dried with cotton wool swabs.

After 4.5 hours the mean percutaneous absorption was 0.08 % of the administered formulation in presence of hair and 0.08 % in absence of hair and 0.08 % in absence of hair + presence of 1,4-diaminobenzene and 0.08 % in presence of hair + presence of 1,4-diaminobenzene.

* Composition of the formulations I and II (vehicle) and III:

	I (g)	II (g)	III (g)
Norantipyrine	0.5	–	0.5
1,4-diaminobenzene	–	–	0.805
sodium disulphite	1.3	1.3	1.3
polyethylene glycol	50	50	50
ammonia, 20%	10	10	10
deionised water	38.2	38.7	37.395

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the acute i.p. study, sensitization study, Ames tests, chromosome aberration assay, micronucleus assay and the mouse lymphoma assay.

General

1-phenyl-3-methyl-5-pyrazolone is used in oxidative hair dye formulations at a maximum concentration of 0.5 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 0.25 % only.

Acute toxicity
The test substance is slightly toxic, on the basis of its acute toxicity (LD_{50} oral, i.p.; rat > 2000 mg/kg b.w.).

Irritation
A concentration of 1 % in the eye and 1 % in the skin irritation study with rabbits, showed no signs of irritation.

Sensitization
No evaluation possible due to the inadequate performance of the test method.

Subacute toxicity
In a 28-day feeding study, Sprague-Dawley rats were fed 0, 40, 200 or 1000 mg Norantipyrine/kg b.w., by gavage once daily. Spleen changes and organ weight changes were observed in the 1000 mg/kg b.w. group. The dose level without effect was 200 mg/kg b.w.

Carcinogenicity
In a 2-year feeding study, mice and rats were fed, respectively, 0, 7500, 15000 and 0, 2500 and 5000 ppm, once daily. No signs of toxicity or neoplastic lesions were observed. The dose level without effect was 15000 ppm, equivalent to 2142 mg/kg b.w. for mice and 5000 ppm, equivalent to 250 mg/kg b.w. for rats.

Teratogenicity
In a teratogenicity study, Sprague-Dawley rats were fed 0, 40, 200 or 1000 mg Norantipyrine/kg b.w. No animal died during the study. No irreversible structural changes were found.

The dose level without maternal toxicity and without embryo/foetotoxicity was 1000 mg/kg b.w.

Genotoxicity
Norantipyrine was tested for its mutagenic potential under *in vitro* conditions in an Ames assay, an E.coli assay, a chromosome aberration assay and a mouse lymphoma assay. Under *in vivo* conditions a micronucleus assay was performed. With the exception of the mouse lymphoma assay with metabolic activation, Norantipyrine was negative in all tests.

On the basis of these results a final conclusion cannot be made; an *in vivo* UDS assay in hepatocytes is necessary to better evaluate the genotoxic potential.

The SCC considered that the *in vivo* UDS test was not necessary since the carcinogenesis test was negative.

Absorption
Norantipyrine was applied to the human breast epidermis, using diffusion Franz cells. The cutaneous absorption was 0.08 % for skin with and without hair and with and without 1,4-diaminobenzene.

Conclusions
Norantipyrine is slightly toxic, on the basis of its acute toxicity.

Norantipyrine, at a concentration of 1 %, showed no signs of irritation. The sensitization test was carried out inadequately.

In the 28-day study with rats, effects were still found in the 1000 mg/kg b.w. group. The dose level without effect is 200 mg/kg b.w.

In the carcinogenicity study no neoplastic lesions were observed.

No adverse effects were reported in an oral teratogenicity study upto 1000 mg/kg b.w. (the highest concentration tested).

With the exception of the mouse lymphoma assay with metabolic activation, Norantipyrine was not genotoxic in all tests.

The cutaneous absorption was 0.08 % for skin with and without hair.

For normal use of hair dye, the following calculation can be made:

0.25 g of Norantipyrine comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 0.25 % Norantipyrine). With a maximal penetration, under normal condition, of 0.08 %, this results in a dermal absorption of 0.2 mg per treatment, which is 0.003 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 66670 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in rats in the 28-day study.

It should be noted that the NOAEL stems from a daily exposure for 28 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

Need for an adequate sensitization test.

The additional information has to be communicated within one year.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

Norantipyrine

Colipa No. A 39

Based on a usage volume of 100 ml, containing at maximum 0.25 %

Maximum amount of ingredient applied:	**I (mg) = 250**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.08**
Dermal absorption per treatment:	**I (mg) x A (%) = 0.2 mg**
Systemic exposure dose (SED):	**SED (mg) = I (mg) x A (%) / 60 kg = 0.003**
No observed adverse effect level (mg/kg):	**NOAEL = 200 mg/kg**

It should be noted that the NOAEL stems from a daily exposure for 28 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

Margin of Safety:	**NOAEL / SED = 66670**

A 98: *AMINOL*

1. General

Summary of the opinion adopted by the plenary session of the SCC of 4/5 November 1991.

Aminol has moderate acute toxicity by the oral route. Limited studies suggest that dermal absorption from hair dye formulations is poor (can be up to 2 %). There was no evidence of skin irritancy in animals using a 5 % solution of aminol and only a mild effect in some humans using a hair dye formulation containing hydrogen peroxide and 2 % aminol and using an occlusive dressing for 24 hours. A 2 % solution produced no significant eye irritation in animals. In a 90-day oral study the no effect level was 20 mg/kg with evidence of bone marrow toxicity at 275 mg/kg and lethality at 550 mg/kg. Aminol has been examined in a range of mutagenicity studies *in vitro* (gene mutation in *Salmonella* and mouse lymphoma cells, metaphase analysis of lymphocytes for clastogenicity) with negative results. Negative results were also obtained in *in vivo* assays for sister chromatid exchange and micronucleus induction in bone marrow. No adverse effects were reported in oral teratogenicity studies in rats at up to 1000 mg/kg or rabbits up to 250 mg/kg.

Safety margins

Permanent hair dye use:

1.5 g of 1-(ß-Hydroxyethylamino)-3,4-methylenedioxybenzene comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1.5 % 3,4-diaminobenzoic acid). With a maximal penetration of 2%, this results in a dermal absorption of 30 mg per treatment, which is 0.5 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 40 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in rats in the 90-day study.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

However, an adequate test for the sensitization potential of aminol is required.

Recommended **Classification: B**

CALCULATION OF SAFETY MARGIN

AMINOL

COLIPA NO. A 98

Based on a usage volume of 100 ml, containing at maximum 1.5%.

Maximum amount of ingredient applied	**I(mg) = 1500**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A(%) = 2**
dermal absorption per treatment	**I(mg) x A(%) = 30 mg**
Systemic exposure dose (SED)	**SED = I(mg) x A(%)/ 60 kg b.w.**
	= 0.5
No observed adverse effect level	**NOAEL = 20 mg/kg**

MARGIN OF SAFETY NOAEL/SED = 40

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

1.1 Primary name

1-(ß-hydroxyethylamino)-3,4-methylenedioxybenzene, hydrochloride

1.2 Chemical names

1-(ß-hydroxyethylamino)-3,4-methylenedioxybenzene, hydrochloride
Aminol

1.4 CAS no.

94158-14-2

1.5 Structural formula

5. Sensitization

Two sensitization studies are provided. The evaluation of the 2 studies (Quality Assurance Declaration was included) is reported below.

A. Magnusson Kligman test:

2 groups of 20 female Hartley guinea pigs (Crl:(HA)BR) were used in this skin sensitization study.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The injections were divided as follows, 2 injections of 0.1 ml of a 5 % solution of the test substance in physiological saline, 2 injections of 0.1 ml of FCA blended with physiological saline (1:1) and 2 injections of 0.1 ml of the test substance (0.005 %) blended with FCA (1:1). The control group received the vehicle (physiological saline); positive control substance was 1,4-phenylenediamine.

Day 1-6: examination local tolerance.

Day 8, an epicutaneous induction of 25 % solution of the test substance in white petrolatum. The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 25 % solution of the test substance in white petrolatum in a 24 hours closed patch test, while the right shoulder was treated with the vehicle (white petrolatum).

On day 24, all animals were sacrificed.

Any sign of erythema and oedema was recorded 24 hours after the intradermal and epicutaneous induction and 24 and 48 hours after the end of the challenge.

No animal died during the study. In 11/20 animals (55 %) light to severe erythema and oedema was observed, after the challenge exposure.

So, the test substance has a sensitizing potential.

B. Buehler test:

20 Female Hartley guinea pigs (Crl:HA(BR)) were used as test substance group and 10 females were used as negative controls, in this sensitization study.

The induction phase consisted of 3 epicutaneous applications of 25 % of the test substance in white petrolatum for 6 hours, to the left flanks of the animals, on days 0, 7 and 14. The control group received the vehicle (white petrolatum) only. On day 28 the challenge exposure started. For the challenge exposure undiluted aminol in KOLESTON 2000 was epicutaneously applied to the right flanks of the test group animals.

Any sign of erythema and oedema was recorded 24 hours after the epicutaneous induction and 24, 48 and 72 hours after the start of the challenge.

The test substance treated skin areas of all animals were coloured yellow/brown so that histopathological examination of these sites was included in the study and was decisive for the rating of an allergenic potency. After challenge exposure, no adverse skin reactions were observed in the negative control group. In the test substance group, spongiosis (intracellular oedema) was detected by histopathological examination in one animal (5 %).

So, the test substance has no sensitizing potential.

11. Conclusions

Overall conclusion over the 2 sensitization studies is:

Aminol has a sensitizing potential.

Classification: A

A 112: *PAROLERSATZ C*

1. General

1.1. Primary name

Parolersatz C

1.2. Chemical names

Parolersatz C
4-amino-2-aminomethylphenol, dihydrochloride
1-hydroxy-2-aminomethyl-4-aminobenzene, dihydrochloride
Oxamitol
GHS 110385

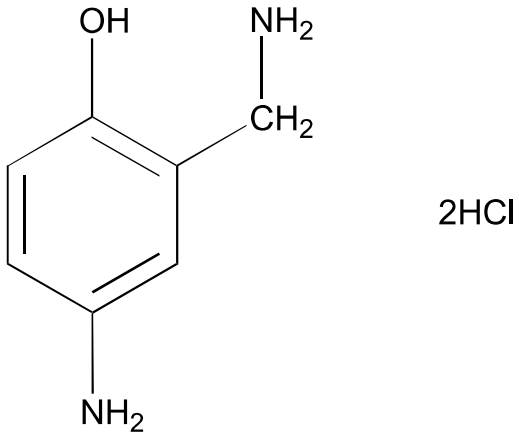
1.3 Trade names and abbreviations

Colipa No.: A112

1.4 CAS no.

79352-72-0

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_7 H_{10} N_2O$. 2 HCl
Mol weight: 211 as dihydrochloride

1.7 Purity, composition and substance codes

sA: 4-amino-2-aminomethylphenol, dihydrochloride (purity: >99%)
fA: 1.25 % 4-amino-2-aminomethylphenol, dihydrochloride

1.8 Physical properties

Subst. code: sA
Appearance: white-pink crystals, light yellow powder
Melting point: 246°C.

1.9 Solubility

The substance exists as a dihydrochloride.

2. Function and uses

4-amino-2-aminomethylphenol is included in oxidative hair dye formulations at a maximum concentration of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1.5 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1. Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	oral	mouse (f)	825	mg/kg b.w.		
sA	oral	mouse (m)	560	mg/kg b.w.		
sA	oral	rat (f)	500	mg/kg b.w.		
sA	oral	rat (m)	625	mg/kg b.w.		

4-amino-2-aminomethylphenol, dihydrochloride dissolved in aqua dest. was administered once via stomach tube to CF1 mice (40/sex) and Wistar rats (18 males and 24 females). Male mice were dosed with 200, 600, 1000 and 1400 mg/kg b.w. and female mice received 500, 750, 1000 and 1250 mg/kg b.w.; rats received doses of 250, 500, 750 and 1000 mg/kg b.w.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

The test substance caused reduced activity and dark discoloration of urines. The test substance is moderately toxic.

3.2 Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	derm	rat	>2000	mg/kg b.w.		

Parolersatz C was administered once dermally to Sprague Dawley rats (5/sex), at a concentration of 2000 mg/kg b.w.

Behaviour, reactions and physical signs of the animals were observed 1, 10 and 30 minutes, 1, 2, 4 and 6 hours after the administration and once daily for 2 weeks. Body weights were recorded weekly. A post mortem examination was carried out on all animals.

In 1 animal chromodacryorrhea was noted 6 hours after administration. In 2 animals white foci on the left kidney were observed.

The test substance is slightly toxic.

3.7 Subchronic oral toxicity

Route: oral	Exposure: 90 days	DWE : 30 mg/kg b.w.
Species: rat	Recov.p. : 4 weeks	LED : 60 mg/kg b.w.
Subst.: sA		

Parolersatz C was administered, by gavage, once daily to 4 groups Wistar rats (15/sex) for 90 days. The test substance was administered at dosage levels of 15 (II), 30 (III) or 60 (IV, V) mg/kg b.w. The control group received the vehicle (distilled water) only. 10 animals/sex of group I (control group) and all animals of group V (60 mg/kg group) were additionally observed for 4 weeks without treatment, for signs of recovery. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality, clinical signs and water consumption. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination was performed. Blood samples were withdrawn from 10 males and 10 females of each test group for haematological and clinical chemistry investigations, during week 6, 12 and 16. Urine samples were taken from 5 males and 5 females of each test group, during week 6, 12 and 16. Organ weights (c. 10) were measured and macroscopy and histopathology (c. 30 organs/tissues) was performed on all animals.

One animal (group V) died during the study (not treatment-related). The urine of the high dose group was discoloured brown, but during week 15 (recovery period) the urine was coloured normal. In the high dose groups histopathologically a slight to moderate active appearance of thyroid glands as well as an astrocyte activation indicating a reinforced liver-clearance were observed.

The dose level without adverse effects was 30 mg/kg b.w.

4. Irritation & corrosivity

4.1. Irritation (skin) (1)

Route: skin	Exposure	Pr.Irr.Index: 0.0
Species: guinea pig	Dose: 1 ml	Effect: not irrit.
Subst.: sA	Concentr: 5.5 %	

One ml of the test substance, dissolved in water, was applied occlusively to the right, clipped back of 5 female Pirbright white guinea pigs for 4 hours. Then the substance remainders were washed off.

Observations for signs of dermal irritation were recorded 1 hour after washing and once daily until the symptoms had subsided (at least for 72 hours).

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.1 Irritation (skin) (2)

Route: skin	Exposure:	Pr.Irr.Index: 0.0
Species: rabbit	Dose: 0.5 g	Effect: not irrit.
Subst.: sA	Concentr: 100 %	

0.5 g of the undiluted test substance (cellulose patch soaked with 1 ml aqua dest.) was applied occlusively to the right, clipped back of 3 female NZW rabbits for 4 hours. The substance residues were washed off.

Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours after patch removal. Additional examinations were performed 6, 8, 10, 13, 15, 17 and 21 days after the end of exposure.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes) (1)

Route: eye	Exposure:	Pr.Irr.Index: 0.0
Species: guinea pig	Dose: 0.1 ml	Effect: not irrit.
Subst.: sA	Concentr: 3 %	

Of a 3 % solution of the test substance in water, 0.1 ml was instilled into the conjunctival sac of the right eye of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls.

The eyes were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after application. After the instillation of one drop of 0.1 % fluorescein-sodium-solution further examinations were carried out after 24 hours and once every following day till no reactions were observed.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2. Irritation (mucous membranes) (2)

Route : eye	Exposure:	Pr.Irr.Index: 0.6
Species: rabbit	Dose : 0.1 ml	Effect: not irrit.
Subst. : sA	Concentr: 100 %	

Of the undiluted test substance, 0.1 ml was instilled into the conjunctival sac of the right eye of 3 female NZW rabbits. The untreated left eyes served as controls.

The eyes were examined 1, 24, 48, 72 hours after application. Additional examinations were carried out after 6, 8, 10, 13, 15, 17 and 21 days post application.

Minimal redness of the conjunctivae was observed in 1 animal, 24 hours after application. The Draize score was 0.6 (not irritating).

5. Sensitization (1)

Subst.: sA Conc.induc.: 0.1 % 40 % Result: positive
Species: guinea pig Conc.chall. : 40 %
Method : Magnusson Kligman

Two groups of 10 female Pirbright white guinea pigs (1 control and 1 test group) were used in this skin sensitization study.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.05 ml of a 0.1 % solution of the test substance in Ringer solution, 2 injections of 0.05 ml of the test substance (0.1 %) in Freund's Complete Adjuvant (FCA) (1:1) and 2 injections of 0.05 ml FCA plus Ringer solution (1:1). The control group received the vehicle (Ringer solution).

Day 1-6: examination on local tolerance.

Day 7, an epicutaneous induction of 40 % aqueous solution of the test substance. The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections.
Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 40 % aqueous solution of the test substance in a 24 hours closed patch test, while the right shoulder was treated with the vehicle.

The control animals were treated the same way, using the vehicle only.

Any sign of erythema and oedema was recorded 1 and 24 hours after the topical induction and 24 and 48 hours after the end of the challenge.

In all test animals (10/10) skin reactions (erythema) were observed. So, the test substance has a sensitizing potential.

5. Sensitization (2)

Subst.: sA Conc.induc.: Result: positive
Species: guinea pig Conc.chall. : 1%
Method : Magnusson Kligman

This study is a cross-sensitization study.

Two groups of 20 female Hartley guinea pigs (Crl:(HA)BR) (1 control and 1 test group) were used in this skin sensitization study, using a hair dye formulation, containing 1.31 % of the test substance diluted with the oxidant Welloxon 9 % at 1:1. The control group was treated only with the vehicle, white petrolatum. 1 group of 20 female guinea pigs was pre-treated with the sensitizer p-phenylenediamine.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.1 ml of a 0.5 % solution of p-phenylenediamine in aqua dest., 2 injections of 0.1 ml of p-phenylenediamine (0.1 %) in Freund's Adjuvant Complete (FCA) (1:1) and

2 injections of 0.1 ml FCA in aqua dest.(1:1). The control group received the vehicle (white petrolatum).

Day 1-6: examination on local tolerance.

Day 7, an epicutaneous induction of 25 % p-phenylenediamine in white petrolatum. The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 10 % p-phenylenediamine in white petrolatum in a 24 hours closed patch test, while the right shoulder was treated with the vehicle.

On day 35, the re-challenge phase started; the left shoulder was treated with 1 % of the test substance in white petrolatum in a 24 hours closed patch test, while the right shoulder was treated with the vehicle.

The control animals were treated the same way, using the vehicle only.

All animals were observed daily for any clinical signs. The body weight of each animal was recorded on days -1, 24 and 38. Any sign of erythema and oedema was recorded 24 hours after the intradermal induction exposure, 24 hours after the epicutaneous induction exposure, 24 and 48 hours after the end of the challenge exposure and 24 and 48 hours after the end of the re-challenge exposure.

There was a decrease in body weight in the test animals at days 24 and 38, due to the treatment with p-phenylenediamine. In the test substance group, all animals showed severe erythema and oedema of the p-phenylenediamine treated sites 24 and 48 hours after the end of the exposure. So, all animals were regarded as sensitized by p-phenylenediamine.

After re-challenge with the test substance, 3 of the 20 pre-sensitised (15 %) animals, had very slight to well defined erythema on the test substance site. These 3 animals were regarded as cros-sensitized.

5. Sensitization (3)

Subst.: sA	Conc.induc.: 100 %	Result: negative
Species: guinea pig	Conc.chall. : 50 % 3 %	
Method : Buehler		

Two groups of Pirbright white guinea pigs (1 control of 10 (5/sex) and 1 test group of 20 animals (10/sex)) were used in this skin sensitization study.

During the induction phase 0.2 g of the undiluted test substance was occlusively applied (via Top-Hill-Chamber) to the shaven shoulder region of the 20 test animals once a week, for 6 hours, for 3 consecutive weeks.

Two weeks after the last treatment the challenge phase started, the left flank was treated with 0.5 ml 50 % aqueous solution of the test substance or 0.5 ml 3 % (right flank) dilution of the test substance in deionised water.

24 hours after the application the test areas were depilated and the skin was inspected 2 and 24 hours later.

After the second and third induction slight erythema was observed in the test animals (9/20). No reactions were observed after the challenge phase. Under the test conditions, the test substance has no sensitizing potential.

5. Sensitization (4)

Subst.: fA	Conc.induc.: 30 %	Result:
Species: guinea pig	Conc.chall. : 5 % 10 %	
Method : Buehler		

Two groups of Pirbright white guinea pigs (1 control of 10 (5/sex) and 1 test group of 20 animals (10/sex)) were used in this skin sensitization study.

A hair dye formulation, containing 2.5 % of the test substance was diluted with the oxidant Welloxon 9 % at 1:1.

During the induction phase 0.5 ml of a 30 % dilution of the formulation in deionised water was occlusively applied (via Top-Hill-Chamber) to the shaven shoulder region of the 20 test animals once a week, for 6 hours, for 3 consecutive weeks.

Two weeks after the last treatment the challenge phase started, the left flank was treated with 0.5 ml 10 % aqueous solution of the formulation or 0.5 ml 5 % (right flank) aqueous solution of the formulation.

24 hours after the application the test areas were depilated and the skin was inspected 2 and 24 hours later.

The signs of irritation can not be assessed, because of oxidation discoloration.

Remark: This test is inadequately performed, because no irritation can be assessed. So, data on the preliminary irritation test are needed.

6. Teratogenicity

Route: oral	Admin.Days: 515	DWE : 90 mg/kg b.w.
Species: rat	LED : mg/kg b.w.	
Subst.: sA		

Parolersatz C was administered, by gavage, to 4 groups of 24 pregnant SPF-Albino Wistar rats (Crl:Wi/Br). The test substance was daily administered at dosage levels of 15, 45 or 90 mg/kg b.w. The control group received the vehicle (aqua dest.) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 5, 10, 15 and 20. Food consumption was measured for the day-intervals 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of

the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

The haircoat of all females in the control and dose groups appeared smooth and brightly. During treatment, females of the mid and high dose group had yellow discoloured urines. In the late gestational phase, a significantly increased food consumption was found in the females of the high dose group. No irreversible structural changes were found.

The dose level without maternal toxicity was 45 mg/kg b.w. and the dose level without embryo/foetotoxicity was 90 mg/kg b.w.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, In vitro mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	*Salm typh*	TA97	frameshift mut	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA98	frameshift mut	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	basepair subst.	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA98	frameshift mut	4-2500 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	basepair subst.	4-2500 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1535	basepair subst.	4-2500 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1537	frameshift mut	4-2500 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1538	frameshift mut	4-2500 µg/pl	-	-	r	AR
*sA	CHO		chrom aber	5-1650 µg/ml	+	-	r	AR

8.2 Mutagenicity (In vivo mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	NMRI	micronuclei	67, 200 and 666 mg/kg b.w.	-

8.3 Mutagenicity tests (text).

Salmonella assays

3 strains of *Salmonella typhimurium* were exposed to Parolersatz C dissolved in distilled water, in the presence and absence of rat liver S9 mix. The dose level tested was 1-10000 µg/plate. The negative control was distilled water; the positive control substances were 2-aminofluorene with and 4-nitro-o-phenylendiamin, 2-nitrofluorene and sodium-azide without S9 mix.

There was no mutagenic effect found in the 3 strains, neither in the absence nor in the presence of S9 mix. Concentrations of ≥ 6000 µg/plate were toxic to the bacteria.

Remark: At least 4 strains are prescribed in the OECD guidelines.

5 strains of *Salmonella typhimurium* were exposed to Parolersatz C dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose level tested was 4-2500 µg/pl. In the second assay the dose level tested was 8-5000 µg/pl with S9 mix. The negative control was DMSO; the positive control substances were 2-aminofluorene with and 4-nitro-o-phenylendiamin, 2-nitrofluorene and sodium-azide without S9 mix.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

Chromosome aberration assay

Parolersatz C was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in distilled water, at dose levels of 5, 16.5, 50 µg/ml -S9mix and 165, 500 and 1650 µg/ml +S9mix. Methylmetanesulphonate and cyclophosphamide served as positive controls. 100 metaphases per culture were analyzed for chromosome aberrations.

Parolersatz C did not induce chromosome aberrations in the presence of S9 mix, but in the absence of S9 mix a significant increased number of chromosome aberrations were induced, at the highest dose only.

Micronucleus assay

Parolersatz C was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of NMRI mice. The test substance, dissolved in distilled water, was administered, by gavage, to the animals (6/sex) at concentrations of 67, 200 and 666 mg/kg b.w. 3 groups (6/sex) were additionally treated by intraperitoneal route at a dose of 333 mg/kg b.w. Cyclophosphamide was the positive control. Samples were taken 24, 48 and 72 hours after administration. In each group 1000 polychromatic erythrocytes of 5 males and 5 females were analyzed for micronuclei and the ratio of polychromatic to normochromatic erythrocytes was estimated.

No cytotoxicity was observed (no increase in NCE; PCE:NCE=1:1). Parolersatz C did not increase frequencies of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse.

Indicator tests (*in vivo* mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	NMRI	SCE	67, 200 and 666 mg/kg b.w.	

Sister Chromatid Exchange assay

Parolersatz C was tested for its potential to induce SCE's in bone marrow cells of NMRI mice. The test substance, dissolved in distilled water, was administered, by gavage, to the animals

(5/sex) at concentrations of 67, 200 or 666 mg/kg b.w. Cyclophosphamide was the positive control. From each animal 25 metaphase cells were scored for SCE's.

Parolersatz C did not induce an increase in SCE's in the bone marrow cells of the mouse.

10. Special investigations

Skin absorption *in vivo*, distribution and elimination [1]:

^{14}C-labelled 4-amino-2-aminomethylphenol-dihydrochloride (purity > 97 %) was applied to the clipped dorsal skin of Sprague Dawley rats (HIM: OFA, SPF) for 30 minutes and then washed off. In the 3 studies 3 rats/sex were used. The test substance was integrated in 2 different hair dye formulations* or was used as a solution in water.

Hair dye formulation IIA was mixed with Welloxon (containing 9 % hydrogen peroxide) (1:1) before application. The study is performed with formulation IIB.

The amount of test substance applied per animal was 30.6 mg of formulation I (3 %) and 29.9 mg of formulation IIB (3 %) and 31.0 mg of the 10 % solution of the test substance.

The content of radioactivity was determined in rinsing water, treated skin areas, faeces, organs and carcass.

The formulation or the solution was left for 30 min and was then scraped off using a spatula, followed by a rinse-off using first about 100 ml of a 3 % solution of a proprietary shampoo and then water of about 37 °C. Rinsing was continued until the rinsing water and the absorbent cellulose tissue which was used to dab the skin dry were free of colour. The rinsings were collected. Than the treated areas were covered with 4 layers of gauze fixed by adhesive tapes. Additional covering by fixation of an air permeable, plastic, truncated cone to prevent licking of the treated area.

98.4-100.5 % of the applied ^{14}C was removed from the skin by rinsing 30 min. after the beginning of the cutaneous application.

The treated area of the skin still contained a small fraction of the applied ^{14}C activity: 0.81 % for formulation I, 2.12 % for formulation IIB and 0.38 % for the solution of the test substance.

Small ^{14}C-concentrations were found in the organs after 72 hours and in most cases in the range of the detection limit.

The mean percutaneous absorption was 0.035 % of the administered ^{14}C for hair dyeing formulation I, 0.037 % for formulation IIB and 0.384 % for the solution.

The absorbed amount of ^{14}C labelled test substance was excreted mainly via urine (69-80 %) and to a lesser extent via faeces (12-18 %). The mean excretion within the first 24 hours was 62-93 %.

* Composition of the formulations I and II:

	I	II	
		A	B
	(%)	cream alone (%)	mixed with Welloxon (%)
- ^{14}C-labelled Parolersatz C	3.00	6.00	3.00
- p-toluylendiamine-sulfate	—	3.50	1.75
- mixture of resorcinol and m-aminophenol	—	1.36	0.68
- mixture of salts	0.70	0.70	0.35
- ammonia, 25%	3.82	2.00	1.00
- isopropanol	3.90	3.90	1.95
- WAS	2.00	2.00	1.00
- deionised water	38.98	25.85	12.92
- formulation base	47.60	47.60	23.80
- ammonia, 25%	—	7.09	3.55
- Welloxon (containing 9% hydrogen peroxide)	—	—	50.00

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the acute oral toxicity test, eye and skin irritation studies with guinea pigs, maximization study with guinea pigs and the test substance and one Ames test.

General

4-amino-2-aminomethylphenol, dihydrochloride is used in oxidative hair dye formulations at a maximum concentration of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1.5 % only.

Acute toxicity

The test substance is moderately toxic, on the basis of its acute oral toxicity (LD_{50} oral; mouse, rat 500-825 mg/kg b.w.). The test substance is slightly toxic, on the basis of its acute dermal toxicity (LD_{50} dermal, rat > 2000 mg/kg b.w.).

Irritation

A concentration of 3 % in the eye and 5.5 % in the skin irritation study with guinea pigs, showed no signs of irritation. A concentration of 100 % in the eye and 100 % in the skin irritation study with rabbits, showed no signs of irritation.

Sensitization

A positive sensitizing effect was observed in guinea pigs in the Magnusson Kligman test. A positive sensitizing effect was observed in guinea pigs in the cross-sensitization study. In one Buehler test, no sensitizing effect was observed in guinea pigs, after administration of the test

substance. No evaluation of the other Buehler test (with the hair dye formulation containing 1.25 % of sA) is possible due to the inadequate performance of the test method.

Semichronic toxicity

In a 90-day feeding study, Sprague Dawley rats were fed 0, 15, 30 or 60 mg Parolersatz C/kg b.w., by gavage once daily. The animals of the 60 mg/kg b.w. group showed the following effects: a slight to moderate activation of thyroid glands as well as astrocyte activation indicating a reinforced liver-clearance. The dose level without effect was 30 mg/kg b.w.

Teratogenicity

In a teratogenicity study, Wistar rats were fed 0, 15, 45 or 90 mg Parolersatz C/kg b.w. No animal died during the study. No irreversible structural changes were found.

The dose level without maternal toxicity was 45 mg/kg b.w. and the dose level without embryo/foetotoxicity was 90 mg/kg b.w.

Genotoxicity

Parolersatz C was tested for its mutagenic potential under *in vitro* conditions in Ames tests and in a chromosome aberration assay. Under *in vivo* conditions a micronucleus assay and a SCE-assay was performed.

With the exception of the chromosome aberration assay without metabolic activation, at the highest dose only, Parolersatz C is considered not genotoxic, based on the provided mutagenicity tests.

Absorption

^{14}C -labelled Parolersatz C was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in water.

Most of the substance was recovered by rinsing (98.4-100.5 %). The cutaneous absorption was 0.035 % for the formulation without hydrogen peroxide, 0.037 % for the formulation with hydrogen peroxide and 0.384 % for the solution.

Conclusions

Parolersatz C is moderately toxic, on the basis of its acute oral toxicity and is slightly toxic, on the basis of its acute dermal toxicity.

A 3 % and 100 % solution of Parolersatz C was not irritating to the eye of guinea pigs and rabbits. A 5.5 % and 100 % solution of Parolersatz C was not irritating to the skin of guinea pigs and rabbits. Parolersatz C has a sensitizing potential.

In the 90-day study with rats, effects were still found in the 60 mg/kg b.w. group. The dose level without effect is 30 mg/kg b.w.

In a teratogenicity study with rats, no irreversible structural changes were observed.

Parolersatz C is considered to be not genotoxic, based on the provided mutagenicity tests.

The cutaneous absorption was 0.035 % for the formulation without hydrogen peroxide, 0.037% for the formulation with hydrogen peroxide and 0.384 % for the solution.

For normal use of hair dye, the following calculation can be made:

1.5 g of Parolersatz C comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1.5 % Parolersatz C). With a maximal penetration, under normal condition, of 0.037 %, this results in a dermal absorption of 0.555 mg per treatment, which is 0.0093 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 3230 can be calculated between the figure for human exposure to this oxidative hair dye and the no effect level found in rats in the 90-day study.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

PAROLERSATZ C

Based on a usage volume of 100 ml, containing at maximum 1.5 %

Maximum amount of ingredient applied:	**I (mg)= 1500**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 0.037**
Dermal absorption per treatment:	**I (mg) x A (%)= 0.555 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg = 0.0093**
No observed adverse effect level (mg/kg):	**NOAEL = 30 mg/kg**
Margin of Safety:	**NOAEL / SED = 3230**

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

A 118: *PAROLERSATZ D*

1. General

1.1 Primary name

Parolersatz D

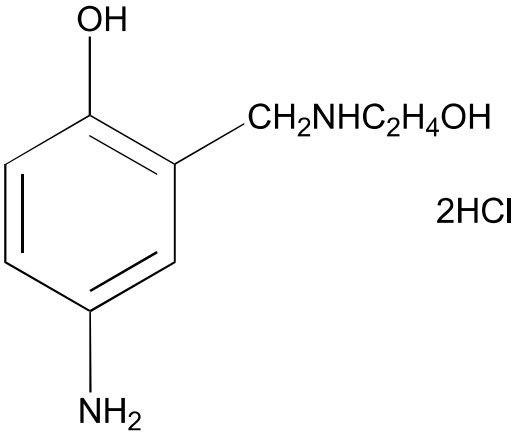
1.2 Chemical names

Parolersatz D
4-amino-2[(2'-hydroxyethyl)-aminomethyl]-phenol, dihydrochloride
1-hydroxy-2[(2'-hydroxyethyl)-aminomethyl]-4-aminobenzol, dihydrochloride
GHS 030585

1.4 CAS no.

110952-46-0

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9 H_{14} N_2 O_2$. 2 HCl
Mol weight: 255 as dihydrochloride

1.7 Purity, composition and substance codes

sA: 4-amino-2[(2'-hydroxyethyl)-aminomethyl]-phenol, dihydrochloride (purity: > 99 %)
fA: 1.25 % 4-amino-2[(2'-hydroxyethyl)-aminomethyl]-phenol, dihydrochloride

1.8 Physical properties

Subst. code: sA
Appearance: white-grey crystalline powder

Melting point: 242°C.

1.9 Solubility

The substance exists as a dihydrochloride.

2. Function and uses

4-amino-2[(2'-hydroxyethyl)-aminomethyl]-phenol, dihydrochloride is included in oxidative hair dye formulations at a maximum concentration of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1.5 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1. Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	oral	mouse (f)				
sA	oral	mouse (m)				
sA	oral	rat (f)				
sA	oral	rat (m)				

Oral studies

4-amino-2[(2'-hydroxyethyl)-aminomethyl]phenol, dihydrochloride dissolved in aqua dest. was administered once via stomach tube to CF1 mice (20/sex) and Wistar rats (10/sex). Male mice were dosed with 400 and 1600 mg/kg b.w. and female mice received 800 and 1600 mg/kg b.w.; rats received doses of 1000 and 1400 mg/kg b.w.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

The test substance caused reduced activity and orange discoloration of urines.

Results: female mice at 1600 mg/kg b.w. 10/10 animals died and at 800 mg/kg b.w. 2/10 animals died; male mice at 1600 mg/kg b.w. 10/10 animals died and at 400 mg/kg b.w. 4/10 animals died; female rats at 1400 mg/kg b.w. 5/5 animals died and at 1000 mg/kg b.w. 2/5 animals died; male rats at 1400 mg/kg b.w. 5/5 animals died and at 1000 mg/kg b.w. 0/5 animals died.

The test substance is moderately toxic.

Remark: The LD_{50} could not be calculated, since only 2 dose levels were used.

3.2. Acute dermal toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	derm	rat	>2000	mg/kg b.w.		

Dermal study
Parolersatz D, moistened with distilled water, was administered once dermally to Sprague Dawley rats (5/sex), at a concentration of 2000 mg/kg b.w.

Behaviour, reactions and physical signs of the animals were observed 1, 10 and 30 minutes, 1, 2, 4 and 6 hours after the administration and once daily for 2 weeks. Body weights were recorded weekly. A post mortem examination was carried out on all animals.

In 1 animal chromodacryorrhoea was noted 6 hours after administration. In 1 animal white foci on the left kidney were observed.

The test substance is slightly toxic.

3.4 Repeated dose oral toxicity

Route: oral Exposure: 28 days DWE: 316 mg/kg b.w.
Species: rat Recov.p.: 14 days LED:1000 mg/kg b.w.
Subst.: sA

Parolersatz D was administered, by gavage, once daily to 4 groups Fischer rats (5/sex) for 28 days. The test substance was administered at dosage levels of 100, 316 or 1000 mg/kg b.w. The control group received the vehicle (distilled water) only. In addition, 2 recovery groups (1 high dosed and 1 negative control) were kept for further 14 days. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination was performed. Blood samples were withdrawn from all animals of each test group for haematological and clinical chemistry investigations, on days 0 and 28 (and 42). Urine samples were taken from all animals of the low and mid dose and the 2 recovery groups, on days 0 and 28 (and 42). Organ weights (c. 6) were measured and macroscopy and histopathology (c. 35 organs/tissues) was performed, on all animals.

One animal died during the study (due to a watering system failure). In several high dose animals, decreased motor activities, disturbed locomotion, piloerection and hunched posture were observed during the first 2 weeks of dosing. All high dose animals had brown coloured urine. Red blood cell count of both high dose females and high dose recovered females was increased significantly. Serum cholesterol was significantly increased in high dose males and high dose recovered males. A significantly decreased spleen weight was observed in high dose females.

The dose level without adverse effects was 316 mg/kg b.w.

3.7. Subchronic oral toxicity

Route: oral	Exposure: 90 days	DWE: 40 mg/kg b.w.
Species: rat	Recov.p.: 4 weeks	LED: — mg/kg b.w.
Subst.: sA		

Parolersatz D was administered, by gavage, once daily to 4 groups Wistar rats (15/sex) for 90 days. The test substance was administered at dosage levels of 10, 20 or 40 mg/kg b.w. The control group received the vehicle (distilled water) only. For recovery observations, satellite groups of 10 male and 10 female rats were attached to the control and high dose groups and observed for 4 weeks without treatment. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic and hearing examinations were performed. Blood samples were withdrawn from 10 males and 10 females of each test group for haematological and clinical chemistry investigations, during week 6, 12 and 16. Urine samples were taken from 5 males and 5 females of each test group, during week 6, 12 and 16. Organ weights (c. 12) were measured and macroscopy and histopathology (c. 30 organs/tissues) was performed, on all animals.

No signs of toxicity were observed. The dose level without adverse effects was 40 mg/kg b.w.

4. Irritation & corrosivity

4.1. Irritation (skin) (1)

Route: skin	Exposure: 4 hr	Pr.Irr.Index: 0.0
Species: guinea pig	Dose: 1 ml	Effect: not irrit.
Subst.: sA	Concentr: 3 %	

One ml of the test substance, dissolved in water, was applied occlusively to the right, clipped back of 5 female Pirbright white guinea pigs for 4 hours. Then the substance remainders were washed off.

Observations for signs of dermal irritation were recorded 1 hour after washing and once daily until the symptoms had subsided (at least for 72 hours).

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.1. Irritation (skin) (2)

Route: skin	Exposure: 4 hr	Pr.Irr.Index: 0.1
Species: rabbit	Dose: 0.5 g	Effect: not irrit.
Subst.: sA	Concentr: 100 %	

0.5 g of the undiluted test substance (cellulose patch soaked with 0.7 ml aqua dest.) was applied occlusively to the right, clipped back of 3 female NZW rabbits for 4 hours. The substance residues were washed off.

Observations for signs of dermal irritation were recorded 1, 24, 48 and 72 hours after patch removal. Additional examinations were performed 6, 8, 10, 13, 15, 17 and 21 days after the end of exposure.

Slight oedema was observed in 1 animal at 24 hr post application The Draize score was 0.1 (not irritating).

4.2 Irritation (mucous membranes) (1)

Route: eye	Exposure:	Pr.Irr.Index: 0.0
Species: guinea pig	Dose: 0.1 ml	Effect: not irrit.
Subst.: sA	Concentr: 1.5 %	

Of a 1.5 % solution of the test substance in water, 0.1 ml was instilled into the conjunctival sac of the right eye of 5 female Pirbright white guinea pigs. The untreated left eyes served as controls.

The eyes were examined 0.5, 1, 2, 3, 4, 6 and 7 hours after application. After the instillation of one drop of 0.1 % fluorescein-sodium-solution further examinations were carried out after 24 hours and once every following day till no reactions were observed.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes) (2)

Route: eye	Exposure:	Pr.Irr.Index: 0.2
Species: rabbit	Dose: 0.1 ml	Effect: not irrit.
Subst.: sA	Concentr: 100 %	

Of the undiluted test substance, 0.1 ml was instilled into the conjunctival sac of the right eye of 3 female NZW rabbits. The untreated left eyes served as controls.

The eyes were examined 1, 24, 48, 72 hours after application. Additional examinations were carried out after 6, 8, 10, 13, 15, 17 and 21 days post application.

Minimal redness of the conjunctivae was observed in 1 animal, 24 hours after application. The Draize score was 0.2 (not irritating).

5. Sensitization (1)

Subst.: sA	Conc.induc.: 10 % 100 %	Result: positive
Species: guinea pig	Conc.chall.: 100 %	
Method: Magnusson Kligman		

Two groups of Pirbright white guinea pigs (20/sex) (1 control and 1 test group) were used in this skin sensitization study.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.05 ml of a 10 % solution of the test substance in aqua dest., 2 injections of 0.05 ml of the test substance (10 %) in Freund's Adjuvant Complete (FCA) and 2 injections of 0.05 ml FCA. The control group received the vehicle (aqua dest.).

Day 1-6: examination on local tolerance.

Day 7, an epicutaneous induction of 0.5 g of the test substance (100 %). The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 0.2 g of the test substance (100 %) in a 24 hours closed patch test, while the right shoulder was treated with the vehicle. The control animals were treated the same way, using the vehicle only.

On day 25, the re-challenge phase started; the right shoulder was treated with 0.5 ml of a 3 % solution of the test substance in aqua dest., in a 24 hr closed patch test, while the left shoulder was treated with the vehicle.

Any sign of erythema and oedema was recorded 24 hours after the intradermal induction exposure, 24 hours after the epicutaneous induction exposure, 24 and 48 hours after the end of the challenge exposure and 24 and 48 hours after the end of the re-challenge exposure.

In 15/20 test animals, slight-moderate erythema was observed, 48 hours after the challenge and re-challenge phase. In 12/20 control animals, slight erythema was observed 48 hours after the re-challenge phase. So, the test substance has a sensitizing potential.

5. Sensitization (2)

Subst.: sA Conc.induc.: 0.63 % 50 % Result:
Species: guinea pig Conc.chall. : 12.5 %
Method: Magnusson Kligman

Two groups of 20 female Dunkin Hartley guinea pigs (1 control and 1 test group) were used in this skin sensitization study.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.1 ml of a 0.63 % solution of the test substance, 2 injections of 0.1 ml of the test substance (1.25 %) in Freund's Adjuvant Complete (FCA) (1:1) and 2 injections of 0.1 ml FCA in aqua dest. (1:1). The control group received the vehicle (aqua dest.).

Day 1-6: examination on local tolerance.

Day 7, an epicutaneous induction of 0.4 ml test substance (50 %) in aqua dest. The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 0.1 ml of 12.5 % test substance in aqua dest. in a 24 hours closed patch test, while the right shoulder was treated with the vehicle.

Skin reactions are evaluated 24 and 48 hours after the end of the challenge exposure.

No skin reactions were observed.

Remark: This test is inadequately performed, because no irritation was observed, neither irritation was induced by SDS-pretreatment. The induction concentrations appears to be too low. So, data on the preliminary irritation test are needed.

5. Sensitization (3)

Subst.: sA Conc.induc.: 100 % Result: negative
Species: guinea pig Conc.chall.: 50 % 3 %
Method: Buehler

Two groups of Pirbright white guinea pigs (1 control of 10 (5/sex) and 1 test group of 20 animals (10/sex)) were used in this skin sensitization study.

During the induction phase 0.2 g of the undiluted test substance was occlusively applied (via Top-Hill-Chamber) to the shaven shoulder region of the 20 test animals once a week, for 6 hours, for 3 consecutive weeks.

Two weeks after the last treatment the challenge phase started, the left flank was treated with 0.5 ml 50 % aqueous solution of the test substance or 0.5 ml 3 % (right flank) dilution of the test substance in deionised water.

24 hours after the application the test areas were depilated and the skin was inspected 2 and 24 hours later.

After the second and third induction slight erythema was observed in the test animals (6/20). No reactions were observed after the challenge phase. Under the test conditions, the test substance has no sensitizing potential.

5. Sensitization (4)

Subst.: fA Conc.induc.: 25 %
Species: guinea pig Conc.chall.: 10 %
Method: Buehler

Two groups of Pirbright white guinea pigs (1 control of 10 (5/sex) and 1 test group of 20 animals (10/sex)) were used in this skin sensitization study.

A hair dye formulation, containing 2.5 % of the test substance was diluted with the oxidant Welloxon 9 % at 1:1.

During the induction phase 0.5 ml of a 25 % dilution of the formulation (without Welloxon) in deionised water was occlusively applied (via Top-Hill-Chamber) to the shaven shoulder region of the 20 test animals once a week, for 6 hours, for 3 consecutive weeks.

Two weeks after the last treatment the challenge phase started, the left flank was treated with 0.5 ml 10 % aqueous solution of the formulation (without Welloxon) and 0.5 ml 10 % (right flank) aqueous solution of the formulation with Welloxon.

24 hours after the application the test areas were depilated and the skin was inspected 2 and 24 hours later.

The signs of irritation can not be assessed, because of oxidation discoloration. In 1/20 animals slight erythema was observed, 48 hours after challenge with the formulation without Welloxon.

Remark: This test is inadequately performed, because irritation cannot be assessed. So, data on the preliminary irritation test are needed.

6. Teratogenicity

Route: oral Admin.Days: 515 DWE: 40 mg/kg b.w.
Species: rat LED: --mg/kg b.w.
Subst.: sA

Parolersatz D was administered, by gavage, to 4 groups of 20 pregnant SPF-Albino Wistar rats (Crl:Wi/Br). The test substance was daily administered at dosage levels of 10, 20 or 40 mg/kg b.w. The control group received the vehicle (aqua dest.) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 5, 10, 15 and 20. Food consumption was measured for the day-intervals 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

In the late gestational phase, a significantly decreased food consumption was found in the females of the high dose group. In the high dose group, females revealed slightly increased uteriweights, at termination. No irreversible structural changes were found.

The dose level without maternal toxicity was 20 mg/kg b.w. and the dose level without embryo/foetotoxicity was 40 mg/kg b.w.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *in vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	*Salm typh*	TA97	frameshift mut	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA98	frameshift mut	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	basepair subst.	1-10000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA98	frameshift mut	8-5000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA100	basepair subst.	8-5000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1535	basepair subst.	8-5000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1537	frameshift mut	8-5000 µg/pl	-	-	r	AR
*sA	*Salm typh*	TA1538	frameshift mut	8-5000 µg/pl	-	-	r	AR
*sA	CHO		chrom aber	0.76-185 µg/ml	+	-	r	AR

8.2 Mutagenicity (*In vivo* mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	NMRI	micronuclei	140, 470 and 1400 mg/kg b.w.	-

8.3 Mutagenicity tests (text).

Salmonella assays
3 strains of *Salmonella typhimurium* were exposed to Parolersatz D dissolved in distilled water, in the presence and absence of rat liver S9 mix. The dose level tested was 1-10000 µg/plate. The negative control was distilled water; the positive control substances were 2-aminofluorene with and 4-nitro-o-phenylendiamin, 2-nitrofluorene and sodium-azide without S9 mix.

There was no mutagenic effect found in the 3 strains, neither in the absence nor in the presence of S9 mix. Concentrations of ≥ 6000 µg/plate were toxic to the bacteria.

Remark: At least 4 strains are prescribed in the OECD guidelines.

5 strains of *Salmonella typhimurium* were exposed to Parolersatz D dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose level tested was 8-5000 µg/pl. The negative control was DMSO; the positive control substances were 2-aminofluorene with and 4-nitro-o-phenylendiamin, 2-nitrofluorene and sodium-azide without S9 mix.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix.

Chromosome aberration assay
Parolersatz D was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in Minimal Essential Medium with Hepes buffer, at dose levels of 0.76, 2.29, 6.86 µg/ml +S9-mix and 20.5, 61.7 and 185 µg/ml +S9-mix. Methylmetanesulphonate and cyclophosphamide served as positive controls. 100 metaphases per culture were analyzed for chromosome aberrations.

Parolersatz D did not induce chromosome aberrations in the presence of S9 mix, but in the absence of S9 mix a significant increased number of chromosome aberrations was found, at the highest dose only.

Micronucleus assay
Parolersatz D was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of NMRI mice. The test substance, dissolved in aqua dest., was administered, by gavage, to the animals (6/sex) at concentrations of 140, 470 and 1400 mg/kg b.w. Cyclophosphamide was the positive control. Samples were taken 24, 48 and 72 hours after administration. In each group 1000 polychromatic erythrocytes of 5 males and 5 females were analyzed for micronuclei and the ratio of polychromatic to normochromatic erythrocytes was estimated.

No cytotoxicity was observed (no increase in NCE; PCE:NCE=1:1). Parolersatz D did not induce higher frequencies of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse.

Indicator tests (In vivo mammalian, Host mediated).

Sub	Species	Strain	Measure endpoint	Test conditions	Res.
*sA	mouse	NMRI	SCE	10-2000 µM	-
*sA	rat	Wistar	UDS	100, 300 and 1000 mg/kg b.w.	-

Sister Chromatid Exchange assay
Parolersatz D was tested for its potential to induce SCE's in bone marrow cells of NMRI mice. The test substance, dissolved in DMSO, was administered, by gavage, to the animals (5/sex) at concentrations of 10-2000 µM. 2-nitro-p-phenylendiamine and 2-acetylaminofluorene were the positive controls. From each animal 30 metaphase cells were scored for SCE's.

Parolersatz D did not induce an increase in SCE's in the bone marrow cells of the mouse.

UDS assay
Parolersatz D, dissolved in DMSO, was administered to groups of Wistar rats (6/sex), by gavage, at concentrations of 100, 300 or 1000 mg/kg b.w. The positive control group received 100 mg/kg methylmethanesulfonate (MMS). The negative control group received distilled water. 14 hours after administration the animals were sacrificed and the livers were removed. Liver preparations were incubated with 3H-thymidine and then washed, fixed onto slides and stained. 100 cells per animal were microscopically examined and grains/nucleus counted.

Parolersatz D did not induce UDS *in vivo*.

10. Special investigations

Skin absorrption, distribution and elimination

^{14}C-labelled 4-amino-2-[(2'-hydroxyethyl)-aminomethyl]-phenol-dihydrochloride (purity > 98%) was applied to the clipped dorsal skin of Sprague Dawley rats (HIM: OFA, SPF) for 30 minutes and then washed off. In the 5 studies 3 rats/sex were used. The test substance was integrated in 2 different hair dye formulations* or was used as a solution in water.

Hair dye formulation IIA was mixed with Welloxon (containing 9 % hydrogen peroxide) (1:1) before application. The study is performed with formulation IIB.

The amount of test substance applied per animal was 15.1 mg of formulation I (3 %) and 14.7 mg of formulation IIB (3 %) and 14.9 mg of the 5 % solution of the test substance and 14.8 mg of the 1.5 % solution of the test substance was used in study D (oral) and 15.3 mg of the 1.5 % solution of the test substance was used in study E (oral).

The content of radioactivity was determined in rinsing water, treated skin areas, faeces, organs and carcass.

The formulation or the solution was left for 30 min and was then scraped off using a spatula, followed by a rinse-off using first about 100 ml of a 3 % solution of a proprietary shampoo and then water of about 37 °C. Rinsing was continued until the rinsing water and the absorbent cellulose tissue which was used to dab the skin dry, were free of colour. The rinsings were collected. Than the treated areas were covered with 4 layers of gauze fixed by adhesive tapes. Additional covering by fixation of an air permeable, plastic, truncated cone to prevent licking of the treated area.

90.9-95.1 % of the applied ^{14}C was removed from the skin by rinsing 30 min. after the beginning of the cutaneous application.

The treated area of the skin still contained a small fraction of the administered ^{14}C-activity: 0.66 % for formulation I, 0.86 % for formulation IIB and 0.36 % for the solution of the test substance.

Small ^{14}C-concentrations were found in the organs after 72 hours and in most cases in the range of the detection limit.

The mean percutaneous absorption was 0.035 % of the administered ^{14}C for hair dyeing formulation I, 0.032 % for formulation IIB and 0.42 % for the solution.

The absorbed amount of ^{14}C-labelled test substance was excreted mainly via urine (90-94 %) and to a lesser extent via faeces (6-10 %). The mean excretion within the first 24 hours was 89-97 %.

After oral administration of the test substance the ^{14}C-labelled test substance was excreted to a larger extent via urine (71 % of the eliminated ^{14}C) and to a lesser extent via faeces (26 %). 98 % of the eliminated ^{14}C was excreted within the first 24 hours. The blood level was highest at 35 minutes post application, it declined with an initial half-time of about 1 hour.

* Composition of the formulations I and II:

	I	II	
		A	B
	(%)	cream alone (%)	mixed with Welloxon (%)
- ^{14}C-labelled Parolersatz D	1.50	3.00	1.50
- mixture of resorcinol and m-aminophenol	—	1.36	0.68
- mixture of salts	0.70	0.70	0.35
- ammonia, 25%	1.20	2.40	1.20
- isopropanol	3.90	3.90	1.95
- WAS	2.00	2.00	1.00
- deionised water	43.10	35.39	17.70
- formulation base	47.60	47.60	23.80
- ammonia, 25%	—	3.65	1.82
- Welloxon (containing 9% hydrogen peroxide)	—	—	50.00

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the acute oral toxicity test, eye and skin irritation assay with guinea pigs, Ames tests and SCE assay.

General
4-amino-2[(2'-hydroxyethyl)-aminomethyl]-phenol, dihydrochloride is used in oxidative hair dye formulations at a maximum concentration of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1.5 % only.

Acute toxicity
The test substance is moderately toxic, on the basis of its acute oral toxicity (LD_{50} oral; mouse, rat 400-1600 mg/kg b.w.). The test substance is slightly toxic, on the basis of its acute dermal toxicity (LD_{50} dermal; rat >2000 mg/kg b.w.).

Irritation
A concentration of 1.5 % in the eye and 3 % in the skin irritation study with guinea pigs, showed no signs of irritation. A concentration of 100 % in the eye and 100 % in the skin irritation study with rabbits, showed no signs of irritation.

Sensitization
A positive sensitizing effect was observed in guinea pigs in the Magnusson Kligman test. In one Buehler test, no sensitizing effects were observed in guinea pigs, after administration of the test substance. No evaluation of another Magnusson Kligman and another Buehler test (with the hair dye formulation containing 1.25 % of sA) is possible due to the inadequate performance of the test method.

Subacute toxicity
In a 28-day feeding study, Fischer rats were fed 0, 100, 316 or 1000 mg Parolersatz D/kg b.w., by gavage once daily. Changed haematological parameters and decreased spleen weight was observed in the 1000 mg/kg b.w. group. The dose level without effect was 316 mg/kg b.w.

Semichronic toxicity
In a 90-day feeding study, Wistar rats were fed 0, 10, 20 or 40 mg Parolersatz D/kg b.w., by gavage once daily. No signs of toxicity were observed. The dose level without effect was 40 mg/kg b.w. (highest dose tested).

Teratogenicity
In a teratogenicity study, Wistar rats were fed 0, 10, 20 or 40 mg Parolersatz D/kg b.w. No animal died during the study. No irreversible structural changes were found.

The dose level without maternal toxicity was 20 mg/kg b.w. and the dose level without embryo/foetotoxicity was 40 mg/kg b.w.

Genotoxicity
Parolersatz D was tested for its mutagenic potential under *in vitro* conditions in Ames tests and a chromosome aberration assay. Under *in vivo* conditions a micronucleus assay, a SCE-assay

and an UDS-assay was performed. With the exception of the *in vitro* chromosome aberration assay without metabolic activation, at the highest dose only, Parolersatz D was negative.

Parolersatz D is considered to be not genotoxic, based on the provided mutagenicity tests.

Absorption
^{14}C-labelled Parolersatz D was applied to the skin of rats in two different hair dye formulations (one of them containing hydrogen peroxide) or as a solution of the test substance in water.

Most of the substance was recovered by rinsing (90.9-95.1 %). The cutaneous absorption was 0.035 % for the formulation without hydrogen peroxide, 0.032 % for the formulation with hydrogen peroxide and 0.42 % for the solution.

Conclusions
Parolersatz D is moderately toxic, on the basis of its acute oral toxicity and slightly toxic, on the basis of its acute dermal toxicity.

A 1.5 % and 100 % solution of Parolersatz D was not irritating to the eye of guinea pigs and rabbits. A 3 % and 100 % solution of Parolersatz D was not irritating to the skin of guinea pigs and rabbits. Parolersatz D has a sensitizing potential.

In the 28-day study with rats, effects were found in the 1000 mg/kg b.w. group. The dose level without effect is 316 mg/kg b.w.

In the 90-day study with rats, no effects were found. The dose level without effect is 40 mg/kg b.w.

In a teratogenicity study with rats, no irreversible structural changes were observed.

Parolersatz D is considered to be not genotoxic based on the provided mutagenicity tests.

The cutaneous absorption was 0.035 % for the formulation without hydrogen peroxide, 0.032 % for the formulation with hydrogen peroxide and 0.42 % for the solution.

For normal use of hair dye, the following calculation can be made:

1.5 g of Parolersatz D comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1.5 % Parolersatz D). With a maximal penetration, under normal condition, of 0.035 %, this results in a dermal absorption of 0.525 mg per treatment, which is 0.0088 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 4550 can be calculated between the figure for human exposure to this oxidative hair dye and the no effect level found in rats in the 90-day study.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

Classification A.

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

PAROLERSATZ D

COLIPA NO. A 118

Based on a usage volume of 100 ml, containing at maximum 0.625 %.

Maximum amount of ingredient applied	**I(mg)=1500**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A(%)=0.035**
Dermal absorption per treatment	**I(mg) x A(%) = 0.525 mg**
Systemic exposure dose (SED)	**SED = I(mg) x A(%)/ 60 kg b.w. = 0.0088**
No observed adverse effect level (mg/kg)	**NOAEL = 40 mg/kg**

MARGIN OF SAFETY NOAEL/SED = 4550

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

B 12: *NEOLANSCHWARZ*

1. General

1.1 Primary name

Neolanschwarz

1.2 Chemical names

1-naphtalenesulfonic acid
3-hydroxy-4-[(2-hydroxy-1-naphthalenyl)azo]-7nitro, sodium salt
1-(2'-hydroxy-4'-sulfo-6'-nitro)-naphtylazo-2-hydroxynaphtalene, sodium salt
Acid Black 52

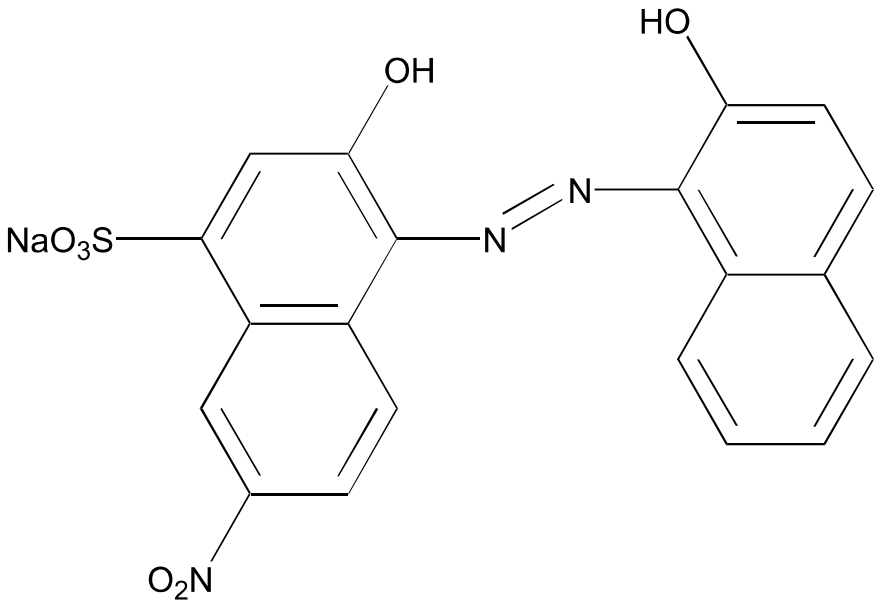
1.3 Trade names and abbreviations

Colipa No.: B 12

1.4 CAS no.

3618-58-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_{20} H_{12} N_3 Na O_7 S
Mol weight: 461.39 as sodium salt

1.7 Purity, composition and substance codes

sA: 1-(2'-hydroxy-4'-sulfo-6'-nitro)-naphtylazo-2-hydroxynaphtalene, sodium salt (purity: 99 %)

1.8 Physical properties

Subst. code: sA
Appearance: brown-black crystalline powder
Melting point: 242°C

1.9 Solubility

The substance exists as sodium salt.

2. Function and uses

1-(2'-hydroxy-4'-sulfo-6'-nitro)-naphtylazo-2-hydroxynaphtalene, sodium salt should be used in oxidative hair dye formulations at a maximum concentration of 2 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Subst	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
sA	oral	rat (f)	>2000	mg/kg b.w.		
sA	oral	rat (m)	>2000	mg/kg b.w.		

Oral studies

Neolanschwarz dissolved in aqua dest. was administered once via stomach tube to Wistar rats (5/sex). Rats received a dose of 2000 mg/kg b.w.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

The test substance is slightly toxic.

3.7 Subchronic oral toxicity

Route: oral	Exposure: 90 days	DWE: 45 mg/kg b.w.
Species: rat	Recov.p.: 4 weeks	LED:100 mg/kg b.w.
Subst.: sA		

Neolanschwarz was administered, by gavage, once daily to 4 groups Wistar Albino rats (15/sex) for 90 days. The test substance was administered at dosage levels of 20, 45 or

100 mg/kg b.w. The control group received the vehicle (distilled water) only. For recovery observations, satellite groups of 10 male and 10 female rats were attached to the control and high dose groups and observed for 4 weeks without treatment. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic and hearing examinations were performed. Blood samples were withdrawn from 10 males and 10 females of each test group for haematological and clinical chemistry investigations, during week 6, 12 and 16. Urine samples were taken from 5 males and 5 females of each test group, during week 6, 12 and 16. Organ weights (c. 15) were measured and macroscopy and histopathology (c. 30 organs/tissues) was performed, on all animals.

One animal in each group and 3 animals of the mid dose group died during the study, due to application faults. Faeces of the high dose group were darkly discoloured. Haematological changes (i.e. Hb, Ht decreased; MCV, MCH increased) were observed in females of the high dose group. After the recovery period, no effects were observed. The dose level without adverse effects was 45 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route: skin	Exposure: 4 hr	Pr.Irr.Index: 0.0
Species: rabbit	Dose: 0.5 ml	Effect: not irrit.
Subst.: sA	Concentr: 2 %	

Dissolved in deionised water, 0.5 ml of the test substance was applied occlusively to the left, clipped back of 6 NZW rabbits for 4 hours. Then the substance remainders were washed off.

Observations for signs of dermal irritation were recorded 0.5 hour after washing and once daily until the symptoms had subsided (at least for 72 hours).

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes)

Route: eye	Exposure:	Pr.Irr.Index: 1.83
Species: rabbit	Dose: 0.1 ml	Effect: not irrit.
Subst.: sA	Concentr: 2 %	

Of a 2 % solution of the test substance in deionised water, 0.1 ml was instilled into the conjunctival sac of the left eye of 3 NZW rabbits. The untreated right eyes served as controls.

The eyes were examined 1, 24, 48 and 72 hours after application. At 24 hours and 72 hours an additional examination was carried out after the instillation of one drop of 1 % fluorescein-sodium-solution.

1 hour postinstillation, slight chemosis was observed in all animals. The Draize score was 1.83 (not irritating).

5. Sensitization

Subst.: sA Conc.induc.: 10 % 100% Result:
Species: guinea pig Conc.chall. : 100 %
Method : Magnusson Kligman

Two groups of Pirbright white guinea pigs (20/sex) (1 control and 1 test group) were used in this skin sensitization study.

The induction phase consisted of 3 series of 2 intradermal injections in the clipped dorsal shoulder region of each animal. The intradermal injections were divided as follows: 2 injections of 0.05 ml of a 10 % solution of the test substance in aqua dest., 2 injections of 0.05 ml of the test substance (10 %) in Freund's Complete Adjuvant (FCA) and 2 injections of 0.05 ml FCA. The control group received the vehicle (aqua dest.).

Day 1-6: examination on local tolerance.

Day 7, an epicutaneous induction of 0.5 g of the test substance (100 %). The occlusive patch application lasted for 48 hours on the surface corresponding to the intradermal injections. Day 12-20: rest period.

On day 21, the challenge phase started; the left shoulder was treated with 0.2 g of the test substance (100 %) in a 24 hours closed patch test, while the right shoulder was treated with the vehicle. The control animals were treated the same way, using the vehicle only.

Any sign of erythema and oedema was recorded 24 hours after the intradermal induction exposure, 24 hours after the epicutaneous induction exposure and 24 and 48 hours after the end of the challenge exposure.

No sign of sensitization was detected in the performed test, but a weak effect could have been masked by the colour of the compound.

6. Teratogenicity

Route: oral Admin.Days: 615 DWE: mg/kg b.w.
Species: rat LED: mg/kg b.w.
Subst. : sA

Neolanschwarz was administered, by gavage, to 4 groups of c. 20 pregnant Albino Wistar rats (Crl:Wi/Br). The test substance was daily administered at dosage levels of 20, 45 or 100 mg/kg b.w. The control group received the vehicle (aqua dest.) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 5, 10, 15 and 20. Food consumption was measured for the day-intervals 0-5, 5-15, 15-20 and 0-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

Females of the mid and high dose group had black discoloured faeces, throughout the application period. No irreversible structural changes were found.

The dose level without maternal and without embryo/foetotoxicity was 100 mg/kg b.w.

Remark: In contrast to the requirements of OECD 414, no maternal toxicity was observed at the highest dose level tested.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *in vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
**sA	*Salm typh*	TA97	frameshift mut	1-3000 µg/pl	-	+	r	AR
*sA	*Salm typh*	TA98	frameshift mut	1-3000 µg/pl	(+)	+	r	AR
*sA	*Salm typh*	TA100	basepair subst.	1-3000 µg/pl	-	+	r	AR
*sA	*Salm typh*	TA98 -NR	frameshift mut	8-5000 µg/pl	(+)	-	r	AR
*sA	*Salm typh*	TA100 -NR	basepair subst.	8-5000 µg/pl	-	-	r	AR
*sA	mouse lymph.	L5178Y	TK+/-	0.00-1666 µg/ml	-	-	r	AR
*sA	human lymph.		chrom aber	0.10-1.00 mg/ml	-	-	r	AR

8.2 Mutagenicity (*in vivo* mammalian, Host mediated).

Sub.	Species	Strain	Meas.endpoint	Test conditions	Res.	Ref.
*sA	mouse	NMRI	micronuclei	2000 mg/kg b.w.	-	

8.3 Mutagenicity tests (text).

Salmonella assays

3 strains of *Salmonella typhimurium* were exposed to Neolanschwarz dissolved in distilled water, in the presence and absence of rat liver S9 mix. The dose level tested was 1-3000 µg/plate. The negative control was distilled water; the positive control substances were 2-aminofluorene with and 4-nitro-o-phenylendiamin, 2-nitrofluorene and sodium-azide without S9 mix.

Neolanschwarz was weakly positive in the absence of S9 mix in strain TA98 and positive in the presence of S9 mix in all 3 strains. However, Neolanschwarz is a nitro- and azo-compound. For these pigments applies that positive results are often seen in the Ames test. This is probably due to nitro- and azo-reductases in the bacteria.

Remark: At least 4 strains are prescribed in the OECD guidelines.

The second Ames test was carried out, due to the positive results obtained with the strains TA98 and TA100 in the first Ames test. In this test the nitroreductase-deficient strains TA 98-NR and TA100-NR, were used. The strains were exposed to Neolanschwarz dissolved in distilled water with and without S9 mix. The dose level tested was 1-6000 µg/plate.

Neolanschwarz showed no mutagenic effect in strain TA98-NR in the presence of S9 mix and in strain TA100-NR (both in the presence and absence of S9 mix). Strain TA98-NR in the absence of S9 showed only a slight increase (about 2-3 times).

Mouse lymphoma fluctuation assay

Neolanschwarz was tested for genotoxicity in the mouse lymphoma fluctuation assay at the $TK^{+/-}$-locus, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, in duplicate at 0.0, 61.72, 185.18, 555.55 or 1666.66 µg/ml. 4-nitroquinoline-N-oxide and benzo(a)pyrene served as positive controls.

Neolanschwarz has no genotoxic activity at the $TK^{+/-}$-locus of L5178Y mouse lymphoma cells, either in the absence or presence of S9 mix.

Chromosome aberration assay

Neolanschwarz was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in DMEM/F12, at dose levels of 0.1-1.00 mg/ml. Ethylmethane-sulfonate and cyclophosphamide served as positive controls. 100 metaphases per culture were analyzed for chromosome aberrations.

Neolanschwarz did not induce chromosome aberrations.

Micronucleus assay

Neolanschwarz was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of NMRI mice. The test substance, dissolved in distilled water, was administered, by gavage, to the animals (5/sex) at a concentration of 2000 mg/kg b.w. Cyclophosphamide was the positive control. Samples were taken 24, 48 and 72 hours after administration. In each group 1000 polychromatic erythrocytes of 5 males and 5 females were analyzed for micronuclei.

Neolanschwarz did not induce higher frequencies of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse.

Indicator tests (in vivo mammalian, Host mediated).

Sub	**Species**	**Strain**	**Measure endpoint**	**Test conditions**	**Res.**
*sA	rat	Wistar	UDS	200, 600 and 1800 mg/kg b.w.	-

UDS assay

Neolanschwarz, dissolved in distilled water, was administered to groups of Wistar rats (6/sex), by gavage, at concentrations of 200, 600 or 1800 mg/kg b.w. The positive control group

received 100 mg/kg methylmethanesulfonate (MMS). The negative control group received distilled water. 24 hours after administration the animals were sacrificed and the livers were removed. Liver preparations were incubated with 3H-thymidine and then washed, fixed onto slides and stained. 100 cells per animal were microscopically examined and grains/nucleus counted.

Neolanschwarz did not induce UDS *in vivo*.

10. Special investigations

Skin absorption of Neolanschwarz

The method used is: *in vitro*, one-chamber flow-through diffusion cell system using pig skin.

The test substance was incorporated into a hair dye gel (Koleston 2000 consists of distilled oleic acid, ethoxylated alkylphenol, ethoxylated alkylcarboxylic acid, isopropanol, ammonia, antioxidants, complexing agents and water; with or without 4.5 % H_2O_2) at a concentration of 1 %. Intact pig skin pieces of 9-10 cm^2 and 1 mm thickness were fixed in permeation cells.

0.1 g of the dye mixture was applied per 1 cm^2 of skin for 30 minutes. Gel residues were then removed by spatula and the skin was washed using warm water and neutral shampoo. Percutaneous permeation was determined after 4 days.

The mean percutaneous absorption was 0.71 % of the administered formulation without H_2O_2 and 0.063 % of the administered formulation with H_2O_2.

The method used is: *in vivo* using rats.

Sprague-Dawley rats (3/sex) were used in each test group. 24 hour after shaving the back of the animals, 0.3-1 g of the hair dye mixture (see above in the *in vitro* study) was applied per 9-11 cm^2 of skin for 30 minutes.

71-98 % of the dose applied was usually washed off. The mean percutaneous absorption was 0.32 % of the administered formulation without H_2O_2 and 0.053 % of the administered formulation with H_2O_2.

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the 2 Ames tests.

General

1-(2'-hydroxy-4'-sulfo-6'-nitro)-naphtylazo-2-hydroxynaphtalene, sodium salt is used in oxidative hair dye formulations at a maximum concentration of 2 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1 % only.

Acute toxicity

The test substance is slightly toxic on the basis of its acute toxicity (LD_{50} oral, rat > 2000 mg/kg b.w.).

Irritation

A concentration of 2 % in the eye and 2 % in the skin irritation study with rabbits, showed no signs of irritation.

Sensitization

In a Magnusson Kligman test, no sensitizing effects were observed in guinea pigs, after administration of the test substance, but a weak effect could have been masked by the colour of the compound.

Semichronic toxicity

In a 90-day feeding study, Sprague Dawley rats were fed 0, 20, 45 or 100 mg Neolanschwarz/kg b.w., by gavage once daily. Haematological changes were observed in the animals of the 100 mg/kg b.w. group. The dose level without effect was 45 mg/kg b.w.

Teratogenicity

In a teratogenicity study, Wistar rats were fed 0, 20, 45 or 100 mg Neolanschwarz/kg b.w. No irreversible structural changes were observed. The dose level without maternal and without embryo/foetotoxicity was 100 mg/kg b.w.

Genotoxicity

Neolanschwarz was tested for its mutagenic potential under *in vitro* conditions in Ames tests, in a mouse lymphoma assay and a chromosome aberration assay. Under *in vivo* conditions a micronucleus and an UDS-assay was performed.

Neolanschwarz was negative in all tests, except for the strains TA98 and TA98-NR, in the Ames tests, in the absence of S9 mix and the strains TA97, TA98 and TA100 in the presence of S9 mix. It is noted that Neolanschwarz is a nitro- and azo-compound.

Neolanschwarz is considered to be not-genotoxic, based on the provided mutagenicity tests.

Absorption

Neolanschwarz was applied to the pig skin (*in vitro*), using diffusion cells and rat skin (*in vivo*). The cutaneous absorption *in vitro* was 0.71 % without and 0.063 % with hydrogen peroxide. The cutaneous absorption *in vivo* was 0.32 % without and 0.053 % with hydrogen peroxide.

Conclusions

Neolanschwarz is slightly toxic, on the basis of its acute toxicity.

Neolanschwarz, at a concentration of 2 %, showed no signs of irritation. Neolanschwarz has no sensitizing potential.

In the 90-day study with rats, effects were found in the 100 mg/kg b.w. group. The dose level without effect is 45 mg/kg b.w.

No adverse effects were reported in an oral teratogenicity study up to 100 mg/kg b.w. (the highest concentration tested).

Neolanschwarz is considered to be not genotoxic, based on the provided mutagenicity tests.

The cutaneous absorption was 0.32 % and 0.71 % for the formulation without hydrogen peroxide *in vivo* and *in vitro* respectively and 0.053 % and 0.063 % for the formulation with hydrogen peroxide *in vivo* and *in vitro* respectively.

For normal use of hair dye, the following calculation can be made:

1 g of Neolanschwarz comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing maximal 1 % Neolanschwarz). With a maximal penetration, under normal condition, of 0.32 %, this results in a dermal absorption of 3.2 mg per treatment, which is 0.053 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 850 can be calculated between the figure for human exposure to this oxidative hair dye and the no effect level found in rats in the 90-day study.

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

NEOLANSCHWARZ

COLIPA NO. B 12

Based on a usage volume of 100 ml, containing at maximum 1 %

Maximum amount of ingredient applied:	**I (mg) = 1000**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.32**
Dermal absorption per treatment:	**I (mg) x A (%) = 3.2 mg**
Systemic exposure dose (SED):	**SED (mg) = I (mg) x A (%) / 60 kg = 0.053**
No observed adverse effect level (mg/kg):	**NOAEL = 45 mg/kg**
Margin of Safety:	**NOAEL / SED = 850**

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more than once a month.

B 51: *1-HYDROXY-3-NITRO-4-AMINOBENZENE*

1. General

1.1 Primary name

Imexine FN

1.2 Chemical names

1-hydroxy-3-nitro-4-aminobenzene
4-amino-3-nitrophenol
4-hydroxy-2-nitroaniline
2-amino-5-hydroxynitrobenzene

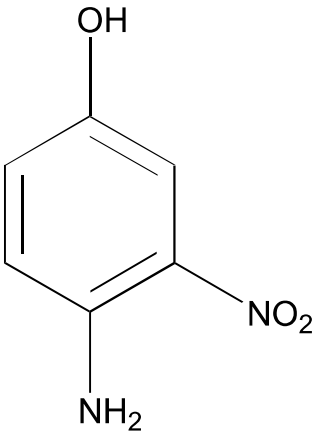
1.3 Trade names and abbreviations

Colipa No.: B 51

1.4 CAS no.

610-81-1

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6 H_6 N_2 O_3$
Mol weight: 154

1.7 Purity, composition and substance codes

sA: 1-hydroxy-3-nitro-4-aminobenzene (purity: 99 %)

1.8 Physical properties

Subst. code: sA
Appearance: red-brown powder, odourless
Melting point: 154°C

1.9 Solubility

The substance exists as a free base.

2. Function and uses

1-hydroxy-3-nitro-4-aminobenzene is included in oxidative hair dye formulations at a maximum of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application the use concentration is 1.5 % only.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}	(unit)	Expos	Remark
*sA	oral	rat	500-1000	mg/kg b.w.		

Oral study

Imexine FN, in 1,2propanediol, was administered once via stomach tube to Sprague-Dawley rats (5/sex) at 3 concentrations, i.e. 500, 1000 and 1500 mg/kg b.w.

During an observation period of 14 days, the mortalities and clinical-toxicological findings were recorded daily and the body weights were noted weekly. A post mortem examination was carried out in all animals.

The test substance caused sedation, dyspnea, tonico-clonic convulsions, ataxia and hypersalivation. The test substance is moderately toxic.

3.4 Repeated dose oral toxicity

Route : oral	Exposure: 4 weeks	DWE : 250 mg/kg b.w.
Species: rat	Recov.p. :	LED : 600 mg/kg b.w.
Subst. : sA		

Imexine FN was administered, by gavage, once daily to 4 groups of Crl:CD-(SD)BR rats (10/sex) for 28 days. The test substance was administered at dosage levels of 100, 250 or 600 mg/kg b.w. The control group received the vehicle (carboxymethyl cellulose). All animals were sacrificed at the end of the study.

All animals were observed daily for mortality and clinical signs. Water consumption was recorded before treatment and during week 3. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination was performed. Blood samples were withdrawn from all surviving animals for haematological and clinical chemistry investigations, during week 4. Organ weights (c. 15) were measured and macroscopy and histopathology (c. 40 organs/tissues) were performed on all control and high dose animals.

Two animals (high dose group) died during the study (laboured respiration). All treated groups showed orange fur-staining, from day one. In the high dose group the following effects were observed: scabbing, perinasal staining, mild convulsions, significantly decreased body weight in the males.

The dose level without adverse effects was 250 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route : skin	Exposure: 4 hr	Pr.Irr.Index: 0.0
Species: rabbit	Dose : 0.5 ml	Effect : not irrit.
Subst. : sA	Concentr: 6 %	

Of the test substance, dissolved in 1,2-propanediol, 0.5 ml was applied semi-occlusively to the right, clipped back of 3 male NZW rabbits for 4 hours.

Observations for signs of dermal irritation were recorded at 1, 24, 48 and 72 hours after application.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

4.2 Irritation (mucous membranes)

Route : eye	Exposure:	Pr.Irr.Index: 0.0
Species: rabbit	Dose : 0.1 ml	Effect : not irrit.
Subst. : sA	Concentr: 6 %	

Of a 6 % solution of the test substance in 1.2-propanediol, 0.1 ml was instilled into the conjunctival sac of the left eye of 3 male NZW rabbits. The untreated right eyes served as controls.

The eyes were examined 1, 24, 48, and 72 hours after application.

No signs of irritation were observed. The Draize score was 0.0 (not irritating).

5. Sensitization

Subst. : sA	Conc.induc.: 3 %	Result:
Species: guinea pig	Conc.chall. : 12.5 %	
Method : epicutaneous maximisation		

Two groups of female Hartley guinea pigs were used in this skin sensitization study (control group 10 animals; test group 20 animals).

On day 0 (induction): 0.5 ml 3 % solution of the test substance was applied under semi-occlusive patch, during 48 hours on days 0, 2, 4, 7, 9, 11 and 14 and 0.5 ml FCA in isotonic NaCl (1:1). Day 1627: rest period.

On day 28, the challenge phase started; the left shoulder was treated with 0.5 ml 12.5 % solution of the test substance in a 48 hours semi-occlusive dressing.

Any sign of erythema and oedema was recorded 1, 6, 24 and 48 hours after the removal of the occlusive patch.

No skin reactions were observed. Under the test conditions the substance showed no sensitizing responses.

Remark: The applied test method is not commonly used. Therefore the sensitizing potential of the substance cannot be evaluated. Reasoned arguments, including validation data, regarding the use of the protocol chosen for the sensitization test should be provided.

6. Teratogenicity

Route : oral	Admin.Days: 615	DWE : 100 mg/kg b.w.
Species: rat		LED : 250 mg/kg b.w.
Subst. : sA		

Imexine FN was administered, by gavage, to 4 groups of 24 pregnant Sprague-Dawley rats (OFA-SD). The test substance was daily administered at dosage levels of 100, 250 or 600 mg/kg b.w. The control group received the vehicle (carboxymethylcellulose) only. All mated females were sacrificed at day 20 of gestation.

The animals were observed daily for clinical signs. Individual body weights were recorded at days 0, 6-15 and 20. Food consumption was measured for the day-intervals 0-6, 6-11, 11-15 and 15-20. Immediately following sacrifice, the uterus was removed, weighed and the number of (non)viable foetuses, early and late resorptions and the number of total implantations and corpora lutea was recorded. A macroscopic examination of the organs was carried out. All foetuses were individually weighed and the sex of the foetuses was determined. Two third of the foetuses was examined for skeletal defects and variations of the ossification process by Alizarin Red staining and one third was evaluated for visceral imperfections (organic defects).

Two females of the high dose group died during the study. Most females of all treated groups had yellow/orange fur staining and yellow/orange stained urine. The high dose females showed significantly reduced body weights. A dose related increase in the number of foetuses exhibiting the skeletal variant of uni-or bilateral vestigial (rudimentary) 14th rib; significant from 250 mg/kg b.w. onwards, was observed. No irreversible structural changes were observed.

The dose level without maternal toxicity was 250 mg/kg b.w. and the dose level without embryo/foetotoxicity was 100 mg/kg b.w.

8.1 Mutagenicity (Bact., Non mammalian eukaryotic, *In vitro* mammalian).

Sb.	Species	Strain	Meas.endp.	Test conditions	res -act	res +act	sp +a	ind +a
*sA	*Salm.typh.*	TA98	frameshift mut.	5-1000 µg/pl	-	-	r	AR
*sA	*Salm.typh.*	TA100	base-pair subst.	5-1000 µg/pl	-	-	r	AR
*sA	*Salm.typh.*	TA1535	base-pair subst.	5-1000 µg/pl	-	-	r	AR
*sA	*Salm.typh.*	TA1537	frameshift mut.	5-1000 µg/pl	-	-	r	AR
*sA	*Salm.typh.*	TA1538	frameshift mut.	5-1000 µg/pl	-	-	r	AR
*sA	CHO		chrom.aber	0.005-0.02 mg/ml	-	-	r	AR

8.2 Mutagenicity (*in vivo* mammalian, Host mediated).

Sub	Species	Strain	Meas.endpoint	Test conditions	Res.
*sA	mouse	Swiss	micronuclei	37.5, 75, 150, 300 mg/kg b.w.	-

8.3 Mutagenicity tests (text).

Salmonella assay

5 Strains of *Salmonella typhimurium* were exposed to Imexine FN dissolved in DMSO, in the presence and absence of rat liver S9 mix. The dose level tested was 5-1000 µg/plate. The negative control was DMSO; the positive control substance was 1,2-diamino-4-nitrobenzene.

There was no mutagenic effect found in the 5 strains, neither in the absence nor in the presence of S9 mix. Concentrations of > 500 µg/plate were toxic to the bacteria.

Chromosome aberration assay

Imexine FN was tested for chromosome aberrations, both in the absence and presence of a rat liver mitochondrial fraction. Cells were treated with sA, dissolved in DMSO, in duplicate at 0.005, 0.01 and 0.02 mg/ml. 100 metaphases per culture were analyzed for chromosome aberrations.

Imexine FN did not induce chromosome aberrations, either in the absence or presence of S9 mix.

Micronucleus assay

Imexine FN was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of male Swiss mice. The test substance, dissolved in DMSO, was administered, by gavage, to the animals (2 males) at concentrations of 37.5, 75, 150 and

300 mg/kg b.w. Samples were taken 24 and 48 hours after administration. In each group 1000 polychromatic erythrocytes of 2 males were analyzed for micronuclei.

No increased micronucleus rate was observed, so Imexine FN was found negative in this assay.

10. Special investigations

Skin absorption of Imexine FN

The method used is: *in vitro*, diffusion cell (Franz cell) using human breast epidermis.

3 % Imexine FN* was applied 8 times, in absence and in presence of hair (adding 10 mg of finely cut tinted hair), using human breast epidermis, for 30 minutes. Then the skin was washed and dried.

The formulation was left for 30 minutes and was then rinsed-off using 10 ml distilled water. The contact area was dried with cotton wool swabs.

After 4.5 hours the *mean percutaneous absorption* was 0.045 % of the administered formulation in presence of hair and 0.017 % in absence of hair.

* Composition of the formulations I and II (vehicle):

	I (g)	II (g)
Imexine FN	3	–
polyethylene glycol (6OE)	50	50
ammonia, 20 %	10	10
deionised water	37	40

11. Conclusions

A Quality Assurance Declaration was included in all tests, except for the Ames test, chromosome aberration assay and micronucleus assay.

General

Imexine FN is used in oxidative hair dye formulations at a maximum concentration of 3 %. Since the permanent hair dyes are mixed with hydrogen peroxide before application, the use concentration is 1.5 % only.

Acute toxicity

The test substance is moderately toxic, on the basis of its acute toxicity (LD_{50} oral, rat 500-1000 mg/kg b.w.).

Irritation

A concentration of 6 % in the eye and 6 % in the skin irritation study with rabbits, showed no signs of irritation.

Sensitization

The applied test method is not commonly used. Therefore the sensitizing potential of the substance cannot be evaluated.

Subacute toxicity

In a 28-day feeding study, rats were fed 0, 100, 250 or 600 mg Imexine FN/kg b.w., by gavage once daily. The animals of the 600 mg/kg group showed the following effects: clinical signs and decreased body weight.

The dose level without effect was 250 mg/kg b.w.

Teratogenicity

In a teratogenicity study, Sprague-Dawley rats were fed 0, 100, 250 or 600 mg Imexine FN/kg b.w. Minor skeletal effects were found in the 250 and 600 mg/kg group. No irreversible structural changes were observed.

The dose level without maternal toxicity was 250 mg/kg b.w. and the dose level without embryo/foetotoxicity was 100 mg/kg b.w.

Genotoxicity

Imexine FN was tested for its mutagenic potential under *in vitro* conditions in an Ames test and a chromosome aberration assay. Under *in vivo* conditions a micronucleus assay was performed. No test for gene-mutations in mammalian cells *in vitro* was performed.

Imexine FN is considered to be not-genotoxic, based on the provided mutagenicity tests.

Absorption

Imexine FN was applied to the human breast epidermis, using diffusion Franz cells. The cutaneous absorption was 0.045 % for skin with hair and 0.017 % for skin without hair.

Conclusions

Imexine FN is moderately toxic, on the basis of its acute toxicity.

Imexine FN, at a concentration of 6 %, showed no signs of irritation. The sensitization test cannot be evaluated.

In the 28-day feeding study with rats, effects were observed in the high dose group. The dose level without effect was 250 mg/kg b.w.

In the teratogenicity study, minor skeletal effects were observed in the foetuses of the rat, after administration of ≥ 250 mg/kg b.w. No irreversible structural changes were observed. The dose level without embryo/foetotoxicity was 100 mg/kg b.w.

Imexine FN is considered not genotoxic, based on the provided mutagenicity tests.

The cutaneous absorption was 0.045 % for skin with hair and 0.017 % for skin without hair.

For normal use of hair dye, the following calculation can be made:

1.5 g of Imexine FN comes in contact with the human skin in permanent hair dye condition (based on a usage volume of 100 ml containing 1.5 % Imexine FN). With a maximal penetration, under normal condition, of 0.045 %, this results in a dermal absorption of 0.68 mg per treatment, which is 0.011 mg/kg b.w. (assuming a body weight of 60 kg).

So a margin of safety of 9090 can be calculated between the figure for human exposure to oxidative hair dye and the no effect level found in rats in the teratogenicity study.

It should be noted that the NOAEL stems from a daily exposure for 10 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Reasoned arguments, including validation data, regarding the use of the protocol chosen for the sensitization test should be provided. The additional information has to be communicated within six months.

Classification: B

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

IMEXINE FN

COLIPA NO. B 51

Based on a usage volume of 100 ml, containing at maximum 1.5 %

Maximum amount of ingredient applied:	**I (mg) = 1500**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.045**
Dermal absorption per treatment:	**I (mg) x A (%) = 0.68 mg**
Systemic exposure dose (SED):	**SED (mg) = I (mg) x A (%) / 60 kg = 0.011**
No observed adverse effect level (mg/kg):	**NOAEL = 100 mg/kg**
Margin of Safety:	**NOAEL / SED = 9090**

It should be noted that the NOAEL stems from a daily exposure for 10 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month.

P 4: *CHLORPHENESIN*

1. General

1.1 Primary name

Chlorphenesin

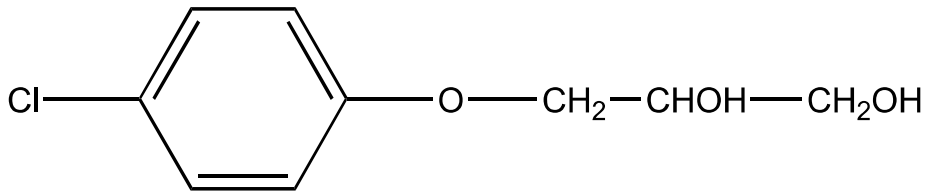
1.2 Chemical names

Chlorphenesin, p-chlorophenyl-glycerol ether
3-(p-chlorophenoxy) propane-1,2-diol
(chlorphenesin, p-chlorophenyl-glycerol ether)

1.4 CAS no.

104-29-0

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_9H_{11}Cl\ O_3$
Mol weight: 202.64

1.9 Solubility

Chlorphenesin is slightly soluble in water (0.6 %), moderately soluble in glycerol (9.5 %) and alcohol (15 %).

2. Function and uses

Used in cosmetics up to 0.3 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

LD_{50} values (in mg/kg) are: oral in rats > 1400, in mice 1060, in guinea pigs 820, i.p. in rats 520, in mice 675 and 911; in guinea pigs 425, s.c. in mice 930.

3.4 Repeated dose oral toxicity

Full details are however available of a 28-day oral toxicity study in rats given doses of 10, 100 and 1000 mg/kg compound by gavage as an aqueous suspension.

Detailed autopsies were performed at the end of the exposure period and in addition serum immunoglobulin levels and B:T lymphocyte ratios in blood and spleen were determined. Compound related mortality was seen at the top dose, 1/5 male animals dying. Other effects noted at this level were reduced weight gain, abnormal posture and gait, reduced haemoglobin levels, reduced spleen and thymus weight and evidence of nephrotoxicity. The only significant effects seen at 100 mg/kg were a slight reduction in haemoglobin levels. No pathology was seen in the spleen, lymph nodes, thymus or bone marrow at any dose level. The no effect level was 10 mg/kg with only marginal effects at 100 mg/kg.

3.7 Subchronic oral toxicity

In an oral 13-week study in rats given doses of 50, 100 or 200 mg/kg b.w./day by gavage, no effect on growth rate or food intake was observed. Examination of vaginal smears provided no evidence of interference with oestrus. No gross changes were observed at autopsy (a detailed report is not available).

Dogs given 75 or 150 mg/kg/day (route not specified) 5 days a week for 18 weeks, did not show any significant changes in behaviour or growth, in haematology or clinical chemistry, and in urine composition (summary report only).

4. Irritation & corrosivity

4.1 Irritation (skin)

A skin irritation test in rabbits was negative (no details). In repeated insult patch tests with 18 humans, application of 0.05 ml of 0.2 % in hand cream, skin lotion and skin soothing milk on 5 successive days was negative, or produced slight erythema in some cases.

4.2 Irritation (mucous membranes)

An eye irritation test in rabbits with 1 % in glycerine, did not provoke corneal irritation.

6. Teratogenicity

S.code	: Chlorphenesin, purity 99.2 %
route	: oral
species	: Sprague Dawley rat
number	: 25 mated females/group
dose	: 0, 10, 50, 100 mg/kg b.w.
exposure	: daily, gavage
am days	: day 0 up to day 15 of gestation
vehicle	: 1 % methylcellulose
According to OECD 414	: yes
GLP-statement	: yes
NOAEL	: 100 mg/kg b.w./day for maternal and developmental toxicity

Effects/Dose (mg/kg b.w.)	**0**	**10**	**50**	**100**
Maternal toxicity				
mortality	-	-	-	-
pregnant animals	23/25	23/25	25/25	24/25
body weight	no treatment-	related	findings	
food intake	no treatment-	related	findings	
clinical signs				
-post-dose salivation	-	-	-	4/25
-fur loss	9/25	13/25	13/25	16/25
Litter response	no treatment-	related	findings	
Examination of fetuses				
-external observations	no treatment-	related	findings	
-skeletal findings	no treatment-	related	findings*	
-visceral findings	no treatment-	related	findings	

* The distribution of skeletal anomalies amongst the litters was higher in all treatment groups than in controls, attaining a statistical significance in the mid-dose group. In the absence of a clear dose-response relationship or of an obvious trend in any specific type of skeletal anomaly, these differences are not considered to be treatment-related.

The study was properly conducted. There was neither evidence of clear maternal toxicity nor of adverse effects on foetal survival, growth and development *in utero* at levels up to 100 mg/kg b.w./day.

7. Toxicokinetics (incl. Percutaneous Absorption)

An *in vivo* study to measure the percutaneous absorption of chlorphenesin has been carried out using a 0.05 % formulation of radiolabelled compound in cold cream and applied under occlusive dressing for up to 96 hours. By that time approximately 50 % of the dosed radioactivity had been excreted in the urine. These data indicate that chlorphenesis is well absorbed through the skin.

8. Mutagenicity

No evidence of mutagenic potential was obtained in a well-conducted Ames test with up to 0.5 mg/plate. Mutagenicity was examined also by the CHO/HGPRT locus bioassay. Treatment of the cells *in vitro* with up to 1.5 mg/ml did not demonstrate mutagenic potential. A chromosomal aberration test with human lymphocytes exposed *in vitro* to up to 0.325 mg/ml was negative.

10. Special investigations

In a repeated intramuscular injection test in mice, with 0.5 ml of a 0.6 % aqueous solution daily for 40 days there were no observable effects on growth or on the state of the organs.

11. Conclusions

Chlorphenesin may affect the immune system: both stimulating and inhibiting properties have been reported. Lymphocyte function *in vitro* was found to be suppressed by 20-50 µg/ml culture medium. However although a reduction in thymus weight was seen at 1000 mg/kg in the 28-day study in the rat, no pathology was noted, nor were there any effects on T:B lympocyte ratios. No effects were seen at 100 mg/kg or below.

Chlorphenesin has low acute toxicity, no significant irritant properties, but no animal data are available on skin sensitization; however experience in use has not suggested significant sensitization properties. It has a relatively low toxicity on repeated (4wk) oral exposure; the no effect level being 10 mg/kg b.w./day but with only a marginal effect at 100 mg/kg b.w./day. In a teratogenicity study in rats, there was neither evidence of maternal toxicity, nor of adverse effects on fetal survival, growth and development at levels up to 100 mg/kg b.w./day.

The compound is well absorbed through the skin (about 50 % under occlusive dressing over 96 hours).

Classification: A

12. Safety evaluation

Assuming extensive use of all cosmetic products total exposure would be to 27.6 g (5.54 g ingested from oral hygiene at 22.06 g skin contact). Assuming that all products contain chlorphenesin at the maximum permitted level (0.3 %), total exposure is to 16.62 mg by ingestion and 66.18 mg by skin contact. Assuming 50 % absorption through the skin this gives a total absorbed dose of 49.80 mg. This is equivalent to 0.83 mg/kg.

$$\text{Safety Margin over marginal effect level} = \frac{100}{083} = 120$$

Since this is based on extreme estimates of exposure this is considered acceptable.

P 84: *SODIUM HYDROXYMETHYLAMINO ACETATE*

1. General

Summary of the previous situation:

Considering the data supplied, CSC has expressed an opinion concerning this preservative during the plenary session on June 25, 1993.

On basis of existing experimental data, it was concluded that:

- NOAEL is 160 mg/kg,
- by lack of information about cutaneous absorption, the preservative must be considered as absorbed at 100 % by cutaneous way,
- the maximal daily exposure dose of finished products which are likely to contain Sodium hydroxymethylamino acetate is of 27.6 g/day,
- considering the will of industry to limit concentration of pure preservative in finished products at 0.5 %, the maximal daily exposure dose is 138 mg/day,
- therefore, this daily dose corresponds to an exposure equivalent to 2.3 mg/kg/day.

and so the security margin is = $\frac{160}{2.3} = 70$

- However, it was noted that data from an *in vivo* assay to measure UDS in the liver were needed to provide adequation reinsurance that activity seen *in vitro* is not expressed *in vivo*.

- **Classification B** was therefore recommended.

Verification regarding nature of the tested ingredient:

Noting that Suttocide A is pointed out as well as a pure substance as an aqueous solution at 50 % of pure substance, we have proceeded to a systematic verification of conditions in which each test was realized, those tests having got to evaluate preservative tolerance and mainly its security margin.

Results of these controls were gathered in the following tables.

Doses which were considered in the 90 day toxicity for the rat are really expressed in pure ingredient (powder) and therefore the calculation of security margin remains acceptable.

On the other hand, studies about reproduction were made with aqueous solution at 50 %, which got to reduce the NOAEL of the two studies at 150 mg/kg/day of pure products; this is in perfect concordance with test by oral way for the rat (160 mg/kg/day); let us remind that these tests didn't display neither embryotoxicity, nor teratogenicity, the sole shown effects corresponding to a maternal toxicity.

1.1 Primary name

Sodium hydroxymethylamino acetate

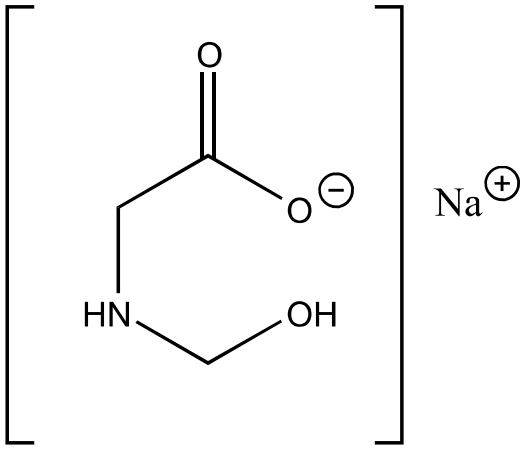
1.2 Chemical names

Sodium hydroxymethylamino acetate
Sodium hydroxymethyl glycinate

1.3 Trade names and abbreviations

Colipa No P 84
Suttocide A

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_3H_6NO_3Na$
Mol weight: 127.1

1.8 Physical properties

Strongly alcaline (pH unknown)

1.9 Solubility

Highly soluble in water. Soluble in methanol, propylene glycol and glycerin. Insoluble in most organic solvents.

2. Function and uses

Sodium hydroxymethylamino acetate is a preservative for use in cosmetics at concentration of 0.05 % to 0.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Initial tested product	powder	powder	50 % aqueous solution
Reported date	12.06.79	20.12.79	28.04.94
Specie	rat	rat	rat
Number of animals	60 6 x 10	60 6 x 10	15 3 x 5
Doses	Range 0.6 to 1.29 g/kg	Range 1.0 to 2.2 g/kg	2000 - 3000 - 4000 mg/kg
Duration	14 days observation	14 days observation	7 days observation
Main observations	gavage as 25 % w/v aqueous solution	gavage as 25 % w/v aqueous solution	gavage
Results	LD_{50} 1.07 g/kg	LD_{50} 1.41 g/kg 1.24 to 1.61	estimated LD_{50} = 2080 mg/kg

3.2 Acute dermal toxicity

Initial tested product	powder
Reported date	4.04.79
Specie	rabbit
Number of animals	10
Doses	2 g/kg
Duration	24 h occlusive
Main observations	6: abraded skin 4: intact skin Moderate to severe burns related to the highly alcaline pH
Results	$LD_{50} > 2$ g/kg

3.3 Subchronic oral toxicity

Initial tested product	powder	powder
Reported date	15.05.84	11.12.90
Specie	rats	rats
Number of animals	80 4 x 20	80 4 x 20
Doses	10 - 40 - 160 mg/kg/d	40 - 160 - 640 mg/kg/d
Duration	90 days	28 days
Main observations	gavage as a 2 % (w/v) aqueous solution	gavage as a 5 % (w/v) aqueous solution
Results	No toxicological or histo-pathological effect at all doses	No toxicological or histo-pathological effect at 40 and 160 mg/kg/d. 640 mg/kg: gastric irritation with biochemical & hematological changes NOEL = 160 mg/kg/d confirmed

4. Irritation & corrosivity

4.1 Irritation (skin)

Primary skin irritation

Reported date	5.07.79	1.10.79	4.4.80	24.8.84	5.07.79	1.10.79	4.4.80	24.8.84	21.5.80	24.8.84
Initial tested product	powder	powder	powder	powder	5 % aqueous solution	5 % aqueous solution	5 % aqueous solution	5 % aqueous solution	0.5 % aqueous solution	0.5 % aqueous solution
Specie	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit	rabbit
Number of animals	6	2	3	3	6	2	3	3	6	3
Doses	0.5g/ patch	0.5g/ patch	0.5g/ patch	0.5g/ patch	0.5ml/ patch	0.5ml/ patch	0.5ml/ patch	0.5ml/ patch	0.5ml/ patch	0.5ml/ patch
Durations	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive	24 h occlusive
Main observation	Abraded and intact skin wetted	Abraded and intact skin wetted	Abraded and intact skin wetted	Abraded and intact skin not wetted	Abraded and intact skin	Abraded and intact skin	Abraded and intact skin	Abraded and intact skin	Abraded and intact skin	Abraded and intact skin
Results	3.71 moderate irritation	1.0 slight irritation	1.0 slight irritation	0 no irritation	4.79 moderate irritation	0 no irritation	0 no irritation	0.5 slight irritation	0.67 slight irritation	1.17 slight irritation

Initial tested product	Aqueous solutions	Powder
Reported date	5.12.84	5.12.84
Specie	guinea pig	guinea pig
Number of animals	4	4
Doses	0.5 ml/patch	0.5 g/patch
Duration	24 h occlusive	24 h occlusive
Main observation	Intact skin Concentration 25-50-75 % w/v	Intact skin wetted
Results	No irritation	No irritation

Repeated exposure skin irritation

Initial tested product	Aqueous solution
Reported date	20.06.85
Specie	guinea pig
Number of animals	4
Doses	0.5 ml/patch
Duration	6 h days 1.3.6 occlusive
Main observation	Intact skin Concentration 0.38 - 0.75 - 7.5 - 50 % w/v
Results	No irritation

4.2 Irritation (mucous membranes)

Initial tested product	Powder	5 % aqueous solution	5 % aqueous solution	5 % aqueous solution
Reported date	1.08.79	1.08.79	4.10.59	15.06.90
Specie	rabbit	rabbit	rabbit	rabbit
Number of animals	9	9	2	9
Doses	100 mg/eye	0.1 ml/eye	0.1 ml/eye	0.1 ml/eye
Duration				
Main observation	no wash out (6) wash out 4 sec. (3)	no wash out (6) wash out 4 sec. (3)	wash out	no wash out (6) wash out 1 mn.
Results	no wash out: moderate irritation wash out: mild irritation	no wash out: mild irritation wash out: no irritation	no irritation	no wash out: mild irritation wash out: mild irritation

5. Sensitization

	Contact Sensitization Landsteiner Jacobs Intradermal	**Magnusson Kligman Maximization Test**	**Buehler Topical Test**
Initial tested product	1 % saline solution	powder and water solutions	5 % aqueous solution
Reported date	22.12.80	5.12.84	20.06.85
Specie	guinea pig	guinea pig	guinea pig
Number of animals	8	**Group A**: 10:induction + challenge **Group B**: 10: challenge (controls) **Group C**: 6: induction + challenge with DNCB (controls)	**Group A**: 10:induction + challenge **Group B**: 3:challenge (controls) **Group C**: 6:induction + challenge with DNCB (controls)
Doses	Injection intradermally: - induction 0.1 ml/day x 10 days - challenge: 0.05 ml	**Induction A**: intradermally 0.1 ml 5 % (D1) + topically (D8) **Induction B**: intradermally 0.1 ml FCA (D1) + topically water (D8) **Induction C**: intradermally 0.1 ml 0.1 % DNCB + FCA (D1) + topically 0.1 % DNCB (D8) **Challenge A and B**: 0.1 ml 50 % solution topically (D 22) **Challenge C**: 0,1 ml 0,1 % DNCB topically (D 22) **Rechallenge A and B**: 0,2 ml 5 % and 0,5 % solution topically (D29) **Challenge C**: 0.1 ml 0,1 % DNCB topically (D29) Concentrate 5 % for intradermal injection	Induction: 3 times/ week topically
Duration	Induction: 10 days Test period: 2 weeks then challenge	Induction: 8 days Test period: 2 weeks then challenge Occlusion: 48 h for induction 24 h for challenge	Induction: 10 times Test period: 2 weeks then challenge Occlusion: 6 h
Main observation		Concentrate 5 % for intradermal injection Powder moistened with water for topical application during induction Concentration 50 % for challenge Concentration 5 % and 0.5 % for rechallenge	
Results	No sensitization	Strong sensitizer at the challenge concentration: 50 % Moderate sensitizer at the challenge concentration: 5 % Mild sensitizer at the challenge concentration: 0.5 % DNCB all positive	No sensitization DNCB: all positive

6. Teratogenicity

	Dose range finding oral developmental toxicity	Oral developmental
Initial tested product	50 % aqueous solution	50 % aqueous solution
Reported date	25.10.1990	
Specie	rat	rat
Number of animals	36 pregnants 6 x 6	108 pregnants 4 x 27
Doses	150 - 300 - 450 - 600 - 750 mg/kg/d	150 - 300 - 450 mg/kg/d
Duration	daily from day 6 to day 15 of gestation	daily from day 6 to day 15 of gestation
Main observations	gavage as a 5 % w/v solution Controls: 15 ml/kg/d deionized water Cesarian day 20	gavage as a 5 % w/v solution Controls: 9 ml/kg/d deionized water Cesarian day 20
Results	Maternal toxicity ≥ 450 mg/kg/d NOEL = 300 mg/kg no embryotoxicity or teratogenicity	Maternal toxicity at 450 mg/kg/d NOEL confirmed = 300 mg/kg developmental toxicity NOEL = 450 mg/kg/d

8. Mutagenicity

	Ames Test	***In vitro* Rat Hepatocyte Primary culture/ DNA repair**	***In Vivo* Micronucleus**	***In Vivo* - *In Vitro* Rat Hepatocyte UDS**
Initial tested product	50 % aqueous solution	50 % aqueous solution	powder	50 % aqueous solution
Reported date	15.09.83	13.09.90	18.05.87	28.04.94
Number of animals			3 sets of 10 mice/ dose	10 groups 5 rats/ dose
Doses	7.5 - 50 - 250 - 375 - 500 µg/ plate	2.5 - 7.5 - 10 - 20 µg/ ml	gavage 375 - 625 - 875 mg/kg	gavage 200 - 700 - 2000 mg/kg
Main observations	Strains: TA 98 TA 100 TA 1535 TA 1537 TA 1538		positive Control: cytophosphamide 60 mg/kg	Positive Controls MMS MMS 200 mg/kg AAF 100 mg/kg
Results	No mutagenic activity	No mutagenic activity	No mutagenic activity	No mutagenic activity

In vivo/in vitro rat hepatocyte unscheduled DNA synthesis assay:

In relation with the CSC advice, the Colipa group has transmitted a 6th submission corresponding to the *in vivo* test of the UDS measurement.

According to the Good Laboratory Practices, purposed test was perfectly managed.

Only the references of used experimental batches along with their vouchers concerning analytic control were missed.

Realized on a whole of 344 male Fisher rats, it includes:

- Two preliminary assessments assigned to agree with the scale of doses to use in definitive tests:

An initial dose range finding assay using the five doses 5000 - 1500 - 500 - 150 - 50 mg/kg rat (5 animals per group).

A secondary assay using the three doses 4000 - 3000 and 2000 mg/kg rat (5 animals per group). Based upon a probing analysis of these results, an LD_{50} of 2080 mg/kg has been estimated.

- The actual *in vivo - in vitro* UDS assay using doses of 2000, 700 and 200 mg/kg rat by oral gavage to approximate the LD_{50}, 1/3 LD_{50} and 1/10 LD_{50} (5 animals per group) according to the following distribution:

A negative control (sterile deionized water 10 ml/kg rat) and two positive controls (methyl methane sulfonate 200 mg/kg rat and 2 acetyl aminofluorene 100 mg/kg rat) were also administrated by oral gavage.

Group Number	**Treatment Article**	**Dose Level**	**Number of Rats treated**	**Number of Rats harvested**	**Post Treatment Harvest Period**
1	Vehicle Control Deionized Water	10 ml/kg	5	3	12 - 18 hours
2	Suttocide A / Integra 44	200 mg/kg	5	3	12 - 18 hours
3	Suttocide A / Integra 44	700 mg/kg	5	3	12 - 18 hours
4	Suttocide A / Integra 44	2000 mg/kg	5	3	12 - 18 hours
5	Positive Control 2-AAF	100 mg/kg	5	3	12 - 18 hours
6	Vehicle Control Deionized Water	10 ml/kg	5	3	2 - 4 hours
7	Suttocide A / Integra 44	200 mg/kg	5	3	2 - 4 hours
8	Suttocide A / Integra 44	700 mg/kg	5	3	2 - 4 hours
9	Suttocide A / Integra 44	2000 mg/kg	5	3 1[1]	2 - 4 hours 12 - 18 hours
10	Positive Control MMS	200 mg/kg	5	3	2 - 4 hours
11[2]	Suttocide A / Integra 44	2000 mg/kg	3	1	12 - 18 hours

[1] One of the five rats treated with 2000 mg/kg Suttocide A/Integra 44 in group 9 was harvested at 12 hours following treatment.

[2] Three animals were assigned to group 11 and treated with 2000 mg/kg Suttocide A/ Integra 44 as a supplement to group 4.

The hepatocytes were harvested 2 to 4 and 12 to 18 hours after test article administration.

The results of the *in vivo* - *in vitro* UDS assay indicate that under the 1st conditions, the 1st article did not induce a significant increase in the mean number of net nuclear grain counts in hepatocytes isolated from treated animals.

Suttocide A is considered to be negative in the *in vivo* - *in vitro* rat hepatocyte unscheduled DNA synthesis assay.

The examination of the data and results of the assay indicate that it has been conducted in compliance with the Good Laboratory Practices, in a sufficient number of animals; the results are clearly negative and give no problem of interpretation.

11. Conclusions

According to the control that we have done on all reports to secure the validity of the previous assessment, and to the negative results obtained with the *in vivo* - *in vitro* UDS assay, a safety margin of 70 is still acceptable.

Nevertheless, it is strongly recommended to the industry to define accurately if Suttocide A is a powder or a 50 % aqueous solution of the powder.

No further assays appear necessary at the present time.

Experimental data demonstrate that this compound is a potential allergen according to the guinea pig maximisation test. At the current usage levels, there is no evidence of unacceptable risk of sensitization to the consumers. However, any background of sensitivity to the compound may be assessed at the latter date if it becomes more widely used as a cosmetic preservative.

Classification: A

OPINIONS ADOPTED DURING THE 62ND PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 18 January 1996

A 14: *O-AMINOPHENOL*

1. General

1.1 Primary name

o-Aminophenol

1.2 Chemical names

2-Aminophenol
o-Aminohydroxybenzene
1-Hydroxy-2-aminobenzene
1-Amino-2-hydroxybenzene
2-Hydroxybenzenamine
o-Hydroxyaniline
2-Hydroxyaniline
o-Hydroxyphenylamine

1.3 Trade names and abbreviations

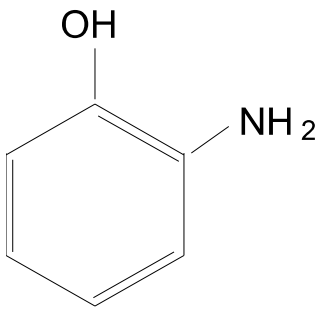
Colipa no.: A 14

Trade names: BASF Ursol 3 GA
Benzofur GG
Fouramine OP
Nako yellow 3 GA
Paradone Olive Green B
Pelagol 3 GA
Pelagol Grey GG

1.4 CAS no.

95-55-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_6H_7NO
CAS formula: not available
Mol weight: 109

1.7 Purity, composition and substance codes

s.A.: 1-hydroxy-2-aminobenzene (purity 99.7 %)
s.B.: 1-hydroxy-2-aminobenzene (unspecified)

Possible impurities may originate from:
- reagents and intermediate products of reaction
  : m-aminophenol and p-aminophenol
  : phenol
  : o-nitrophenol
  : aminophenoxazone
- solvent : ethanol
- other : : NaCl

1.8 Physical properties

Subst. code: s.A.
Appear.: a light-beige powder, almost odourless

1.9 Solubility

The substance is insoluble in water, but soluble in ethanol (96 %) and in dimethylformamide (DMF).

2. Function and uses

1-hydroxy-2-aminobenzene is used in oxidation hair dye formulations at a maximum concentration of 2 % which, after mixing 1:1 with H_2O_2 just prior usage, corresponds to 1 % upon application.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.4 Repeated dose oral toxicity (1) (OECD No 407)

Route:	oral	Exposure:	30 days	NOAEL (DWE): -
Species:	rat	Recov.period.:		
Subst.:	s.A.			

Description:

The test substance (dissolved in a hydrogel of 0.2 g polysorbate 80 and 0.2 g sodium carboxymethylcellulose) was administered by gavage for 30 consecutive days to groups of 20 Sprague-

Dawley (OFA) rats (10/sex) at doses 0 (group I), 20 (group II), 80 (group III) and 320 (group IV) mg/kg/day. The age of the animals at the start of the study was approximately 6 weeks and the body weight range was 164 - 186 g for males and 134 - 169 g for females. Food and water ad libitum.

Examinations: Clinical observation and mortality daily. Ophthalmoscopic examination before and at the end of the study. Body weights, food and water consumption twice weekly.

Haematology (RBC, WBC, thrombo, Diff., Hb, Hct, MCV, MCH, MCHC, reticulocytes, prothrombin time) and blood biochemistry (bil, gluc, total protein, albumin, SGOT, SGPT, GLDH, AP, CK, Na, K, Cl, PO_4, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the end of the study. Urinanalysis (color, nitrite, leuco, pH, prot, glu, ketones, urobil, bil, blood, sed, spec. weight) at the end of the study. Necropsy, main organ weights, histopathological examination (at sacrifice).

Results: Orange discoloration of the urines throughout the study groups III and IV and orange discoloration of the fur in group IV females from day 7 on.

Signs of regenerative macrocytic anaemia in group IV males and females. Increase of GOT activity in group IV (males and females) and group III (males). Increase of blood urea nitrogen in group IV females. Increase of urinary proteins in group IV males and females. Renal cells in the urine in males of group II.

Increased relative liver and kidney weights in group IV males and females. Kidneys were pale or mottled at macroscopic examination and showed renal tubular lesions at histopathological examination in males of group III and IV. Increased vacuolisation of the urothelium of the bladder in males and females in group II and III.

Conclusion: None of the three tested doses could be considered as NOAEL.

3.4 Repeated dose oral toxicity (2)

Route:	oral	Exposure:	28 days	NOAEL (DWE): 5 mg/kg b.w.
Species:	rat	Recov.period:		
Subst.:	s.A.			

Description:

The test substance (dissolved in 0.5 % w/v aqueous carboxymethylcellulose) was administered by gavage for 28 consecutive days to groups of 20 Sprague-Dawley rats (strain crl: CD [SD] BR) (10/sex) at doses 0 (group I), 2 (group II), 5 (group III) and 15 (group IV) mg/kg/day. The age of the animals at the start of the study was approximately 4 - 5 weeks and the body weight range was 141 - 188 g for males and 111 - 157 g for females. Food and water ad libitum.

Examinations: Clinical observation and mortality daily. Ophthalmoscopic examination before and during week four. Body weights, food and water consumption weekly.

Haematology (RBC, WBC, thrombo, Diff., Hb, Hct, MCV, MCH, MCHC, reticulocytes, prothrombin time) and blood biochemistry (bil, gluc, total protein, albumin, SGOT, SGPT, GLDH, AP, CK, Na, K, Cl, PO_4, Fe, Ca, urea, uric acid, creat, chol, triglyceride) and

urinanalysis (color, nitrite, leuco, pH, prot, glu, ketones, urobil, bil, blood, sed, spec. weight) during week four. Necropsy, main organ weights, histopathological examination (at sacrifice).

Results: Reduced bodyweight gains in males and females group IV and in females group II and III. Increased plasma glucose level in males group IV. Increases in absolute (not significant) and relative (significant) thyroid weights in females group IV.

Conclusions: The reduced bodyweight gains are not considered to be related to treatment as there is no dose relationship. The thyroid weight changes are also considered of no toxicological importance as no histopathological evidence was related. Thus a NOAEL of 5 mg/kg b.w. can be accepted.

6. Teratogenicity

Route:	oral	Admin. Days: 6-15	NOAEL (DWE):	70 mg/kg b.w. for maternal toxicity
Species:	rats			70 mg/kg b.w. for fetal development
Subst.:	s.A.			(embryotoxicity)

Description:

The test substance (dissolved in a hydrogel of 0.2 g polysorbate 80 and 0.2 g sodium carboxymethylcellulose) was given daily from day 6 - 15 of gestation by oral administration of doses of 0 (group I), 20 (group II), 70 (group III) and 250 (group IV) mg/kg b.w. to pregnant Sprague-Dawley (OFA) rats (11 - 12 weeks old and with a mean bodyweight of 270 g on day 0 of gestation).

Examinations: Clinical signs, mortality daily. Bodyweights were taken at the beginning of the study and at day 6, 7, 10, 15, 21. Food consumption on days 6, 15, 21.

At sacrifice, hysterectomy (number of live, dead or absorbed fetuses in each uterine horn; position of the fetuses in the uterus; number of corpora lutea, of early and late resorptions, of implantation sites) after gross examination of all animals and of placentas.

Weight, sex and gross external examination of each fetus, skeletal (2/3 of each litter) or visceral (the remaining 1/3) anomalies.

Results: Brown discoloration of urines in all treated animals 24 hours after the first administration of the test substance. Reduced bodyweight gain (from days 7 to 10) and food consumption (from days 6 to 15) in group IV. Decreased mean weight of the fetuses in group IV (maternal toxicity).

2 fetuses with bilateral anophthalmies in one litter of group IV animal. Slight ossification retardation in group IV.

Conclusions: NOAEL for maternal toxicity and for fetal development (embryotoxicity) was 70 mg/kg/day.

8. Mutagenicity - Indicator tests (in vivo mammalian, host mediated)

In vivo/in vitro *unscheduled DNA synthesis (UDS) test*

The test substance (dissolved in 0.01M HCl) was given in doses 400 and 2000 mg/kg b.w. to male Wistar (Crl[Wi]BR) rats (47 - 66 days old, weighing 178 - 232 g). After 4 or 12 hours the rats were sacrificed and their livers were perfused with collagenase. The primary hepatocyte cultures were treated with ^{3}H thymidine for 4 hours. The hepatocytes were examined for number of grains present in the nucleus minus the highest number of grains in an equivalent area of cytoplasm. Negative (vehicle) and positive (2-acetylaminofluorene and dimethylnitrosamine) controls were included.

Results: The test substance had no genotoxic activity in this test.

10. Special investigations

In vitro *penetration*

A formulation containing 2 % of the test substance, mixed with 1:1 H_2O_2, was applied to a diffusion cell (Franz cell) using human breast epidermis in absence or presence of hair. After 30 minutes the upper part of the skin was washed and dried. The mean quantity of test compound in the receiving chamber 4.5 hours after application was measured. Approximately 40 mg of the test solution were applied on the skin, which corresponds to a mean quantity of 0,2 mg of the test compound/cm^2. Two Franz cells were set up with a control formulation which did not contain the test compound.

Results: In absence of hair, 500 ng/cm^2 and 87 ng/cm^2, which correspond to 0.24 % and 0.04 % respectively of the test compound applied on the skin, were measured in the receiving chamber. In presence of hair, 221 ng/cm^2 and 99 ng/cm^2, which correspond 0.105 % and 0.043 % respectively of the test compound applied on the skin, were measured in the receiving chamber.

A formulation containing 2 % of the test substance and 1.98 % of p-phenylene-diamine, mixed with 1:1 H_2O_2, was applied to a diffusion cell (Franz cell) using human breast epidermis in absence or presence of hair. After 30 minutes the upper part of the skin was washed and dried. The mean quantity of test compound in the receiving chamber 4.5 hours after application was measured. Approximately 40 mg of test solution were applied on the skin, which corresponds to a mean quantity of 0.2 mg of test compound/cm^2. Two Franz cells were set up with a control formulation which did not contain the test compound nor p-phenylene-diamine.

Results: In absence of hair, 80 ng/cm^2, which corresponds to 0.037 % of the test compound applied on the skin, was measured in the receiving chamber. In presence of hair, 60 ng/cm^2, or 164 ng/cm^2, which correspond 0.025 % and 0.077 % of the test compound applied on the skin, was measured in the receiving chamber.

A formulation containing 2% of the test substance and 2 % of 1-hydroxy-3-aminobenzene, mixed with 1:1 H_2O_2, was applied to a diffusion cell (Franz cell) using human breast epidermis in absence or presence of hair. After 30 minutes the upper part of the skin was washed and dried. The mean quantity of test compound in the receiving chamber 4.5 hours after application was measured. Approximately 40 mg of test solution were applied on the skin, which

corresponds to a mean quantity of 0.2 mg of test compound/cm^2. Two Franz cells were set up with a control formulation which did not contain the test compound nor 1-hydroxy-3-aminobenzene.

Results: In absence of hair, 64 ng/cm^2, which correspond to 0.028 % of the test compound applied on the skin, was measured in the receiving chamber. In presence of hair, 50 ng/cm^2, which corresponds 0.024 % of the test compound applied on the skin, was measured in the receiving chamber.

11. Conclusions

The oral LD_{50} of ortho-aminophenol in the rat was approximately 1000 mg/kg bodyweight.

A 1 % solution was found slightly irritant to the rabbit eye one hour after instillation without rinsing. The effect was reversible within 24 hours.

When topically applied a 1 % solution was not irritant to the rabbit skin and no cutaneous sensitizing reaction was observed in the guinea-pig.

Ortho-aminophenol was found very slightly toxic in the rat after a repeated oral administration of 50 mg/kg/day for 3 months.

Topical application of an oxidation hair-dye mixture containing 0.3 % of ortho-aminophenol in rabbit for 13 weeks has not shown signs of systemic toxicity.

A 9 month oral study with ortho-aminophenol hydrochloride at 0.117 % in the diet showed no tumorigenic effects in rats.

A two year study by topical application in mice with an oxidation hair-dye mixture containing 0.3 % of ortho-aminophenol showed no toxicological nor carcinogenic effects.

No mutagenic potential was detected *in vitro* in an Ames test on *Salmonella typhimurium* or in vivo in a Sex-Linked Recessive Lethal Test and in a Sister-Chromatid Exchange test.

Dermal application in the pregnant rat of an oxidation hair-dye mixture containing 0.3 % of ortho-aminophenol (days 1, 4, 7, 10, 13, 16 and 19 of gestation) failed to elicit teratological effects.

Ortho-aminophenol failed to produce tumors when implanted as a pellet (12.5 % in a cholesterol pellet) in the bladder of mice.

A topical two-year study in rats of an oxidation hair-dye mixture containing 0.3 % of ortho-aminophenol showed neither toxicological nor carcinogenic effects.

Ortho-aminophenol used in an Ames test was found mutagenic in only one (TA 100) out of five strains of *Salmonella typhimurium*.

Ortho-aminophenol was not found mutagenic on the yeast *Schizosaccharomyces pombe* (strain P1).

Ortho-aminophenol did not induce chromosome aberrations in CHO cells.

Ortho-aminophenol gave conflicting results for SCEs in various mammalian cell cultures.

Ortho-aminophenol was found inactive in most *in vivo* tests including *Drosophila melanogaster*, micronucleus induction and cytogenetic analysis on different strains of mouse and SCEs induction in Chinese hamsters.

Ortho-aminophenol was moderately toxic in an *in vivo* hen'egg test and had no teratogenic potential in this test.

Ortho-aminophenol administered intraperitoneally on day 8 of gestation to Syrian golden hamsters at 100, 150, 200 mg/kg b.w. was found teratogenic.

A reproduction study on three generations with formulations containing 0.3% of the test substance showed no effect on the reproduction systems of rats.

After dermal administration to the back of hairless rats, ortho-aminophenol was poorly absorbed (2.91 µg/cm^2) and excreted mainly via the urines.

Ortho-aminophenol was not considered mutagenic in an Ames reversion test (strains TA1535, TA1537, TA1530, TA98, TA100).

Ortho-aminophenol was considered slightly active in a DNA repair test by the liquid micromethod procedure.

Ortho-aminophenol was not found mutagenic in the Sex-Linked Recessive Lethal assay.

A three-month oral toxicity study was performed in rats and submitted previously. As a single dose level of 50 mg/kg/day was tested, results were inconclusive.

A Quality Assurance Declaration was included at the two one-month oral toxicity studies, the teratogenicity study and the UDS test.

1-hydroxy-2-aminobenzene was given over 30 days to groups of 20 SD (OFA) rats at doses 0, 20, 80, 320 mg/kg b.w. None of the three tested doses could be considered as a NOAEL.

In the following one-month oral toxicity study 1-hydroxy-2-aminobenzene was given over 28 days to groups of 20 Crl:CD (SD) BR rats at doses 0, 2, 5, 15 mg/kg b.w. 5 mg/kg are considered to be the NOAEL.

1-hydroxy-2-aminobenzene was tested for teratogenicity by oral gavage to groups of 20 pregnant SD (OFA) rats on days 6 - 15 of gestation at doses 20, 70, 250 mg/kg b.w. Pathological findings were only observed in 250 mg/kg treated group; a brown discoloration of urines occurred in all treated animals 24 hours after the first administration of the test substance. Thus a NOAEL for maternal toxicity and for fetal development was 70 mg/kg b.w.

1-hydroxy-2-aminobenzene was investigated for its ability to induce unscheduled DNA synthesis using an *in vivo*/*in-vitro* test model. The test substance had no genotoxic activity in this test system.

1-hydroxy-2-aminobenzene was investigated for *in vitro* percutaneous penetration rates using a diffusion cell (Franz cell) with human breast epidermis. Given the test substance alone the penetration rates were 0.04-0.24 % in absence of hair and 0.043 - 0.105 % in presence of hair. Given the test substance together with p-phenylene-diamine the penetration rates were 0.037 % in absence and 0.025-0.077 % in presence of hair. Given the test substance together

with 1-hydroxy-3-aminobenzene the penetration rates were 0.028 % in absence and 0.024 % in presence of hair.

1-hydroxy-2-aminobenzene is used in permanent hair coloration with a maximum concentration of 2 %. 50 ml of this formulation are applied to hair after mixing with an equal amount of oxidant. The use of this maximum concentration thus corresponds to the application of 1 g of 1-hydroxy-2-aminobenzene. The mixture is left on the hair for about 30 minutes. After this period of time the product in excess is removed by rinsing.

Classification: A

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

1-HYDROXY-2-AMINOBENZENE

COLIPA A 14

OXIDATION OR PERMANENT

Based on a usage volume of 100 ml, containing at maximum 1 %

Maximum amount of ingredient applied	**I(mg) = 1000**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.04 - 0.24 % = 0.14** **0.043 - 0.105 % (+ hair) = 0.07**
Dermal absorption per treatment	**I(mg) x A(%) = 1.4** **0.7**
Systemic exposure dose (SED)	**SED(mg) = I(mg) x A % / 60 kg b.w.** **0.02** **0.01**
No observed adverse effect level (mg/kg)	**NOAEL = 5 mg/kg b.w.** **(rat, oral gavage, 90 d)**

Margin of Safety NOAEL / SED = 250 - 500

B 31: *KARDINALROT*

1. General

1.1 Primary name

4-N,N-bis((-hydroxyethyl)-2-nitro-p-phenylenediamine

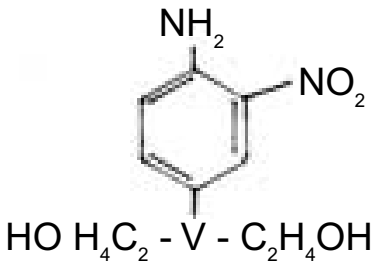
1.2 Chemical names

2-nitro-4-bis((-hydroxyethyl)-p-phenylenediamine
1-amino-4-bis((-hydroxyethyl)amino-2-nitrobenzene
1-amino-2-nitro-4-bis((-hydroxyethyl)-aminobenzene
2-nitro-4-bis((-hydroxyethyl)aminoaniline
4-bis((-hydroxyethyl)amino-2-nitroaniline
4-amino-N,N-bis((-hydroxyethyl)-3-nitroaniline
2,2′-(4-amino-3-nitroanilino)bisethanol

1.3 Trade names and abbreviations

Colipa no.: B 31
trade name: Kardinalrot

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_{15}N_3O_4$
Mol weight: 241

1.7 Purity, composition and substance codes

C.P.: Kardinalrot: (Commercial Product)
84 % 1-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene*,
13 % 1-(ß-hydroxyethyl)-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene**
3 % 1-amino-2-nitro-4-(ß-hydroxyethyl)-amino-benzene***

* s.A.: investigated in different specifications:

s.A_1: purity 99 %

s.A_2: unspecified

s.A_3: 1-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene, hydrochloride

** s.B.: identical with B37

***s.C.: identification when tested as such.

1.8 Physical properties

Subst. code: s.A_1 Appear.: black powder

Subst. code: C.P. Appear.: black pasty mass having a slight characteristic odour

1.9 Solubility

The substance exists as a free base (s.A_1) or as its hydrochloride (s.A_3).

The substance (C.P.) is soluble in water, methanol, ethanol and acetone.

2. Function and uses

C.P. is used in semipermanent hair tinting products, colouring setting lotions and permanent hair dye formulations at an applied maximum concentration of 2.5% and 1.25 %, respectively.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Route: oral Species: rat LD_{50}: >2000 (mg/kg b.w.)

Subst.: C.P.

The test substance was administered once as a 10 % suspension in aqueous gum tragacanth (0.5%) by oral intubation at doses 1000, 1600, 2500 and 4000 mg/kg b.w. to groups of 10 (5/sex) CFY rats (weight range was 100-120 g). Rats treated with the vehicle alone served as controls.

During the following observation period of 14 days a record was kept of all mortalities and signs of toxicity. Autopsy of death was carried out for all rats that died. At the end of the observation period all surviving animals were sacrificed and gross necropsies performed.

Results: As substance-related effects were observed lethargy, piloerection, diuresis and purple staining of the urine in all exposed groups shortly after dosing, accompagnied by ataxia in rats treated with 1000, 2500, or 4000 mg/kg b.w. and by increased lacrimation, decreased respiratory rate and purple staining of external extremities in rats treated with 2500 and 4000 mg/kg b.w. Fine body tremors were observed within two hours of treatment in male rats at 2500 mg/kg b.w. During the first week of observation depressed body weight gains were noted in the surviving rats at 2500 mg/kg b.w.

Necropsy of mortalities revealed slight haemorrhage of the lungs and purple staining of all internal organs except the lungs. Terminal autopsy findings of survivors showed no extraordinary results. LD_{50} was calculated to be 2120 (1810 - 2480) mg/kg b.w.

3.7 Subchronic oral toxicity

Route:	oral	Exposure:	90 days	NOAEL (DWE): -
Species:	rat	Recov.period:	4 weeks	
Subst.:	$s.A_3$			

The test substance (dissolved in water) was administered by oral gavage once daily to groups of 30 Wistar rats (15/sex) (Bor: (Wi)W, SPF) at doses 0 (group I), 75 (group II), 150 (group III) and 300 (group IV) mg/kg/day for 90 days.

19 rats (10 f, 9 m) of the control group and additional 30 rats (15 f, 15 m), which were treated at the highest dose for the same period were examined for signs of reversibility after 4 weeks without treatment.

The age at the start of the study was approximately 7-8 weeks and the average body weight was 134.4 g for females and 154.8 g for males. Food and water ad libitum. After 13 weeks (group V: 16 weeks) the animals were sacrificed.

***Examinations*:** - Clinical signs and mortality daily.
- Ophthalmoscopic examination of groups I, IV and V at the start and at the end of the study.
- Body weights, food and water consumption weekly.
- Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC) and
- Clinical Chemistry: (bil, glu, total protein, SGOT, SGPT, LDH, AP, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the start of the study and after 6 and 12 weeks (group I-V) and after 16 weeks (group I and V).
- Urinanalysis (nitrite, leuco, pH, prot, gluc, ketones, urobil, bil, blood, sed) at the start of the study and after 5 and 11 weeks (group I-IV) or after 6 and 12 weeks (group V) and after 15 weeks (group I and V).
- Relative and absolute organ weights, gross pathology and histopathology of 10 animals/group (5/sex).

Results: - Two animals dosed at 300 mg/kg/day died.
- *Haematology:* Hb-values were significantly reduced in the females of groups II, IV and V after 12 weeks of treatment. After 6 weeks eosinoph. granulocytes were reduced in the females in groups II-V, and after 12 weeks leukocytes were reduced significantly in the same groups. In males lymphocytes were significantly reduced in groups II, IV and V after 6 weeks of treatment and also in group V after 4 weeks of recovery.
- *Clinical Chemistry:* Alkaline Phosphatase was significantly reduced in the females after 6 weeks (group II, IV, V) and after 12 weeks of treatment (groups II and III). Ca-values were reduced in the males and in the females of groups III-V after 6 and after 12 weeks of treatment, as well as in the females of group V (after 4 weeks of recovery). After 12 weeks of treatment Fe was reduced significantly in groups IV and V in males as well as in females. After

4 weeks of recovery in the high dose females (group V) significantly increased GOT-values were observed.

- *Urinalysis:* The urines of the groups treated at 150 and 300 mg/kg/day and some animals of group II were discoloured since the 3rd or 4th week of treatment. The discoloration disappeared after the reversibility period.
- *Organ weights:* Increased organ weights were noted for spleen (group III, females (abs.) and II, III, IV, females (rel.)) and kidneys (group IV, males, (rel.) and IV, females (rel.)). Brain weights were reduced significantly in the females of groups II-IV (V?).
  [Statistical comparison of relative and absolute organ weights after 4 weeks of recovery (group I and V) was not performed.]
- *Gross pathology:* Gross pathology revealed a dark discoloration of the thyroids in all groups except the control group. The number of affected animals increased with the dose level (1 in group II, 7 in group III, 16 in group IV; 9 in group V after recovery period.).
- *Histopathology:* The histopathological examination showed a transformation of thyroid epithelium at doses of 150 and 300 mg/kg/day with increased intensity, accompagnied by an augmentation of epithelium cell nuclei at the higher dose. This substance-related effect was restricted to males (4 in group III, 5 in group IV and 4 in group V).
  Furthermore a liver cell hypertrophy was observed in animals treated with 300 mg/kg/day (8 in group IV and 5 in group V after recovery period). In group V an increase of lipocytes in bone marrows was noted (5/10 animals), which could possibly be a consequence of the advanced age of these animals.

According to acknowledged rules a NOAEL could not be established.

Route:	oral	Exposure:	90 days	NOAEL (DWE): -
Species:	rat			
Subst.:	$s.A_3$			

The test substance (dissolved in water) was administered by oral gavage once daily to a group of 24 Wistar rats (Bor (Wi)W, SPF) (12/sex) at 10 mg/kg/day (group II) for 90 days, 24 animals served as control (treated with the vehicle alone)(group I). The age at the start of the study was approximately 6-7 weeks and the average body weight was 113 ± 6 g for females and 115 ± 5 g for males. Food and water ad libitum. After 14 weeks the animals were sacrificed.

Examinations: Clinical signs and mortality daily. Ophthalmoscopic examination at the start and at the end of the study. Body weights weekly.

Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC) and clinical chemistry (bil, glu, total protein, SGOT, SGPT, LDH, AP, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the start of the study and after 8 and 14 weeks. Urinanalysis (nitrite, leuco, pH, prot, glu, ketones, urobil, bil, blood, sed) at start of the study and after 6 and 12 weeks. Relative and absolute organ weights, gross pathology and histopathology.

Results:

- *Haematology:* After 7 weeks of treatment erythrocytes, haematocrit and MCV, MCH and MCHC were significantly reduced in the females of the test

group. At the same time the haematocrit and the MCV were significantly reduced in the male rats of the test group as well. After 13 weeks leukocyte values in the test group females were significantly increased.
- *Clinical chemistry:* Fe-values were reduced significantly in the males of the test group after 7 weeks of treatment.
- *Urinalysis:* The urine of the test group animals was discoloured.
- *Organ weights:* Absolute and relative organ weight of the spleen was reduced in the females of the test group, whereas the relative spleen weight and the rel. weight of the kidney were increased significantly in the corresponding males.
- *Histopathology:* The histomorphological examination revealed a slight activation of the thyroids in 10 male and 1 female rat of the test group. As another substance-related effect lymphatic enteritis was observed in 10 animals of group II.

According to acknowledged rules a NOAEL could not be established.

Route: oral Exposure: 90 days NOAEL (DWE): 5 mg/kg b.w.
Species: rat
Subst.: C.P.

The test substance (dissolved in water) was administered by gavage once daily to a group of 20 Sprague-Dawley (CFY) rats (10/sex) at dose 5 mg/kg/day (group II) on 90 consecutive days, 20 animals served as control (Aqua dest.) (group I). The age at the start of the study was approximately 8 weeks and the body weight was 138-185 g for females and 144-190 g for males. Food and water ad libitum. After 14 weeks the animals were sacrificed.

Examinations: Clinical signs and mortality daily. Ophthalmoscopic examination at the start and at the end of the study. Body weights and food weekly. Water consumption by visual inspection daily.

Haematology (RBC, WBC, Diff., Hb, Hct, MCV, MCH, MCHC) and clinical chemistry (bil, gluc, total protein, albumin, γ-GT, AP, Na^+, K^+, Cl^-, Ca^{2+}, inorganic phosphorus, urea, creat, ALAT, ASAT, albumin/globulin ratio) during the last week of the study. Relative and absolute organ weights, gross pathology and histopathology.

Results:
- *Haematology*: The MCV was decreased in both males and females of the test group, whereas the MCH was decreased only in males. Furthermore prothrombin time was increased in the treated males.
- *Clinical chemistry:* In the females of group II decreased glucose values, decreased albumin/globulin ratios and increased creatinine values were observed. Examination of the males of the test group revealed decreased Na^{+-} and serum alanine aminotransferase values.

All the values of these parameters were considered to fall into the normal range.

- *Organ weight:* No statistically significant absolute weight changes. Mean relative kidney weight (% of body weight) was elevated in treated males ($p \leq 0.05$), but none of the individual values was considered abnormal.

- *Other signs:* Purple-coloured urine was noted in approx. 60% of the females during the first week and on isolated occasions up to 34 days after the start of the study.

No toxicologically significant treatment-related effects were observed. The NOAEL was therefore considered to be 5 mg/kg/day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Route:	skin	Exposure:	24 hrs.	Pr.Irr. Index:
Species:	albino rabbits	Concentr.:	2.5%	Effect: neg.
Subst.:	C.P.			

A 2.5% aqueous test solution was applied with a patch test technique to one intact and one abraded site of the clipped dorsum (6.54 cm^2) of three albino rabbits [no strain data given] for 24 hours. Records were taken after an exposure time of 24 and 72 hours.

Results*:* No effects were seen.

Remark: Compared with OECD/CEC-requirements the application time of the test procedure (acc. to Code of Federal Regulations, Title 16, Section 1500.41) is relatively long.

4.2 Irritation (mucous membranes)

Route:	eye	Exposure:	10 sec.	Pr.Irr. Index:
Species:	albino rabbits	Dose:	0.1 ml	Effect: neg.
Subst.:	C.P.	Concentr.:	2.5 %	

0.1 ml of a 2.5 % aqueous solution of the test compound was instilled into one eye of each of three albino rabbits [no strain data given], the other eye served as control. The treated eye was irrigated 10 seconds after instillation and the grade of ocular reaction was recorded at a 7-day observation period.

Results: No effects were seen.

Remark: Compared with OECD/CEC-requirements the application time of the test procedure (acc. to Code of Federal Regulations, Title 16, Section 1500.42) is relatively short.

5. Sensitization

Subst.:	C.P.	Conc. induc.:	1 %	Result: neg.
Species:	guinea pig	Conc. chall.:	1 %	
Method:	Landsteiner and Draize			

Inducing procedure was performed by intracutaneous application of 0.1 ml of a 1 % test substance dilution (in Ringer solution) into the shaven shoulder areas of 15 female Pirbright guinea pigs, 3 times daily on 5 consecutive days (10 animals served as control).

Four weeks later test and control animals were challenged by an intracutaneous injection of 0.1 ml of the test solution (1 %) into the untreated flanks.

Results: No allergic reaction was observed.

Remark: The concentration used for induction is relatively low.

Subst.:	$s.A_1$	Conc. induc.:	3 %	Result: neg.
Species:	guinea pig	Conc. chall.:	1 %, 2 %, 3 %	
Method:	Magnusson/Kligman			

Induction was performed by pairwise intracutaneous injections on the clipped shoulder region of 20 female Pirbright guinea pigs (Hoe:DHPK (SPF-LAC)/Boe) in the following sequence:

- 2 x 0.05 ml of Freund's Complete Adjuvant (FCA) (1:1 in aqua deion.)
- 2 x 0.05 ml of the test substance at 3 % in aqua deion.

10 animals treated with 1-Chlor-2,4-dinitrobenzene (DNCB) served as positive control. They received 4 pairwise intracutaneous injections in the following order:

- 2 x 0.05 ml FCA (1:1 in aqua deion.)
- 2 x 0.05 ml 0.005 % DNCB dil. in aqua deion.

Negative control consisted of a group of 10 animals:

- 2 x 0.05 ml FCA (1:1 in aqua deion)
- 2 x 0.05 ml aqua deion.

On the next day and 6-8 hours before the first dermal treatment all animals were pre-treated with sodium laurylsulfate (10 % in white vaseline). Induction by percutaneous route was carried out by application of 0.5 ml of the test substance at a concentration of 3 % in white vaseline (24 h closed patch). Positive controls were treated with 0.025 % DNCB (in white vaseline) (0.5 ml), negative controls with 0.5 ml 3 % aqua deion. (in white vaseline).

The second intradermal treatment was carried out 48 hours after the first one:

Test group:	- 2 x 0.05 ml test substance 3 % in FCA (dil. in ol. arach. 1:1)
Positive control:	- 2 x 0.05 ml DNCB 0.005 % ”
Negative control:	- 2 x 0.05 ml aqua deion. ”

14 days after the last exposure test and control animals were challenged by a cutaneous application of (24 h closed patch):

Test group:	- 0.5 ml each of 3 %, 2 %, 1 % test subst. in FCA (dil. in ol. arach. 1:1)
Positive control:	- 0.5 ml each of 1 %, 0.5 % , 0.01 % DNCB in FCA (dil. in ol. arach. 1:1)
Negative control:	- 0.5 ml aqua deion.

Challenge sites were evaluated for cutaneous reactions 24 and 48 hours p.a.

Results: No primary skin irritations and no allergic reactions were observed, thus the substance was classified as non-sensitizer.

6. Teratogenicity

Route:	oral	Admin. Days:	5-15 dpc	NOAEL (DWE): 30 mg/kg b.w.
Species:	rat	Subst.:	C.P.	

The test substance (dissolved in water) was given daily from day 5-15 of gestation to groups of 24 pregnant Wistar rats, respectively, (Bor:Wisw-SPF TNO strain) by oral gavage of doses of 5 (group I), 15 (group II) and 30 (group III) mg/kg b.w. 24 pregnant females treated with aqua deion. served as controls. Prior to treatment females were 14 weeks old and had a body weight range from 160-220 g. Food and drinking water ad libitum. According to sperm found in vaginal smear (day 0 of gestation), the females were sacrificed after 20 days post conceptionem.

Examinations:

- Clinical observations daily. Bodyweights were taken at the beginning of the study and at day 5, 10, 15 and 20. Food consumption was measured for days 0-5, 5-15, 15-20 as well as for 0-20.
- Complete autopsy of the dams and a macroscopic evaluation of the organs were carried out on day 20.
- Determination of the number of: dead and alive fetuses, distribution and site in the uterus, early and late resorptions, placentae, implantations, sex determination, corpora lutea. Determination of the weight of fetuses, placentae, graved uteri, uteri without fetuses.
- Externally visible deviations in fetuses, organic imperfections (in 1/3 of all fetuses) and skeletal defects (in 2/3 of all fetuses) were evaluated.

Results: No maternal abnormalities and no signs of maternal toxicity were observed. No abnormalities were found in the fetuses. Thus the NOAEL is 30 mg/kg.

7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption :

A hair dye formulation at an average amount of 43.16 g containing 2.3 % of the test substance ($s.A_2$) (= dose of 15.13 mg/kg b.w.) was applied to 5 healthy female volunteers's washed hair for 15 minutes. Blood samples were taken 0, 10, 20, 30, 45 minutes and 1, 2, 3 and 24 hours after the application. Blood and urine samples were examined via HPLC (detection limit 20 ng/ml in serum, 6 ng/ml in urine).

Results: The test substance could not be detected neither in the serum nor in the urine of the test persons. Therefore it was concluded that the amount of $s.A_2$ absorbed was nil or, at any rate, less than 0.13 % of the amount applied (less than 0.0195 mg/kg b.w.).

Toxicokinetics :

^{14}C-labelled Kardinalrot (C.P.) was applied to the dorsal skin of groups of 6 (3/sex) Sprague Dawley rats (Him:(OFA), b.w. approx. 200 g) for 30 minutes and then washed off. The test substance was integrated in two different hair dyeing formulations containing 2 % (I) and 4 % (II), respectively. Additional test substance was used as a 6.66 % solution in water/DMSO 1/2.

Hair dying formulation II was mixed with Welloxon (containing 9 % H_2O_2) before application. Oral application of the test substance was used as a reference. An additional experiment was performed to determine the blood level after peroral application.

Composition of the formulations:

Ingredient	Concentration in formulation *I*	*II*	
		cream alone	mixed with Welloxon
	(%)	*(%)*	*(%)*
KARDINALROT (^{14}C)	2.00	4.00	2.00
p-toluylenediamine-sulfate	/	3.50	1.75
mixture of resorcinol and m-aminophenol	/	1.36	0.68
mixture of salts	0.70	0.70	0.35
ammonia, 25%	0.36	2.00	1.00
isopropanol	3.90	3.90	1.95
WAS	2.00	2.00	1.00
water, deionised	43.44	31.30	15.65
formulation base	47.60	47.60	23.80
ammonia, 25%	/	3.65	1.83
Welloxon (containing 9% H_2O_2)	/	/	50.00

Treatments:

Group A: hair dye formulation I, containing 2 % of the test substance
cutaneous application

Group B: hair dye formulation II, mixed with Welloxon (containing 9 % H_2O_2) (1:1); final concentr. of the test substance: 2 %
cutaneous application

Group C: solution of the test substance in water/DMSO (1:2), containing 6.66 % of the test substance
cutaneous application

Group D: solution of the test substance (2 %) in water/DMSO (1:2)
oral application (reference)

Group E: solution of the test substance (2 %) in water/DMSO (1:2)
oral application

Animals of groups A-D were sacrificed 72 hours after the application of the test substance, animals of group E (kinetic parameters in blood) were sacrificed 24 hours p.a.

Examination: radioactivity in rinsings, treated skin areas, urine, faeces, blood, organs, carcass (using a liquid scintillation counter).

Results:

- Percutaneous absorption, mode and rate of elimination:

Percutaneous absorption (as the amount eliminated during 72 hours after application and the amount present in the carcass) was:

- 0.043 % for hair dyeing formulation I,
- 0.047 % hair dyeing formulation II plus hydrogen peroxide and
- 0.050 % for the test substance solution.

The treated skin areas contained mean ^{14}C-activities of 0.57 % for formulation I, 1.14 % for formulation II plus H_2O_2 and 0.06 % for the solution of the test substance. In studies A and B the mean ^{14}C-amount in the application site was statistically significantly higher in males than in females.

The absorbed amount of the test substance was excreted via urine (56-62 % of the eliminated ^{14}C) and via faeces (38-44 %). The mean excretion within the first 24 hours was 71-90 % of the eliminated ^{14}C.

After oral administration 46 % of the eliminated radiolabelled compound was excreted via urine and 54 % via faeces. 92 % of the eliminated ^{14}C was excreted within the first 24 hours.

The blood level was highest at 35 min. post application, it declined with an initial half-life of approximately 2.3 hours.

- Distribution into the organs 72 hours after application:

Mean ^{14}C-concentrations of blood and analysed organs in studies A, B and C were all near or below the detection limit after 72 hours. Only liver and fat were above the detection limit in all three studies, additionally kidney in study B and blood in study C. Detection limits were from approx. 0.0004 % dose/g for thyroids to 0.00002 % dose/g for large organs.

After *dermal* application (groups A, B and C) the relatively highest concentrations were found in thyroid and adrenals (both below detection limit), followed by fat, ovaries and liver. Lowest concentrations were detected in brain, femur and muscle.

The remaining mean amount in the carcass 3 days after cutaneous application was 0.0009 % (A), 0.0002 % (B) and 0.0001 % (C) of the administered ^{14}C-amount.

After *oral* application highest ^{14}C-concentrations were detected in thyroid, liver and kidney, the concentrations were 5-9 times higher in these organs than in blood.

Lowest concentrations were found in brain, femur, muscle.

The remaining mean amount of ^{14}C in the carcass 3 days after oral application was 0.34 % of the administered ^{14}C-amount.

Skin painting test:

Test formulations containing 0.013 %, 1.0065 % and 2.0 % of s.A_2 and 0.5023 % and 1.0 % of 1-amino-2-nitro-4-ß-hydroxy ethylamino-5-chloro-benzene in a vehicle mixture were

administered dermally to the back areas of groups of 150 NMRI (75/sex) mice (Han: NMRI) three times weekly for 18 months. The application volume was 0.05 ml/animal/day. Water only and the vehicle mixture without both substances were included as a negative and a vehicle control respectively.

Results: The treatment had no effect on the survival ratio. Body weight gain in the males of all test groups was reduced during the first three month in a dose-related manner and continued to be reduced in the males of the high dose group (III) during the whole study.

The histomorphological examination of the organs did not show any substance-affected formation of neoplasms and non neoplastic alterations.

8. Mutagenicity

Subst.	**Species Strain**	**Parameter**	**Dose Range**	**result metabolic act. - / +**		**species inducer**
$s.A_2$	*Salm.typh.*, TA 1535	base pair mutation	10-10000 µg/plate toxic ≥ 10000 µg/plate	-	+	rat, Aroclor
$s.A_2$	*Salm.typh.*, TA 1537	frameshift mutation	10-10000 µg/plate toxic ≥ 10000 µg/plate	-	-	rat, Aroclor
$s.A_2$	*Salm.typh.*, TA 1538	base pair mutation	10-10000 µg/plate toxic ≥ 10000 µg/plate	+	+	rat, Aroclor
s.B.	*Salm.typh.*, TA 98-NR	frameshift mutation	300-10000 µg/plate toxic ≥ 10000 µg/plate	-	-	rat, Aroclor
s.B.	*Salm.typh.*, TA 100-NR	frameshift mutation	300-10000 µg/plate toxic ≥ 10000 µg/plate	-	-	rat, Aroclor
$s.A_2$,B.	*Salm.typh.*			No exact data given*		rat, Aroclor
$s.A_2$,B.	*Salm.typh.*			No exact data given*		rat, Aroclor
s.C.	*Salm.typh.*, TA 98/100	base pair mutation		No exact data given*		rat, Aroclor
s.C.	*Salm.typh.*, TA 1535/37/38	base pair mutation		No exact data given*		rat, Aroclor
$s.A_2$	*E. coli* 343/113			1, 10, 100 µg/ml (survival rate at 100 µg: 100%)		neg.
$s.A_1$	mouse lymph L5178Y	mutat. HGPRT and ouabain resist.	12.5-200 µg/ml (toxic ≥ 200 µg/ml)	-	-	rat, Aroclor
s.B.	mouse lymph L5178Y	mutat. HGPRT and ouabain resist	12.5-200 µg/ml (toxic ≥ 200 µg/ml)	-	-	rat, Aroclor

s.A_1	CHO cells	chrom. aberr.	50, 250, 500 µg/ml toxic ≥ 500 µg/ml	-	-	rat, Aroclor
s.B.	CHO cells	chrom. aberr.	0.5, 2.5, 5 µg/ml toxic ≥ 5 µg/ml	-	-	rat, Aroclor
s.A_2	CFY rats	micronucleated polychromatic erythrocytes	3200 mg/kg b.w.			neg.

*Remark: Exact test results should be demanded.

Mutagenicity (bact.), description

Ames tests:

Kardinalrot was tested for mutagenicity in three strains of *Salmonella typhimurium* in the absence and presence of metabolic activation. The dose levels tested were 10-10000 µg/ plate (toxic conc. was 10000 µg/plate). The negative control was DMSO and the positive were ß-naphtyl-amine, neutral red and 2-acetyl-aminofluorene.

The accessory component was tested for mutagenicity in two strains of *Salmonella typhimurium* in the absence and presence of metabolic activation. The dose levels tested were 300-10000 µg/plate. The negative control was DMSO and the positive were 2-nitrofluorene and 2-aminofluorene.

The main and the accessory component of Kardinalrot were assayed in *Salmonella typhimurium* (strain(s) not given) in the absence and in the presence of metabolic activation. Test conditions (dose levels, controls, exact results etc.) were not given.

The second accessory component (s.C.) of Kardinalrot was tested for mutagenicity in five strains of *Salmonella typhimurium* in the absence and presence of metabolic activation. Test conditions (dose levels, controls, exact results etc.) were not given.

E. coli assay:

Kardinalrot (dissolved in DMSO) was tested for mutagenicity in *E.coli* bacteria strain 343/113. The dose levels tested were 1, 10, 100 µg/ml.

Remark: Metabolic activation and control substances were not included.

Mutagenicity (*in vitro* mammalian), description

Mouse lymphoma assays:

1-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene (dissolved in DMSO) was tested for 6-thioguanine resistance (HGPRT genetic locus) and ouabain resistance (Na^+/K^+ cell membrane ATPase locus) in a mouse lymphoma fluctuation assay with L5178Y cells, both in the absence and the presence of metabolic activation (S-9 mix of Aroclor 1254-induced male Wistar rats). The dose levels were 12.5-200 µg/ml (200 µg/ml proved to be a toxic concentration). Negative (DMSO) and positive controls (benzo(a)pyrene with and 4-nitroquinoline-1-oxide without metabolic activation) were included.

1-(2-hydroxyethyl)-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene (= B37) (dissolved in DMSO) was tested for 6-thioguanine resistance (HGPRT genetic locus) and ouabain resistance (Na^+/K^+ cell membrane ATPase locus) in a mouse lymphoma fluctuation assay with L5178Y cells, both in the absence and the presence of metabolic activation (S-9 mix of Aroclor 1254-induced male Wistar rats). The dose levels were 12.5-200 µg/ml (200 µg/ml proved to be a toxic concentration). Negative (DMSO) and positive controls (benzo(a)pyrene with and 4-nitroquinoline-1-oxide without metabolic activation) were included.

Chromosome aberration assays:

The main component of Kardinalrot (dissolved in DMSO) was tested for chromosome damaging potential using duplicate cultures of chinese hamster ovary cells both in the presence and absence of metabolic activation. Cells were treated with 50, 250, 500 µg/ml (500 µg/ml was a toxic concentration). Cyclophosphamide with and ethyl-methane-sulphonate without metabolic activation were used as positive control substances. The mitotic index at each test dose was determined. 100 metaphases from each culture were analysed for chromosome aberration including and excluding gaps.

Results: Kardinalrot did not produce statistically significant increase in chromosome aberrations neither in the presence nor in the absence of metabolic activation.

The accessory component of Kardinalrot (dissolved in DMSO) was tested for chromosome damaging potential using duplicate cultures of chinese hamster ovary cells both in the presence and absence of metabolic activation. Cells were treated with 0.5, 2.5, 5 µg/ml (5 µg/ml was a toxic concentration). Cyclophosphamide with and ethyl-methane-sulphonate without metabolic activation were used as positive control substances. The mitotic index at each test dose was determined. 100 metaphases from each culture were analysed for chromosome aberration including and excluding gaps.

Results: Kardinalrot did not produce a statistically significant increase in chromosome aberrations neither in the presence nor in the absence of metabolic activation.

Mutagenicity (*in vivo* mammalian), description

Micronucleus test:

Kardinalrot (dissolved in 0.5% gum tragacanth) was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of each of 10 CFY rats (5/sex) (130-160 g b.w.).

A total dose of 3200 mg/kg b.w. of the test compound suspended in 0.5% gum tragacanth was administered by oral gavage in two equal doses separated by an interval of 24 hours. The rats were sacrificed six hours after the second dose. 10 animals served as negative control (vehicle), and 6 animals (3/sex) were treated with Mitomycin C (total dosage: 14 mg/kg b.w.) by oral gavage and served as positive control group.

In the bone marrow smear of the femurs 2000 polychromatic erythrocytes per animal were examined.

Results: Kardinalrot was not mutagenic in the micronucleus test at a dose of 3200 mg/kg b.w.; this dose was near the toxic dose range.

Indicator tests (in-vitro mammalian)

Subst.	Species Strain	Parameter	Dose Range	result metabolic act. - / +		species inducer
s.B.	HeLa cells	unscheduled DNA-synthesis	0.125-250 µg/ml (toxic≥ 250 µg/ml)	-	-	rat, Aroclor
s.B.	CHO K1 cells	SCE (sister chromatid exchanges)	10-10000 µM	-	-	rat, Aroclor

Unscheduled DNA-synthesis:

The accessory component of Kardinalrot (dissolved in DMSO) was assayed with and without metabolic activation at dose values 0.125-250 µg/ml in a cell culture medium containing 3H-thymidine. DMSO served as a negative control, 3,3'-dichlorobenzidine with S9 and 4-nitroquinoline-1-oxide without S9 were used as positive control.

Results: The tested substance did not induce unscheduled DNA-synthesis in HeLa cells neither in the presence nor in the absence of metabolizing system.

Sister chromatid exchange (SCE) assay:

The accessory component of Kardinalrot (dissolved in DMSO) was assayed for SCE using duplicate cultures of CHO K1 cells with and without metabolic activation at dose values 10-10000 µM. DMSO served as a negative control, 2-nitro-p-phenylene-diamine without and 2-acetylaminofluorene with metabolic activation served as positive controls.

Results: The tested substance did not raise the frequency of sister chromatid exchanges.

9. Carcinogenicity

Carcinogenicity (s.B. = accessory component):

The accessory component (1-(ß-hydroxyethyl)-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene) was given for two years to groups of 100 Fisher 344/N rats (50/sex), respectively, and 100 (50/sex) B6C3F1 mice (male C57 BL/6N, female C3H/HEN) by mixing into the diet. Male rats received 5000 and 10000 ppm, female rats were dosed with 10000 and 20000 ppm. Male mice were treated with 1500 and 3000 ppm, respectively, while female mice were exposed to 3000 ppm and 6000 ppm, respectively. One untreated control group was run for each species and sex.

Examinations: Observations twice daily. Clinical signs were recorded once weekly. Body weights once weekly for the first 13 weeks and once every 4 weeks for the remaining 91 weeks. A complete necropsy was carried out in all animals. All organs and tissues were inspected for grossly visible lesions. For each animal 32 organs of tissues were examined histologically.

Results: Male and female rats and female mice showed a dose-related tendency of body weight decrease throughout the duration of the study. Dose-related increased marked hyperostosis of the skull was observed in rats of both sexes. In the female mice of the high dose group survival was lower than in the control group.

In the female rats neoplastic nodules or carcinomas of the liver were observed (control: -/50 ; group I: 2/50; group II: 3/50), which occur relatively infrequently in Fisher rats. Hepatocellular adenomas plus carcinomas (combined) were observed in the mouse studies (males: control: 10/50; I: 16/48; II: 18/49) (females: control: 4/50; I: 1/50; II: 7/49).

In 2 top dose female rats malignant mixed mesenchymal tumours of the kidney were noted, which have not been observed in more than 2000 control female F344 rats.

11. Conclusions

General:

Kardinalrot, which is a mixture of the three components s.A., s.B. and s.C., is used in semipermanent hair tinting products, colouring setting lotions and permanent hair dye formulations at an applied maximum concentration of 2.5% and 1.25 % respectively.

The single components, with used abbreviations in the text, are:

C.P.: Kardinalrot: (Commercial Product)
- 84 % 1-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene*,
- 13 % 1-(ß-hydroxyethyl)-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene**
- 3 % 1-amino-2-nitro-4-(ß-hydroxyethyl)-amino-benzene***

* s.A.: investigated in different specifications:
- $s.A_1$: purity 99 %
- $s.A_2$: unspecified
- $s.A_3$: 1-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene, hydrochloride

** s.B.: identical with B 37
*** s.C.: identification when tested as such.

Acute Toxicity:

The oral LD_{50} value for the rat is 2120 mg/kg (1810-2480 mg/kg) (C.P.).

Irritation/sensitization:

The eye irritation test (C.P.) was carried out with a relatively short exposure time (10 sec. only), but according to the described procedure (Fed. Reg.). The skin irritation test (C.P.) was carried out with a relatively long exposure time (24 hrs), while observation period was only 72 hrs.

Together with the sensitization tests (C.P. (only 1%) and $s.A_1$ (up to 3%)) the substance can be classified as non sensitizer.

Semichronic toxicity:

In a 90-day study, Wistar rats were fed 0, 75, 150, 300 mg/kg/d $s.A_3$ by gavage once daily. Two animals at 300 mg-dose level died. The urine of the group treated at 150 and 300 mg/kg/day was discoloured. Dark coloration of the thyroids occurred in a dose-related manner in all test groups except the control group; transformation of thyroid epithelium was only seen at 150 and 300 mg/kg/day. Increased relative organ weights were noted for spleen and kidney mainly in the high dose groups. Liver cell hypertrophy was noted in animals treated with 300 mg/kg/day.

In a following 90-day study, Wistar rats were fed ($s.A_3$) 10 mg/kg b.w. by gavage once daily. The histomorphological examinations of the organs showed a slight activation of the thyroids and lymphatic enteritis.

In a following 90-day study, Wistar rats were fed 5 mg/kg b.w. (C.P.) by gavage once daily. The investigations did not show any treatment-related effects. Based on these results the dose of 5 mg/kg/day is considered to be the marginal NOAEL.

Teratogenicity:

In a teratogenicity study, Wistar rats were fed 0, 5, 15, 30 mg/kg/d Kardinalrot (C.P.). No signs of maternal toxicity or adverse effects to the fetal development were observed, thus the NOAEL was 30 mg/kg b.w.

Carcinogenicity:

The accessory component (s.B.) (1-(ß-hydroxyethyl)-amino-2-nitro-4-bis-(ß-hydroxyethyl)-amino-benzene) was given for two years to groups of 50 male and female Fisher 344/N rats and male and female B6C3F1 mice (male C57 BL/6N, female C3H/HEN) by mixing into the diet. Male rats received 5000 and 10000 ppm, female rats were dosed with 10000 and 20000 ppm. Male mice were treated with 1500 and 3000 ppm, respectively, while female mice were exposed to 3000 ppm and 6000 ppm, respectively. Liver carcinomas and adenomas were observed in control and test animals of both species, kidney tumours were noted in two females of the high dose group.

Mutagenicity:

In an Ames-test using *Salmonella typhimurium* strain 1538 s.A_2 showed positive results both with and without metabolic activation. It showed also mutagenic properties in presence of S9-mix in Salm. typh. TA 1535. Reevaluation in a second Ames-test (s.B.) using Salmonella typhimurium TA 98 NR and 100 NR yielded negative results. Assays with E. coli (s.A_2), mouse lymphoma cells (s.A_1,B) and CHO-cells (s.A_1,B) yielded also negative results; the results from the E. coli assay are questionable because of methodological reason.

The micronucleus test (s.A_2) showed negative results.

Absorption:

^{14}C-radiolabelled C.P. was applied to the skin of rats in two different hair dye formulations or as a solution in water/DMSO. The cutaneous absorption was 0.043 % and 0.047 % respectively for the formulations I (without H_2O_2) and II (with H_2O_2) and 0.050 % for the test substance solution in water/DMSO.

In another absorption study a hair dye formulation containing s.A_2 was applied to volunteers's washed hair for 15 minutes. The amount absorbed was less than 0.13 % (detection limit) of the amount applied.

A Quality Assurance Declaration was included at the sensitization test, the toxicokinetic study, the three semichronic toxicity studies, the teratogenicity, carcino-genicity and the mutagenicity studies with mouse lymphoma, CHO cells and HeLa S3 cells.

FINAL CONCLUSIONS:

The irritation tests showed no harmful effects.
The substance (C.P.) can be classified as non sensitizer.
In the 90-day studies with rats, 5 mg/kg b.w. C.P. is considered to be the NOAEL.
In the teratogenicity study, no signs of maternal or fetal toxicity were observed in the rat after administration of 30 mg/kg b.w. (NOAEL).

It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Percutaneous absorption of a formulation was about 0.05 %.

Classification: A.

12. Safety evaluation

See next pages.

CALCULATION OF SAFETY MARGIN

KARDINALROT

COLIPA B 31

OXIDATION OR PERMANENT

Based on a usage volume of 100 ml, containing at maximum 1.25 %

Maximum amount of ingredient applied	**I(mg) = 1250**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.05 %**
Dermal absorption per treatment	**I(mg) x A(%) = 0.625 mg**
Systemic exposure dose (SED)	**SED(mg) = I(mg) x A % / 60 kg b.w. 0.01 mg/kg b.w.**
No observed adverse effect level (mg/kg) (rat, oral gavage, 90 d)	**NOAEL = 5 mg/kg b.w.**
Margin of Safety	**NOAEL / SED = 500**

CALCULATION OF SAFETY MARGIN

KARDINALROT

COLIPA B 31

SEMIPERMANENT

Based on a usage volume of 35 ml, containing at maximum 2.5 %

Maximum amount of ingredient applied	**I(mg) = 875 mg**
Typical body weight of human	**60 kg**
Maximum absorption through the skin	**A (%) = 0.05 %**
Dermal absorption per treatment	**I(mg) x A(%) = 0.438 mg**
Systemic exposure dose (SED)	**SED(mg) = I(mg) x A % / 60 kg b.w. 0.0073 mg/kg b.w.**
No observed adverse effect level (mg/kg) (rat, oral gavage, 90 d)	**NOAEL = 5 mg/kg b.w.**
Margin of Safety	**NOAEL / SED = 685**

B 89: *ETHYLCHLOROORANGE*

1. General

1.1 Primary name

2-Chloro-6-ethylamino-4-nitrophenol

1.2 Chemical names

1-Chloro-3-ethylamino-2-hydroxy-5-nitrobenzene
1-Hydroxy-2-ethylamino-4-nitro-6-chlorobenzene
3-Chloro-N-ethyl-2-hydroxy-5-nitroaniline

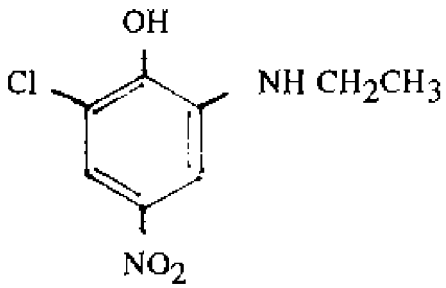
1.3 Trade names and abbreviations

Ethylchloroorange
Trade name: ROT CO (COS 552)

1.4 CAS no.

131657-78-8

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_9ClN_2O_3$
CAS formula: not available
Mol weight: 216.6

1.7 Purity, composition and substance codes

s.A.: 2-chloro-6-(ethylamino)-4-nitro-phenol (purity > 99 %)

1.8 Physical properties

Appearance: s. A.: brown-ochre crystals or orange powder
Melting point: 136-138 °C

1.9 Solubility

The substance exists as a free base. The substance is soluble in water at pH > 7; at pH < 7 solubility is about 0.04 % by weight. It is soluble in DMSO and propyleneglycol without pH change and suspendable in methylcellulose or gum arabic.

2. Function and uses

2-chloro-6-(ethylamino)-4-nitro-phenol is intended to be used in permanent and semi-permanent hair dye formulations at a maximum concentration of 3 %. As for use as oxida-tive hair dye a prior to use mixture with hydrogen peroxide (1:1) is made, in this case the applied concentration is 1.5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Sub.	Route	Species	LD_{50}/LC_{50}
s.A.	oral	rat	$\geq$ 1728 mg/kg b.w.
			(m 2026, f 1461 mg/kg b.w.)

The test substance (s.A.) (dissolved in 0.5 % carboxymethylcellulose solution) was administered once by oral intubation at doses of 1000, 1500, 2000 and 2500 mg/kg b.w. to treatment groups consisting of 10 (5/sex) Wistar rats Crl:(Wi)BR, respectively (weight range: 160-198 g for females and 184-227 g for males).

After administration the animals were observed for 14 days. A post mortem examination was carried out in mortalities immediately after finding. At the end of the observation period all surviving animals were sacrificed and gross necropsies were performed.

Results: In appropriate doses the substance caused reduced activity (apathia), discoordination, abnormal posture and position, piloerection and reduced prehension- and limb-tonus. A red staining of the saw dust was observed.

The post mortem examination of mortalities revealed residues of the test-substance in stomach, small and large intestine and colouring of the spleen, liver, kidneys and serosa. Terminal autopsy findings of survivors were normal. LD_{50} was calculated to be 1728 mg/kg b.w. (for males 2026 mg/kg b.w. and for females 1461 mg/kg b.w.).

Remark: Body weights were recorded on day 0 and on day 14 post applicationem. Body weight gain was reduced in a dose-dependent manner.

There was no control group included in this test.

3.7 Subchronic oral toxicity

Route:	oral	Exposure:	13 weeks	Species:	rat
Recov.p.:	(4 weeks)	Subst.:	s.A.		
NOAEL (DWE):	10 mg/kg b.w.				

Ethylchloroorange (dissolved in sodium carboxy methylcellulose) was administered by oral gavage once daily (7 days/week) to groups of 30 Wistar rats (15/sex) (Crl: (Wi) BR) at doses 0 (group I), 10 (group II), 30 (group III) and 90 (group IV) mg/kg/day for 13 weeks. The age of the animals at the start of the study was approximately 6 weeks and the body weight range was 98 - 136 g for females and 117 - 151 g for males. Food and water ad libitum. After 13 weeks the animals were attached to the control and the high dose group. They were deprived of treatment after 13 weeks and subjected to a subsequent 4 weeks recovery period.

Examinations:
- Clinical observation and examination daily.
- Ophthalmoscopic examination, hearing- and reflex-examination (with special regard to awareness, emotion, coordination and autonomic functions) at the start and at the end of the study.
- Body weights, food and water consumption weekly.
- Haematology (RBC, WBC, thrombocytes, Hb, Hct, MCV, MCH, MCHC, diff. blood count, reticulocytes, inclusion bodies, prothrombin time).
- Clinical chemistry (bil, gluc, total protein, albumin, SGOT, SGPT, GLDH, AP, CK, Na, K, Cl, PO_4, Fe, Ca, urea, uric acid, creat, chol, triglyceride) at the start of the study and after 6 and 13 weeks.
- Urinanalysis (colour, nitrite, leuco, pH, prot, glu, ketones, urobil, bil, blood, sed, spec. weight) at the start of the study and after 6 and 13 weeks.
- Autopsy, determination of organ weights and histomorphological examination.

Results: As treatment-related effects were observed:

- Significantly reduced body weights in the high-dosed males (group IV) over the entire course of the study.
- Haematological investigation revealed a slight increase of reticulocyte- and total leucocyte values in the high-dosed females at the end of the study.
- Clinical chemistry values indicated slightly increased total bilirubin and uric acid values in males and females as a dose-related tendency.
- Organ weight data revealed significantly increased liver weights in the males of the mid- and high-dose groups and a concomitant tendency in the females. Kidney weights showed a dose-related tendency towards increase (not statistically significant).
- An orange-red discoloration of the urines in all treated groups.

All findings described above were found to be completely reversible at termination of the recovery period.

10 mg/kg b.w. (group II) was considered to be the NOAEL.

4. Irritation & corrosivity

4.1 Irritation (skin)

(I)

Route:	skin	Exposure:	4 hrs.	Pr.Irr.Index:	
Species:	albino rabbits	Dose:	0.5 ml	Effect:	neg.
Subst.:	s.A.	Concentr.:	3 %		

In a closed patch test 0.5 ml of a 3 % dilution of the test substance in propyleneglycol were applied to scarified and intact skin areas (2.5 cm x 2.5 cm), respectively, of the clipped dorsum of six albino rabbits (White New Zealand). After 4 hours the substance was washed off and skin reactions (erythema and edema) were recorded 0.5, 1, 24, 48 and 72 hours later.

Results: The substance did not cause any adverse skin reaction, thus it was classified as not irritating.

Remark: Because of the colouring of the skin, erythema, if existent, may be difficult to observe.

(II)

Route:	skin	Exposure:	4 hrs.	Pr.Irr.Index:	
Species:	albino rabbits	Dose:	0.5 g	Effect:	neg.
Subst.:	s.A.	Concentr.:	100 %		

In a closed patch test 0.5 g of the test substance were applied to scarified and intact skin areas (6.25 cm^2) of the clipped dorsum of six albino rabbits (White New Zealand). After 4 hours the substance was washed off. Skin reactions (erythema and edema) were recorded after 0.5, 1, 24, 48 and 72 hours.

Results: No skin reactions were noticed in any of the animals, thus it was classified as not irritating.

Remark: It is not mentioned whether the substance was moistened sufficiently, as recommended in current OECD-guidelines.

4.2 Irritation (mucous membranes)

(I)

Route:	eye	Dose:	0.1 ml	Pr.Irr.Index:	
Species:	albino rabbits	Concentr.:	3 %	Effect:	neg.
Subst.:	s.A.				

0.1 ml of a 3 % dilution of the test substance in propyleneglycol was instilled into the left eye of each of six albino rabbits (White New Zealand), the right eye served as control, respectively. In three animals the treated eye was irrigated 4 seconds after instillation, in the remaining three the substance was not washed off. The grade of ocular reaction was recorded 1, 24, 48 and 72 hours after the application.

Results: Hyperemia (injection) was observed one hour after the application in the conjunctiva of the 3 animals, in which the substance had not been washed off. Other eye reactions were not noted. Thus the substance was classified as not irritating under the conditions of this test.

(II)

Route:	eye	Dose:	0.1 g	Pr.Irr.Index:	
Species:	albino rabbits	Concentr.:	100 %	Effect:	neg.
Subst.:	s.A.				

0.1 g of the test substance was instilled into the left eye of each of six albino rabbits (White New Zealand), the right untreated eye served as control, respectively. In three animals the treated eye was irrigated 4 seconds after instillation, in the remaining three the substance was not washed off. The grade off ocular reaction was recorded 1, 24, 48 and 72 hours after the application.

Results: The substance was classified as not irritating to mucous membranes (acc. to 83/467/EWG).

Remark: Hyperemia of the conjunctiva was observed in all treated animals; redness of the conjunctiva as well as affection of the iris were observed in most of the treated animals, but it should be noted that the test substance itself has a redish colouring effect.

5. Sensitization

Method:	Magnusson/Kligman	Conc. induc.:	10 %	Result:	neg.
Species:	guinea pigs	Conc. chall.:	1 %	Subst.:	s.A.

Induction was performed by pairwise intracutaneous injections on the clipped shoulder region of 20 Pirbright guinea pigs (Bor: DHPW (SPF)) in the following sequence:
- 2 x 0.05 ml of the test substance at 10 % in deionised water + Cremophor
- 2 x 0.05 ml of the test substance at 10 % in Freunds Complete Adjuvant (FCA)
- and 2 x 0.05 ml FCA.

20 animals served as controls. They received 6 pairwise intracutaneous injections in the following order:
- 2 x 0.05 ml FCA
- 2 x 0.05 ml Aqua deion. + Cremophor at 10 % in FCA
- 2 x 0.05 ml Aqua deion. + Cremophor.

Induction by percutaneous route was carried out 7 days later by application of 0.5 ml of the test substance at a concentration of 1 % in deionised water (48 h closed patch) (controls: Aqua deion.).

On day 21 test and control animals were challenged by a cutaneous application of 0.5 ml test substance at a concentration of 1 % in deionised water (24 h closed patch).

Evaluation of the cutaneous reaction at the challenge site was carried out 24 and 48 hours after removal of the patch.

Results: No allergic reaction was observed, thus the substance was classified as non sensitizer.

6. Teratogenicity

Route:	oral	Species:	rat	Subst.:	s.A.
Admin. Days:	5-15 dpc				
NOAEL (DWE):	90 mg/kg b.w.				

Ethylchloroorange (dissolved in sodium-carboxy methylcellulose) was given daily from day 5-15 of gestation by oral administration to groups of 20 pregnant Wistar rats (Crl:(Wi) BR) of doses of 0 (group I), 10 (group II), 30 (group III) and 90 (group IV) mg/kg b.w. Prior to treatment females were approximately 8 weeks old and had a bodyweight range from 160-245 g. Food and drinking water ad libitum. According to sperm found in vaginal smear, the females were sacrificed on day 20 post conceptionem.

Examinations:
- Clinical observations daily.
- Bodyweights were taken at the beginning of the study and at day 5, 10, 15 and 20.
- Food consumption was measured for days 0-5, 5-15, 15-20 as well as for 0-20.
  Complete autopsy of the dams and a macroscopic evaluation on the organs were carried out on day 20.
- Determination of the number of: dead and alive fetuses, birth position and site in the uterus, early and late resorptions, placentae, implantations, sex determination, corpora lutea. Determination of the weight of: fetuses, placentae, grav. uteri, uteri without fetuses.
  Externally visible deviations in fetuses were evaluated, visceral imperfections were studied in 1/3 of all fetuses and skeletal defects in 2/3 of all fetuses.

Results:
- Skeletal examinations of fetuses revealed wavy ribs at comparable intergroup frequencies.
- Two fetuses of the high-dosed group IV showed malformations (one exencephalus, one with complexed ribs).
- One fetus of group II and one of group IV were found with head/headneck edemas.

All observed incidences in fetuses were considered to be within the spontaneous variation range for this strain of rats.

Thus the highest studied dose: 90 mg/kg b.w. was considered to be the NOAEL for teratogenicity/embryotoxicity.

(A substance and dose related orange-red discolouration of urine was observed in all groups during the application period.)

7. Toxicokinetics (incl. Percutaneous Absorption)

^{14}C-labelled ethylchloroorange (integrated in two different hair dye formulations or used as a solution in water/DMSO) was applied dermally on the clipped dorsal skin of groups of 6 (3/sex) Sprague Dawley rats (Him:(OFA), SPF, b.w. approx. 200 g). After 30 min. the test solutions were washed off.

Oral application of the test substance was used as a reference. An additional experiment was performed to determine the blood level after oral application.

Treatments:

Group A: hair dye formulation I, containing 2 % of the test substance
cutaneous application
Group B: hair dye formulation II, mixed with Welloxon (containing 9 % H_2O_2) (1:1);
final concentr. of the test substance: 2 %
cutaneous application
Group C: solution of the test substance in water/DMSO (3:7), containing 6.66 % of the test substance
cutaneous application
Group D: solution of the test substance (2 %) in water/DMSO (4:6)
oral application (reference)
Group E: solution of the test substance (2 %) in water/DMSO (4:6)
oral application

Animals of groups A-D were sacrificed 72 hours after the application of the test substance, animals of group E (kinetic parameters in blood) were sacrificed 24 hours p. a.

Examination:

Radioactivity in rinsings, application site, urine, faeces, blood, organs and carcass (using a liquid scintillation counter).

Results:

Percutaneous absorption, mode and rate of elimination:

Percutaneous absorption (as the amount eliminated during 72 hours after application and the amount present in the carcass) was:

- 0.14 % for hair dyeing formulation I,
- 0.10 % hair dyeing formulation II plus hydrogen peroxide and
- 2.87 % for the test substance solution (water/DMSO).

The absorbed amount of the test substance was excreted via urine (85-88 %) and via faeces (12-15 %). The mean excretion within the first 24 hours was 90 % of the eliminated ^{14}C in studies A-C.

The treated skin areas contained mean ^{14}C-activities of 0.18 % for formulation I, 0.47 % for formulation II plus H_2O_2 and 0.50 % for the solution of the test substance.

After oral administration 67 % of the eliminated radiolabelled compound was excreted via urine and 33 % via faeces. 77 % of the eliminated ^{14}C was excreted within the first 24 hours.

The blood level was highest at 35 min. post application, it declined with an initial half-life of approximately 1 hour.

Distribution into the organs 72 hours after application:

Mean ^{14}C-concentrations of blood and analyzed organs in studies A and B were all near or below the detection limit, in study C mean ^{14}C-concentrations were higher, only thyroids were below the detection limit.

After *dermal* application the relatively highest concentrations were found in:

Group A: - kidneys, thyroids and adrenals
Group B: - thyroids, kidneys, adrenals and ovaries
Group C: - kidneys, adrenals and ovaries.

After *oral* application highest ^{14}C-concentrations were detected in:
- kidneys, adrenals, liver and thyroids.

8. Mutagenicity

Species, Strain	**Parameter**	**Dose Range**	**result metabolic act. - / +**	**Species**	**Inducer**
Salm.typh., TA 97	frameshift mutation	1-3000 µg/plate toxic ≥ 3000 µg/plate	- -	rat	Aroclor
Salm.typh., TA 98	frameshift sub. mut.	1-3000 µg/plate toxic ≥ 1000 µg/plate	(+) -	rat	Aroclor
Salm.typh., TA 100	base pair mutation	1-3000 µg/plate toxic ≥ 1000 µg/plate	- -	rat	Aroclor
Salm typh., TA 98 NR	frameshift mutation	10-6000 µg/plate toxic ≥ 1000	- np	rat	Aroclor
Mouse lymph L1578Y	mutation HGPRT	2.2-5000 µg/ml toxic ≥ 185 µg/ml + S9 toxic ≥ 1666 µg/ml -S9	- -	rat	Aroclor

Species	**Parameter**	**dose**	**result**
NMRI mice	micronucleated polychromatic erythrocytes	2500 mg/kg b.w.	neg.

Mutagenicity (bact.)

Ames tests:
Rot CO (dissolved in DMSO) was tested for mutagenicity in three strains of Salm. typh. with and without metabolic activation. The dose levels tested were 1-3000 µg/plate (eight concentrations, toxic conc. was 3000 µg/plate for strain TA 97 and 1000 µg/plate for strains TA 98 and 100). The negative control was DMSO and the positive were 2-amino-fluorene for plates with metabolic activation and sodium azide, 2-nitro-fluorene and 4-nitro-o-phenyldiamine, respectively, for those without metabolic activation.

Rot CO (dissolved in DMSO) was tested for mutagenicity in Salm. Typh. strain TA 98 NR (nitroreductase deficient) in the absence of metabolic activation only. The dose levels tested were 10-6000 µg/plate (six concentrations, toxic conc. was 1000 µg/plate). The negative control was DMSO and the positive was 2-nitrofluorene.

Mutagenicity (*in vitro* mammalian)

Mouse lymphoma assay:
Rot CO (dissolved in DMSO) was tested for 6-thioguanine resistance (HGPRT genetic locus) in a mouse lymphoma fluctuation assay with L5178Y cells, both in the absence and the presence of metabolic activation (S-9 mix of Aroclor 1254-induced male Wistar rats). The dose levels were 2,2-5000 µg/ml (eight concentrations, toxic conc.: 185 µg/ml with S9 and 1666 µg/ml without S9). Negative (DMSO) and positive controls (benzo(a)pyrene with and 4-nitroquinoline-N-oxide without metabolic activation) were included.

Mutagenicity (*in vivo* mammalian)

Micronucleus test:
Rot CO (dissolved in DMSO) was tested for its potential to induce micronuclei in polychromatic erythrocytes in the bone marrow of 3 groups of 10 (5/sex) NMRI mice (Crl:(NMRI) BR, 31.5 - 37.8 g b.w.).

A total dose of 2500 mg/kg b.w. of the test compound was administered once via stomach intubation. The animals were sacrificed 24, 48, 72 hours after the application.

One negative control group (DMSO, 10 animals, (5/sex), killed 48 hours p. a.) and one positive control group (cyclophosphamide (40 mg), 10 animals, (5/sex), killed 24 hours p.a.) were included in the study.

In the bone marrow smear of the femurs 1000 polychromatic erythrocytes per animal were examined.

Results:

The test substance did not show any evidence of mutagenic potential.

A cytotoxic effect, emphasized by a reduction of the number of nucleated cells, was observed 24 hours after the application.

Indicator tests (*in vitro* mammalian)

Species, Strain	**Parameter**	**Dose Range**	**Result metabolic act. - / +**	**Species**	**Inducer**
CHO K1 cells	sister chromatid exchange	10-10000 µM (toxic ≥ 10000 µM)	- -	rat	Aroclor

Sister chromatid exchange (SCE) assay:
Rot CO (dissolved in DMSO) was assayed for SCE using cultures of CHO K1 cells with and without metabolic activation at dose values 10-10000 μM. DMSO served as a negative control, 2-nitro-p-phenylene-diamine without and 2-acetyl-amino-fluorene with metabolic activation served as positive controls. At each concentration 100 metaphases were evaluated.

Results:

It is concluded that the test substance was not able to produce a mutagenic effect.

The result of this test is questionable, and so far as the positive control substance was obviously not able to produce a significant rise of number of SCE.

11. Conclusions

General:
Ethylchloroorange is used in permanent and semipermanent hair dye formulations; permanent: 1.5 %, semipermanent: maximum 3 %.

Acute toxicity:
The oral LD_{50} value for the rat is 1728 mg/kg ethylchloroorange.

Irritation/sensitization:
Slight eye reactions were observed, but the substance can be classified as not irritating to mucous membranes.

The skin irritation tests were carried out appropriately. However, erythema, if occurring, might be difficult to diagnose, because of the colouration of the skin.

The sensitization test revealed that the substance can be classified as not sensitizer.

Semichronic toxicity:
In a 90-day study, Wistar rats fed 0, 10, 30 and 90 mg/kg b.w. ethylchloroorange by oral gavage once daily. Treatment-related effects were significantly reduced body weights in high dosed males (90 mg/kg b.w.) and significantly increased liver weights in males of the 30 and 90 mg/kg b.w. dosed groups (concomitant tendency in the females). A slight increase of reticulocyte- and leucocyte values in the high-dosed females was observed at the end of the study, as well as a dose-related slight increase in total bilirubin and uric acid values in males and females. An orange-red discolouration of the urines was observed in all treated groups.

Based on these results the dose of 10 mg/kg/day was considered to be the NOAEL.

Teratogenicity:
In a teratogenicity study, pregnant Wistar rats were fed 0, 10, 30 and 90 mg/kg b.w. ethylchloroorange. A substance- and dose-related orange-red discolouration of urine was observed in all groups during the application period. Two fetuses of the high dosed group (90 mg/kg b.w.) showed malformations (one with exencephalus, one with complexed ribs), one low dose group fetus (10 mg/kg b.w.) and one high dose group fetus (90 mg/kg b.w.) were found with

head/head-neck edemas. All observed incidences in the fetuses were considered as being within the spontaneous variation range for this strain of rats. Thus 90 mg/kg b.w. is stated to be the NOAEL.

Mutagenicity:

The test substance did not show any mutagenic potential in *Salmonella thyphimurium* strains TA 97 and TA 100, neither in the presence nor in the absence of metabolic activation. Without S9-mix ethylchloroorange caused a slightly elevated number of revertant colonies in strain TA 98 at a higher concentration. Re-evaluation in a second Ames-test using TA 98 NR (without S9-mix) yielded negative results. Other strains (i.e. TA 1535/37) were not tested.

Treatment of mouse lymphoma cells with the test substance did not induce gene mutations at the HGPRT locus, with and without S9-mix.

The sister chromatid exchange test with CHO Kl cells was inadequate.

The micronucleus test did not show any evidence of mutagenic potential of ethylchloroorange.

Toxicokinetics:

^{14}C-radiolabelled ethylchloroorange was applied to the skin of rats in two different hair dye formulations or as solution. Oral administration of the test substance served as a reference.

Percutaneous absorption was 0.14 % and 0.10 %, respectively, for the formulation I and formulation II (containing H_2O_2) and 2.87 % for the test substance solution (6.66 % in water/DMSO). The absorbed amount of the test substance was excreted via urine (85-88 %) and via faeces (12-15 %). After oral administration 67 % of the radiolabelled compound was excreted via urine and 33 % via faeces.

72 hours after application the relatively highest concentrations of ^{14}C-radiolabelled ethylcloroorange were found in kidneys, adrenals, thyroids, liver and ovaries.

A Quality Assurance Declaration was included for the acute toxicity test, all irritation and sensitization tests, the toxicokinetic, the semichronic toxicity and the teratogenicity study.

In the mutagenicity test QAU declarations are missing, except for the micronucleus test.

FINAL CONCLUSIONS:

The substance can be classified as non irritating to mucous membranes and as non-sensitizer.

In the 90-day studies with rats, 10 mg/kg b.w. is considered to be the NOAEL. In the teratogenicity study, no signs of maternal or fetal toxicity were observed after administration of 90 mg/kg b.w. in rats. It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month.

Percutaneous absorption of a formulation was 0.14 % without and 0.10 % with H_2O_2.

In general the test substance did not show any mutagenic potential.

Classification: A.

12. Safety evaluation

See next pages.

CALCULATION OF SAFETY MARGIN

ETHYLCHLOROORANGE

OXIDATION OR PERMANENT

Based on a usage volume of 100 ml, containing at maximum 1.5 %

Maximum amount of ingredient applied:	**I (mg) = 1.500 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.10 % (+ H_2O_2)**
Dermal absorption per treatment:	**I (mg) x A (%) = 1.5 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg b.w. = 0.025 mg/kg b.w.**
No observed adverse effect level (mg/kg): (rat, oral gavage, 90 d)	**NOAEL = 10 mg/kg b.w.**
Margin of Safety:	**NOAEL / SED = 400**

CALCULATION OF SAFETY MARGIN

ETHYLCHLOROORANGE

SEMIPERMANENT

Based on a usage volume of 35 ml, containing at maximum 3 %

Maximum amount of ingredient applied:	**I (mg) = 1050 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.14 %**
Dermal absorption per treatment:	**I (mg) x A (%) = 1.470 mg**
Systemic exposure dose (SED):	**SED (mg) = I (mg) x A (%) / 60 kg = 0.0245 mg/kg b.w.**
No observed adverse effect level (mg/kg): (rat, oral gavage, 90 d)	**NOAEL = 10 mg/kg b.w.**
Margin of Safety:	**NOAEL / SED = 400**

P 93: *SILVER CHLORIDE, TITANIUM DIOXIDE (COMPLEX)*

1. General

1.1 Primary name

Silver chloride, Titanium dioxide (complex)

1.2 Chemical names

Silver chloride, Titanium dioxide (complex)

1.3 Trade names and abbreviations

JMAC (Johnson Matthey Antimicrobial Composite)

1.5 Structural formula

1.7 Purity, composition and substance codes

Composition of silver chloride (I) deposited on titanium oxide (IV)
20 % AgCl (m/m) on 80 % TiO_2

1.8. Physical properties

Appearance: Insoluble white powder.
Melting point: 1825°C

2. Function and uses

Function

Preservative

Categories and concentrations of use

Various types of cosmetics: skin creams, shampoos, baby lotions etc.: < 0.02 % excepted oral applications such as toothpastes, mouthwash and lip products.

Other uses/other categories of products

Biocide for household detergents, paper coming in contact with foodstuffs, paints, etc.

Annual tonnage

250 to 1000 kg.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral route
Sprague-Dawley rats (5 males, 5 females).
Method: OECD 401
LD 50 > 2000 mg/kg pc.

Oral route
Sprague-Dawley albino rats (10 males)
Method: Federal Hazardous Substance Act regulations
16 CFR 1500 (USA)
LD 50 > 5000 mg/kg pc.

Intravenous route
CF 1 albino mice (10 males)
Method: injection into the lateral vein of the tail.
0.17 mg a.i. in suspension in 1 ml of a solution containing 0.9 % NaCl.
The dose is not lethal at 8.6 mg/kg pc.

Intraperitoneal route
Albino mice (5 test, 5 control, sex?)
Method: intraperitoneal injection of 50 ml/kg pc of a saline solution, at 228 mg/ml.
The dose is not lethal at 11.4 mg/kg pc.

Method: OECD 404
Substance/concentration: JMAC (no vehicle).
Species/number: white New Zealand rabbits (3males).

Semi-occlusive application during four hours of 400 mg a.i. on the intact shaven skin of the flank near the hindmost rib (over a surface of 2.5 cm^2).

Readings 1, 24, 48 and 72 hours after removal of the patch.

Result:
Non irritant.

Method: (FHSA) 16 CFR 1500
Substance/concentration: 500 mg JMAC in 0.5 ml mineral oil.
Species/number: 6 white New Zealand rabbits (sex ?).

Application under occlusion during 24 hours on the hind quarters over 1 $inch^2$ of intact skin and 1 $inch^2$ of shaved skin.

Reading 30 minutes and 72 hours after termination of contact.

Result:
Non irritant under the test conditions.

Primary irritation index less than 5.
Very mild erythema and very mild oedema in one animal at the two sites after 72 hours.

3.3 Acute inhalation toxicity

Wistar albino rats (5 males, 5 females).
Method: 200 mg/lt air during one hour.
LC 50 > 200 mg/lt/ 1 h.

3.4 Repeated dose oral toxicity

Method: Application by gavage
Protocol (JMU I/C) GLP

Species/number: Sprague-Dawley rates. 4 groups of 5 males and 5 females.
Substance/concentration: JMAC in suspension in aqueous solution of 1 % methylcellulose 0, 300, 750, 1500 mg of JMAC/kg/j.

One daily application over seven days.

Observations: clinical symptoms, body weight, food consumption.
After autopsy, weighing of essential organs and macroscopic examination.

Results:
All the animals survived. No signs of intoxication at the three doses used. A dose level of 1500 mg/kg/j may be used in a more long-term study.

Method: 28 day oral study, gavage

Species/number: Sprague-Dawley rats + Charles River rats. 40 (4 groups of 5 males and 5 females).

Substance/concentration: JMAC in suspension in aqueous solution at 1 % methyl cellulose 0, 300, 750, 1500 mg JMAC/kg/j.

Daily application over 28 days.

Observations: Mortality, clinical symptoms, body weight, food consumption, blood sampling at end of test and full range of serum biochemistry and haematological examinations.

After autopsy, weighing of essential organs, macroscopic examination and observation of the tissues in situ, histopathological examination of the organs of the controls and the rats tested at 1500 mg/kg/j dose.

Results:
Non-lethal.
No clinical symptoms of poisoning at the three doses used.
Significant drop in the average weight of males in lot 4 (1500 mg/kg/j).
No variation in food consumption.

No essential variation in haematological parameters (all values lying within the normal range defined by the test laboratory) despite a relative reduction in leucocytes in females at the 1500 mg/kg/j dose.

Serum biochemistry:
- significant increase in blood alkaline phosphatase at the three doses in the males and at the two higher doses in the females;
- significant increase in blood sugar in the males at the 1500 mg/kg/j dose and significant reduction in the females at the two higher doses;
- significant increase in blood transamine at the two highest doses, only in the males;
- the other parameters remain normal.

Reduction in weight of the thymus in males and to a lesser extent in females at the 1500 mg/kg/j dose.

Method: 28 days oral study, gavage

No visible lesions in macroscopic and microscopic examinations of the different organs and abnormal content (firm dark material) throughout gastro-intestinal tract at the 1500 mg/kg/d dose.

Method: complementary histological evaluation

of the gastro-intestinal tract after 28 day oral treatment, gavage.

Observations: Histological evaluation of organs associated with 1500 mg/kg/d.
Treatment; stomach, jejunum, caecum, ileum, colon + mesentric lymphnodes, liver and spleen.
N.B.: No complementary evaluation at 300 and 750 mg/kg/d because of absence of macroscopic injuries.

Results:
- Brown discoloration of caecum and ileum mucosis consistent with silver deposit (males and females).
- Material deposit (probably silver salts or silver and titanium salts) in ileum macrophages and material deposit in mesentric lymphnodes, associated with macrophages migration (males and females). No deposit in the spleen.
- Except the reduction of clear (glycogen) hepatic cells in the males, no microscopic hepatic injuries. No deposit in Kupfer cells.

4. Irritation & corrosivity

4.2 Irritation (mucous membranes)

Method: OECD 405
Substance/concentration: preparation at 1% a.i. in a base for hand cream.
Species/number: white New Zealand rabbits, 3 females.

Application of 0.1 ml of the preparation in the conjunctival sac of an eye. No rinsing.

Reading after 1, 24, 48 and 72 hours.

Result:
Non-irritant under the test conditions (in accordance with the Kay and Calandra modified scale).

Method: (FHSA) Fed. Haz. Subst. Act. regel. (USA) 16 CFR 1500
Substance/concentration: JMAC (no vehicle).
Species/number: white New Zealand rabbits (sex?).

Instillation into the conjunctival sac of an eye of 100 mg of the substance, without vehicle. No rinsing.

Reading after 24, 48 and 72 hours.

Result:
Light to moderate irritation in accordance with the Kay and Calandra scale.
Irritant in accordance with the FHSA guidelines.
Recuperation after 48 and 72 hours.

Method: vaginal application (no standard protocol)

Substance/concentration: suspension at 44 mg JMAC in 2 ml solution of 0.9 % ClNa.
Species/number: white New Zealand rabbits. 5 females (3 tests, 2 controls).

One vaginal application per day over five days in a rubber catheter, dose 17.6 mg/kg pc.

Reading: Daily examination of general health and signs of irritation. Macroscopic and microscopic examinations of the vaginas after autopsy.

Result:
Non-irritant under the test conditions.
No macro or microscopic differences between tests and controls.

5. Sensitization

Method: OECD 406, Magnusson Kligman (GLP)

Species/number: Dunkin Hartley guinea pigs. 30 females (20 tests, 10 controls).

Substance/concentration:	*Induction*	(1) 5 % JMAC in water and in FCA
		(2) 50 % JMAC in water
	Challenge	(3) 25 % JMAC in water
		(4) 50 % JMAC in water.

On day 1, *induction* by intradermic injection between the shoulders (two sites) of 0.1 ml of

- FCA/water emulsion (50/50)
- suspension of 5 % JMAC in water (non-irritant dose, intradermic route)
- suspension of 5 % JMAC in FCA/water emulsion.

On day 8, *induction* by topical occlusive application during 48 hours at the intradermic injection sites of a filter paper saturated with 50 % JMAC in water.

14 days after topical application, *revelation* by application under occlusion during 24 hours of a suspension of 50 % in water (3) on the left side and a suspension of 25 % in water (4) on the right flank.

Reading after 24 and 48 hours.

Result:
No evidence of hypersensitization (type IV).

Method: Buehler patch test (abbrev.)
Species/number: Hartley albino guinea pigs. 15 males (10 tests, 5 controls).
Substance/concentration: 500 mg JMAC in 0.5 ml mineral oil.

Induction by three occlusive applications on days 1, 7 and 14 on the shaved right flank for six hours of a suspension of 500 mg JMAC in 0.5 ml mineral oil.

Revelation 14 days after the last induction application, by occlusive application during six hours on the left flank of the same test material.

Readings 24, 48 and 72 hours after removal of the patch.

Result:
No evidence of sensitization.
NB: The light reddenings observed in two test animals during induction are not considered as significant.
A control animal presented erythema 24 hours after application of the revelation.

Sensitization in man

Method: Adapted RIPT (Shelansky and Shelansky)
Species/number: volunteers: 104.
Substance/concentration: suspension of 1 % JMAC in a cream base (0.2 ml).

Induction: nine occlusive applications for 24 hours of 0.2 ml suspension of 1 % to the upper arm over a three-week period.

Examination of the skin 48 or 72 hours after each application.

Revelation: two weeks after the end of the induction period, a new application for a duration of 24 hours of the same test material on the two arms.

Readings after 48 and 96 hours after application.

NB: Same treatment with the placebo (cream base).

Result:
According to the author, no significant differences between the test material and the placebo in 99 subjects who continued the test to its conclusion.
However, there are some doubts as to the test´s validity (positive responses in placebo?).

Photosensitization

Method: Photo allergy test (GLP)

Species/number: Dunkin Hartley guinea pigs, 41 males (10 tests, 10 positive controls, 9 vehicle controls, 12 irritation controls).

Substance/concentration:	*Induction*	1 % JMAC in ethanol
	Revelation	1 % JMAC in acetone.

Induction: After intradermic injection of 0.1 ml of FCA, topical non-occlusive application behind the shaved neck of 0.1 mg of a 1 % suspension in ethanol for 3 hours, prior to irradiation at 350 nm (10 J/cm^2 per exposure) for 0.5 to one hour.

Five applications altogether on days 1, 3, 6, 8 and 10.

The treated sites were examined (erythema) 24 hours after each application.

Positive control: 3 % TSCA in ethanol.

Revelation: 18 days after the last induction treatment, non-occlusive application on the hind quarters (8 symmetrical sites) of 0.1 ml JMAC at 1 % in acetone and dilutions of this solution at 0.1 % and at 0.01 % in acetone (i.e. three applications of the test material plus three applications of the positive controls: 1 %, 0.5 % and 0.25 % TSCA in the acetone and two applications of the vehicle). After three hours of contact, irritation of the left sites under the same conditions as during induction.

Result:

No primary irritation or photo-irritation response in the negative controls (vehicle) 24, 48 and 72 hours after revelation.

Positive irritation response at all irradiated and non-irradiated sites in the positive controls (induction and challenge).

No sensitization or photosensitization at a concentration of 1 % JMAC.

7. Toxicokinetics (incl. Percutaneous Absorption)

Method: Penetration of human epidermis in diffusion cell (GLP)

Substance/concentration: 1 % JMAC in a handscream base.

The test was performed on the excised abdominal epidermis after autopsy, minus skin and sub-epidermic fat, whose integrity was verified. Application of approximately 5 mg/cm^2 of a cream at 1 % JMAC.

Determination of Ag in the host liquid (phosphate buffer containing 3 % albumin and 0.05 mg/ml silver nitrate) by ICP-MS (validated method) after 8, 16 and 24 hours of contact.

Result:

According to the author, permeation speed of the silver is 23.3 mg/cm^2, corresponding to a penetration of 0.31 % of the administered JMAC dose in the worst case.

8. Mutagenicity

Mutagenicity in bacteria *in vitro*

Method: Ames test (GLP).
Sam. typh. TA98, TA100, TA1535, TA1537, TA1538 with and without metabolic activation.

Result:
After preliminary tests to determine the non-toxic doses, no positive results were observed in any of the strains tested at doses of 0.33, 1.0, 3.3, 10, 20 mg/50 ml in the absence of the S-9 mixture and at doses of 3.3, 10, 33, 100 and 200 mg/50 ml in the presence of the S-9 mixture.
Positive controls (2-nitrofluorene, sodium azide and ICR-191 in the absence of S-9, 2-amino-anthracene in the presence of S-9).

Clastogenicity in mammalian cells *in vitro*

Method: OECD 473.
Metaphase analysis in CHO cells with and without metabolic activation.

Results:
After two preliminary tests, no clastogenic effect was observed in the ovary cells of the Chinese hamster at doses of 1, 3 and 5 mg/ml with or without metabolic activation.
Positive controls: Mitomycin in absence of S-9, cyclophosphamide in presence of S-9.

Mutagenicity in bacteria *in vitro*

Method: OECD 45 (1992).
Salm. typh. TA100, TA1535, TA98, TA 1537, with and without metabolic activation.
Esch. Coli WP2 uvr A with and without metabolic activation.

Result:

JMAC is cytotoxic for all tested strains with or without S9, with inconsistant results attributed to silver erratic release from the test material, during the incubation period.

(Following cytotoxicity doses are noted in the finding range test and in the main test:

- Without S9: > 50 to > 150 µg/plate in TA100, TA98, WP2 uvr A and TA1537
  > 150 µg/plate in TA 1535
- With S9: > 500 to > 1500 µg/plate in TA100, TA98, WP2 uvr A and TA1537
  > 50 to > 1500 µg/plate in TA1535.)

In the main study (7 doses, triplicate) JMAC does not induce increase of the number of revertant colonies, in any of the tested strains at the following (non cytotoxic) doses:

- 0.5 - 1.5 - 5- 15 - 50 µg/plate without S9 in all tested strains,
- 5 - 15 - 50 - 150 - 500 µg/plate with S9 in TA 1537,
- 1.5 - 5 - 15 - 50 - 150 - 500 µg/plate with S9 in the other strains.

Positive controls: N-ethyl-N'-nitro-N-nitrosoguanidine, 9 aminoacridine, 4 nitroquinoline-1-oxyde without S9, and 2 amino-anthracène with S9.

Clastogenicity in mammalian cells *in vitro*

Method: Metaphase analysis on CHO cells with and without metabolic activation (GLP).

Result:
Two cytogenic tests are performed at doses inducing a 50 % reduction of mitotic index.
Excepted cultures dosed at 50 µg/ml with S9 in the first test (showing the highest frequency of aberrant cells with a statistic signification at 50 % non confirmed in the second test), there is no statistically significant increase of aberrant cells frequency at the following doses:

- 1 - 5 and 10 µg/ml (1st test) and 5 - 7 - 10 and 12 µg/ml (2nd test) without S9
- 40 and 45 µg/ml (1st test) and 40 - 45 - 47 and 50 µg/ml (2nd test) with S9.

Positive controls: mitomycin C without S9, cyclophosphamide with S9.

Mutagenicity on mammalian cells *in vitro*

Method: OECD 476. Mutation in mouse lymphoma cells TK +/- locus with and without metabolic activation.

Result:
With and without S9, JMAC shows a marked cytotoxicity inducing cell viability reduction on day 0, confirmed on day 2 at 15, 20 and 30 µg/ml without S9 and 30 µg/ml with S9.

In two tests with respective doses of:
- 0.31 to 30 µg/ml (8 doses) with and without metabolic activation (first test),
- 1.25 to 20 µg/ml (6 doses) with S9 (second test).

JMAC does not induce increase of mutant frequency in L5178Y TK +/- locus cells (at no cytotoxic doses).

N.B.: A small significative increase of mutant frequency at 10 µg/ml without S9, is observed in the 2nd test but not in the 1st test and there is no dose relationship. The biological significance of this result is not clearly defined.

Positive controls: ethylmethanesulfonate without S9 and cytophosphamide with S9.

11. Conclusions

According to the 18th Adaptation of Directive 67/548/EEC (93/21/EEC) JMAC is not harmful if swallowed in the acute toxicity studies. It is well tolerated by the skin and the mucosa even when administered undiluted. It does not cause allergic sensitization in animals in the Magnusson and Kligman maximization test and in a repeated topical application test (Buehler). Likewise, in a study on volunteers, no significant differences are observed between the repeated application - RIPT in accordance with Shelansky - of the test material at 1 % in a cream base and application of the cream as a control.

JMAC does not lead to photosensitization in the guinea pig in a test conducted at a 1 % concentration for induction and for revelation.

With or without metabolic activation, JMAC does not induce genetic mutation in 2 Ames tests, in Escherichia Coli, and in lymphoma mouse cells, nor chromosomial aberrations in CHO cells.

In the subchronic toxicity study (oral route), a convergence of symptoms recorded after 28 days of treatment at a dose of 1500 mg/kg - notably the slow-down in weight growth, increases in blood phosphatase, blood alanine and aspartate aminotransferases, and fluctuations in blood sugar - points to a low-level toxic syndrome, affecting the blood, with no anatomopathological lesions after 28 days treatment. Microscopic metallic deposit in the gastro-intestinal at 1500 mg/kg/d are consistent with a local irritation.

Alteration of the serum parameters observed at the 750 mg/kg dose suggests a dose effect, with males being more sensitive.

300 mg/kg/d was a marginal effect level.

Cutaneous penetration measured *in vitro* via the human epidermis is 0.3 % of the administered dose in the worst case.

Classification: A

Since risk of argyria cannot be excluded JMAC should not be used where there is a possibility of accumulation (e.g. baby products, oral preparations, eye and lip cosmetics).

12. Safety evaluation

Daily use in cosmetics	27.6 g/d
Average body weight	60 kg
Percutaneous absorption	0.3 %
Maximum concentration	0.02 %
Systemic exposure =	0.000276 mg/kg

Taking a no adverse effect dose of 300 mg/kg/j, one obtains

$$\text{safety factor} = \frac{300}{0.000276} = 1\,087\,000.$$

Applying an uncertainty factor of 10 (or 100) - since the results are taken from a very short term study (28 days) - we obtain respectively

$$\text{safety factor} = \frac{300}{10 \times 0.000276} = 108\,700$$

$$\text{(or safety factor} = \frac{300}{100 \times 0.000276} = 10\,870\text{).}$$

S 13: *2-ETHYLHEXYLSALICYLATE*

1. General

1.1 Primary name

2-ethylhexylsalicylate

1.2 Chemical names

2-ethylhexylsalicylate

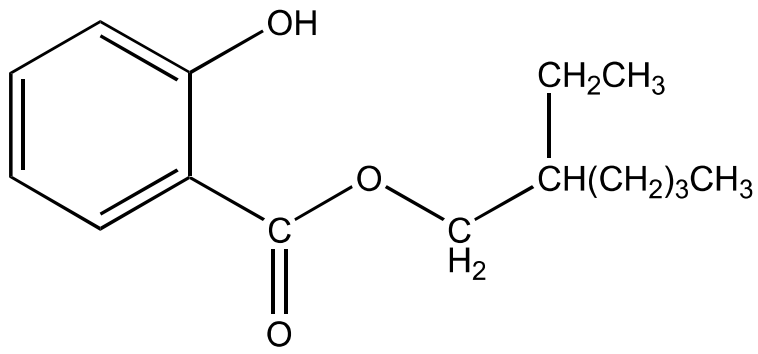
1.3 Trade names and abbreviations

Sunarome

1.4 CAS no.

118-60-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{22}O_3$
Mol weight: 250.33

1.8 Physical properties

Appearance: clear odourless liquid
Density: 1.013 to 1.022
Absorbance maximum at 306.8 nm

1.9 Solubility

Immiscible with water; miscible with ethanol, mineral oil, and other organic solvents.

The compound dissociates only slightly at pH values greater than 8 (about 0.5 %) and not at all below that value.

2. Function and uses

Proposed use level up to 5 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. Oral. Following range finding studies, 5 male and 5 females were used. The LD_{50} was estimated at 4.8 +/- 0.3 gms/kg b.w.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study (diet).

The study was carried out according to GLP. Groups of 10 male and 10 female animals were used, except that the control group and the top dose group contained 20 male and 20 female animals. In these larger groups, 10 animals per sex per group formed recovery groups, which were maintained without the active ingredient for 4 weeks. During the test, animals were caged separately. The doses chosen were 0, 50, 100, and 250 mg/kg b.w./day. Tests for homogeneity and stability of the active ingredient in the diet were carried out, and the concentration of active ingredient in the diet was estimated at weeks 1, 2, 3, 4, 8 and 12. The results showed that the target concentrations were not quite attained (92 % to 99 %). The average consumption of active ingredient by the animals was very close to the amount laid down by the protocol.

Animals were observed twice daily, and subjected to detailed examination once a week. Body weight, water consumption and food consumption were measured once a week. Haematological, biochemical and urinary variables were measured at the end of the treatment period or at the end of the recovery period, as appropriate.

Ophtalmoscopy was carried out before the experiment began and again before sacrifice.

All animals of the test groups were subjected to necropsy, as well as animals of the control and top dose groups of the recovery animals. All major organs of the animals subjected to necropsy were inspected and weighed; histological preparations of a large number of tissues were made, and those specified by the protocol were subjected to histological examination (control and top dose animals only in the recovery animals), in addition to sections from any organ found to be macroscopically abnormal.

Results. No animal died. There were no abnormalities on clinical examinations. No differences were found in body weights, or in body weight gain, between control and test animals. Food consumption was not affected. Water consumption during the test was increased in males at the top dose and in females at the intermediate and top doses; in the recovery animals, this increase

was also found at the top dose, more marked in females than in males. The increases varied from 6 % to 13 %. The authors state that the biological significance of this is uncertain.

Haematological examination showed some variation in the values examined, but these were not dose related or uniform in the directions of the changes, and were not regarded as biologically significant.

Biochemical estimations (including liver enzymes) showed some variations, but the changes were not dose related and not uniform in the direction of the changes, and were regarded as not of biological significance.

There were some differences in the measurements of urinary changes, but these were inconstant and not dose related, and so were regarded as not of biological significance.

All animals sacrificed at 13 weeks were subjected to necropsy, and animals of the control and high dose groups in the case of the recovery animals. No significant gross lesions were found. Organ weights were generally unaffected, but certain changes were found.

In males sacrificed at week 13, there was a general tendency for the absolute and relative weights of the thyroid/parathyroid glands to be reduced (the changes varied from - 11 % to - 34 %). These changes reached significance in a few cases, but there was no dose relationship, and histological examination was normal. The brain weights were increased significantly (6 % and 9 %) at the intermediate and top doses; the relative weights (brain/body) also showed an increase, but the increases did not reach significance. There was a tendency for the kidney weights in this group of animals to increase also, but the changes did not reach significance. In males of the recovery group, reductions in thyroid/parathyroid weights were found to a less extent (- 3 % to - 7 %) than in the animals sacrificed at 13 weeks; brain and kidney weights were normal.

In females sacrificed at 13 weeks, there was an absolute and relative increase in kidney weights in all dosed animals, but these never reached significance, and were not dose related. The absolute and relative weights of the thyroid/parathyroid glands were reduced in all dosed animals, but these changes never reached significance and were not dose related. Brain weights were not affected. In recovery animals, there was no effect on kidney weights, but again the thyroid/parathyroid weights were reduced, though not significantly (- 5 % to - 8 %). The authors state that, as histological examinations of these organs were normal, and there was no dose relationship, these findings were not of biological significance.

Histological examinations revealed no abnormalities.

The investigation seems to have been carefully carried out, to a satisfactory protocol; no NOAEL was determined, but might be greater than 250 mg/kg b.w./day.

3.8 Subchronic dermal toxicity

Rat. Dermal. A 13 week study was carried out in 40 male and 40 female rats. The active ingredient was applied as an 18.5 % ethanolic solution. The doses were 0, 55.5, 277 and 555 mg/kg b.w./day. These doses were chosen in the light of a survey showing average human use to be 4.1 mg/kg b.w./day, so that the doses used were 14, 68 and 135 times higher. The solutions were applied daily, 5 days a week, to the shaved skin of the back. At the end of the

experiment, all animals were sacrificed and subjected to necropsy. Various observations and analyses were carried out during the experiment.

All animals survived. There was clinical evidence of skin irritation in all animals, particularly at the intermediate and top doses. Body weight and body weight gain were reduced in males at the intermediate and top doses; in females, this occurred at the top dose only.

Blood was taken for examination at weeks 7 and 13. Haematological values showed changes, some statistically significant, but having regard to dose relationship and the variable direction of the changes, only an increase in the neutrophil/lymphocyte ratio seemed biologically significant. This change was found at week 7 in males and females at the top dose and in males of the intermediate dose; at week 13, the change was found in top dose males only. These changes may have been related to inflammatory changes in the skin. Biochemical estimations showed some changes. Glucose levels were reduced in all dosed animals at weeks 7 and 13; this may have been due to poor feeding because of the irritation of the skin. In males, SGPT was increased in males at weeks 7 and 13 at the intermediate and top doses; in females, this effect occurred only in week 7 at the top dose. In both males and females, the SAP was increased at the intermediate dose in week 7, and at the intermediate and high doses at week 13. There was some proteinuria, but it was not dose related, and was absent in some treated animals.

Necropsy: no significant dose related changes were found on macroscopic examination.

Organ weights. At the low dose, in males the relative weight of the lungs was increased; in females, the absolute and relative weights of the kidneys and spleen were increased, and the absolute weight of the heart. At the intermediate dose, in males the absolute weight of the liver was decreased, but the relative weights of brain, lung, kidney and testis were increased; in females, the absolute kidney weights and the relative weights of the brain, heart, liver, kidney and uterus were increased.

Histological examination showed no evidence of dose related changes except for the skin in the area of application. There, the low dose was associated with slight hyperkeratosis; the intermediate dose with rather more marked hyperkeratosis and occasional inflammatory foci; and at the high dose, hyperkeratosis was more severe, and inflammatory changes more common, than at the lower doses.

The study seems to have been well conducted. Apart from local irritation, the NOAEL may be 55.5 mg/kg b.w./day; if the changes in liver enzymes are thought not to be biologically significant, since the histological appearances of the liver were normal, it might be set higher.

4. Irritation & corrosivity

4.1 Irritation (skin)

Test for irritant and sensitizing effects on the skin.

Man. Ten subjects were tested by applying a 5 % dilution in mineral oil to the skin, under occlusion, for 24 hours. Reading was at removal and 72 hours after removal. After 7 days rest,

the procedure was repeated (whether at the same site or at a different one is not stated). No reaction was found to any application.

Man: Ten male and 15 female subjects were tested, using 0.5 ml of active ingredient (possibly undiluted, but more probably a 5 % formulation; the report is not clear) under occlusive patches on the skin of the forearm. The first patch was allowed to remain for 48 hours. After a day's rest, a patch was applied for 24 hours, and this was repeated (probably every second day) until 10 such patches had been applied. The same site was used for each application. Readings were made at each removal of a patch. After a rest period of 10 to 14 days, the 48 hour exposure was repeated. No reaction was found at any time.

4.2 Irritation (mucous membranes)

Test for capacity to injure mucous membranes.

Rabbit: Nine animals were used in groups of three. The first group had no rinsing, the second group had rinsing after 2 seconds and the third rinsing after four seconds. It is not stated what dilution of active ingredient was used; it may have been used undiluted. The mean scores of the groups were: group 1: 6; group 2: 0.7; group 3: 1.3. Conjunctival changes only were found. According to the protocol employed, the test was regarded as negative.

5. Sensitization

Test for capacity to produce photocontact allergy

Man. Tests were carried out on 2 male and 23 female subjects. The test material was hydrophilic ointment USP containing 15 % of active ingredient. Occlusive patches containing 10 µl/cm^2 of the formulation were applied to sites on the back with occlusion for 24 hours. The area treated is not stated. After removal of the patches, the sites were exposed to 3 MED of SSR from a xenon arc. This sequence was repeated after 48 hours and thereafter twice weekly for 3 weeks, to the same site. After a 10 day rest, 5 µl/cm^2 was applied to a new site and occluded for 24 hours. The site was then irradiated with 4 J/cm^2 of UVA. An irradiated area of untreated skin was used as a control. Reading was at 48 and 72 hours after irradiation. No reactions were seen.

7. Toxicokinetics (incl. Percutaneous Absorption)

Study of percutaneous absorption.

Human skin *in vitro*. Female abdominal skin obtained at autopsy was maintained at -20° until prepared. The experiments were carried out according to GLP. Preparation was by first removing subcutaneous fat; under water, the stratum corneum and epidermis were removed from the dermis, and the sheets thus prepared were kept flat, at -20°, until used. Between 4 and 6 donors were used for each experiment.

The experiments were carried out using Franz Cells; the exposed surface of epidermis used varied somewhat between cells, but this was allowed for by measuring the actual area exposed in each case and applying a correction.

All applications contained ^{3}H sucrose to control the integrity of the membranes. The applications used were as follows:

(a) 5 % of active ingredient, labelled with ^{14}C, formulated in an o/w emulsion, and containing trace amounts of ^{3}H sucrose;
(b) the same, except that the active ingredient was made up in a hydro-alcoholic formulation;
(c) 2.7 % ^{14}C salicylic acid with trace amounts of ^{3}H sucrose in an o/w formulation;
(d) ^{3}H sucrose in o/w formulation;
(e) ^{3}H sucrose in hydro-alcoholic formulation. (The concentration of sucrose is not given, but is stated to be "trace"; it appears to have been in the low nanogram range.)

In the case of (a) above, applications were made at either 5 mg formulation/cm^2 ("finite") or 100 mg/cm^2 ("infinite"); in the case of (b), 5 µl/cm^2 or 100 µl/cm^2; in the case of (c) "finite" applications only, and in the case of (d) and (e) "infinite" applications only.

The receptor fluid was phosphate saline buffered at pH 7.4, with 6 % "Volpo N20" (polyoxyethylene oleyl ether). The solubility of the active ingredient in this medium was shown to be adequate. The cells were maintained in a water bath at 37°, giving a temperature at the skin of 32°. Samples of receptor fluid (200 µl) were taken at (hours from beginning of the experiment) 2, 4, 8, 20, 24, 30, 44, 48. At the end of the experiment, for all samples except the "infinite" hydro-alcoholic one, the skin was rinsed 3 times with receptor fluid, and the rinsings counted; then the skin was solubilised, and counted. In the case of the infinite hydro-alcoholic formulation, since this formulation was not a liquid at room temperature, an extraction in ethanol was used to obtain the samples. Replicates: 6 experiments were carried out in the sucrose-only tests; 12 experiments were carried out in each of the other tests, except that in the case of the "infinite" application of active ingredient + sucrose in hydro-alcoholic formulation, the experiments were carried out in 11 chambers only, as the amount of formulation available was insufficient.

The results were as follows:

(a) the integrity of the membranes was satisfactory throughout.
(b) "Finite" in o/w: percentage permeated 0.65 +/- 0.16; recovery in washings 36.66 %, skin 17.18 %. "Infinite" in o/w: percentage permeated 0.47 +/- 0.22; recovery in washings 34.13 %, skin 11.49 %.
(c) "Finite" in hydro-alcoholic vehicle: percentage permeated 0.59 +/- 0.09; recovery washings 36.21 %; recovery skin 32.77 %.
(d) Because total recoveries of radioactivity were unsatisfactory in respect of the active ingredient (52.28 +/- 3.8 %), the technique used for washing in the experiment involving "infinite" application of active ingredient in a hydro-alcoholic formulation was altered to include washing the cap of the donor chamber, and other modifications (not specified). This gave an overall recovery of 82.93 %. The authors argue that this involves only the input side of the experiment, and does not affect the amount permeating. This seems reasonable. The results of this experiment were: total permeating 0.23 +/- 0.05 %; recovery washings 65.28 %; skin 17.92 %.

In sum, the percutaneous penetration of the active ingredient of this experiment seems low, and the differences between the various formulations and amounts applied may represent little

more than experimental variations. It is possible that most of the radioactivity permeates as salicylic acid. A fair amount of the active ingredient is found in the skin, suggesting a reservoir effect. It may be reasonable, provisionally, from these experiments, to take about 0.5 % as the overall percutaneous absorption.

8. Mutagenicity

A standard Ames test and a test using *Saccharomyces cerevisiae* were carried out, with and without activation, over a range of concentrations of 0.001 to 5 µl per plate. Owing to poor reproduction in the microfiche, the tables could not be fully read. The authors state, however, that the test showed no evidence of mutagenicity.

A test for the production of chromosomal aberrations *in vitro* was carried out according to GLP, using a culture of Chinese hamster ovary cells. Activation was by means of an "Aroclor" induced S9 mix from rat liver. The solvent was DMSO. Without activation, cells were exposed to the active ingredient for 18 or 42 hours, after which the cultures were refed for 2 hours before harvest. With activation the organisms were exposed to active ingredient and S9 mix for 4 hours; the cultures were then refed and incubated for 14 to 38 hours.

Relative cloning efficiency (number of colonies in test/ number of colonies in control x 100) at various concentrations of a.i. was estimated in preliminary experiments. Without activation, the relative cloning efficiency was reduced to 1 % at 40 µg/ml with 20 hour harvesting, and to less than 1 % at 20 µg/ml with 44 hour harvesting. With activation, 50 µg/ml produced relatively little reduction in relative cloning efficiency at 100 µg/ml with 20 hour harvesting; the relative cloning efficiency was reduced to 2 % at 100 µg/ml with 44 hour harvesting.

Following preliminary range finding studies, the following concentrations were tested (µg/ml) in the definitive experiment: without activation, 2.5, 5, 10, 20; positive control triethylene-melamine; with activation, 6.3, 12.5, 25, 50, 100; positive control cyclophosphamide. Harvesting was at 20 and 44 hours. The studies were carried out in duplicate, and 200 cells in all were examined whenever practicable.

There was no evidence of production of chromosomal aberrations by the active ingredient; the positive controls produced marked effects. The report is somewhat difficult to interpret.

10. Special investigations

Test for capacity to produce phototoxicity.

Man. Ten subjects were tested. A 5 % solution of active ingredient in ethanol was applied for 1 hour on stripped skin and for 24 hours on unstripped skin, followed in each case by exposure to ultraviolet radiation, 320 to 410 nm. Over 24 hours there was no adverse effect on the skin. Positive control sites, treated with 3 % demethylchlortetracycline, showed positive reactions.

11. Conclusions

Acute toxicity is low. Subchronic toxicity studies show a NOAEL of 250 mg/kg b.w./day or above. The compound does not appear to be irritant to the mucous membranes, but the concentration used for the test is not clear. Tests in man for skin irritation, phototoxicity and

photoallergy were negative. A study of percutaneous absorption, using human skin *in vitro*, showed an absorption of about 0.5 % of applied active ingredient. An Ames test, and a chromosomal aberration test *in vitro* using CHO cells, were negative.

Tests for photomutagenicity have not been carried out. The results of these tests should be presented within 12 months.

Classification: B

S 46: *UROCANIC ACID*

1. General

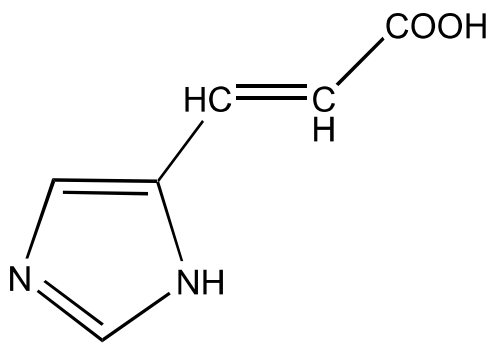
1.1 Primary name

Urocanic acid.

1.2 Chemical names

Urocanic acid.
4-imidazole acrylic acid.
(Note that Colipa submissions include (variously) the ethyl ester and the ethyl ether of urocanic acid as active ingredients.)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_6H_6N_2O_2$
Mol weight: 138.2

1.8 Physical properties

The compound absorbs maximally at 268 nm *in vitro*. However, when it is applied to the skin, reflection spectrophometry shows a bathochromic shift of the absorption maximum to 310 nm. The same change can be shown to occur when the compound is applied to silk, and it is suffested that the shift may be due to protein binding of the urocanic acid.

1.9 Solubility

Poorly soluble in water; insoluble in alcohol, ether.

2. Function and uses

Earlier authorised for use as a sunscreen at concentrations up to 2 %, expressed as acid.

This substance occurs naturally in the skin and cornea. It is said to represent about 0.7 % of the dry weight of negro skin, and about 0.2 % of the dry weight of white skin. It is produced by deamination of histidine, and is metabolised to formiminoglutamic acid, which is excreted. In the skin, however, it is converted to the *cis*-isomer under the influence of UV radiation, in which form it is not metabolised. The amount found in the skin increases with increasing exposure to UV radiation. In one investigation, it was found that in white subjects, there was a progressive increase in the skin content of urocanic acid in summer, reaching a maximum in autumn, and a decrease in winter. These investigators also found a reduction in the skin content of urocanic acid in subjects suffering from atopic dermatitis, nickel contact allergy, and psoriasis. They believe that these findings support the view that urocanic acid is an endogenous protection agent in human skin.

The use of urocanic acid as a sunscreen was proposed by Zenisek and Kral.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The LD_{50} is reported to be greater than 3 gm/kg b.w. intraperitoneally. No further details are given.

3.2 Acute dermal toxicity

Guinea pig. In a preliminary screen for the production of primary irritation, 4 animals were shaved and 0.1 ml of an emulsion (nature not specified) applied to (presumably) 5 areas of the skin. The concentrations of *trans*-urocanic acid used were (%): 0, 0.02, 0.2, 0.5, 1 & 2. No irritation was produced, and 2 % was taken to be the maximum non-irritating concentration (although no concentration high enough to produce irritation had been used).

In the main test, 3 groups of female animals were used: 10 test, 10 vehicle controls, and 5 positive controls. The skin of the dorsum was chemically depilated and then stripped with tape. Areas of 2 x 2 cm were delineated, and 0.1 ml of the test solution or of the vehicle were applied to each site. The positive control was an ethanolic solution of 0.01 % 8-methoxypsoralen. After this, one of the sites, and an area of the dorsum, were protected by aluminium foil, and the remainder of the dorsum irradiated for 3 hours with UVA at 1.12×10^8 ergs/cm^2. The spectral range of the light source was 320 to 400 nm, and the dose was monitored by a UV meter. Reading was at 24 and 48 hours, and 7 days. A Draize scoring system was used.

There was no primary irritation of the skin. There was no evidence of phototoxicity in any of the test animals, or in the vehicle control animals; nor was there any evidence of clinical abnormality or weight loss. The positive control animals showed effects on the skin in all animals at 24 hours, and at 4/5 animals at 48 hours and 7 days (mean scores, 2.2, 2.0 and 2.4 respectively).

4. Irritation & corrosivity

4.1 Irritation (skin)

An ointment containing an unspecified concentration of a.i. was applied daily to the abdominal skin of guinea pigs for 14 days. No irritation was produced.

A similar preparation was applied to the abdominal skin of 10 rabbits and 6 guinea pigs, daily for 21 days. No abnormality was produced.

4.2 Irritation (mucous membranes)

A solution of 10 % was made up in water with 1 % triethanolamine as a solubiliser. This was placed in (presumably) rabbit eye, without rinsing. No further details are given. No adverse effect was produced.

5. Sensitization

Test for capacity to produce photosensitization.

Guinea pig. Female animals of the Dunkin-Hartley strain were used. The a.i. used was *trans*-urocanic acid.

(i) A primary skin irritation screen was carried out. It is stated that 4 sites were prepared on the skin of 2 animals; however, 5 concentrations of a.i. were used in an emulsion at concentrations (%): 0.02, 0.2, 0.5, 1 and 2. Reading was at 1, 2 and 24 hours. No abnormality was seen at the sites of application or in clinical appearances. The maximum non-irritant concentration was taken to be 2 %.

(ii) Primary phototoxicity screen. Six animals had the same concentrations of an emulsion of a.i. applied to 2 sites on the dorsum. On the left side, the areas were exposed to 30 J/cm^2 of UVA (70 minutes). The right side was shielded. Reading was at 1, 2 and 24 hours. No abnormal local or clinical changes were seen. A concentration of 2 % was taken to be the maximum non-phototoxic concentration.

(iii) For the main test, 3 groups each of 10 female animals were used. Animals of the first group were treated with an emulsion of 2 % a.i.; of the second group with vehicle only, and of the third group with the positive control substance, 5 % 6-methylcoumarin. The sequence was as follows:

Day 1: a. Four injections of 0.1 ml emulsified FCA in the nuchal region;
b. Skin stripped with tape;
c. 0.1 ml of test (or control) sample applied;
d. UV irradiation at a dose of 10 J/cm^2 for about 24 minutes. The flux was subject to monitoring to ensure a correct dose. Reading was at 24 hours.

Days 2 to 5: Procedure of day 1 repeated, with reading at about 24 hours after the applications.

Day 19: Dorsum stripped in all animals, and 0.1 ml of the appropriate solution applied to both sides. The right side was shielded, and the left side irradiated with UV as before. Reading was at 24, 48 and 72 hours.

A numerical scoring system was used. There was no reaction in the irradiated or non-irradiated animals except for a slight reaction in one non-irradiated animal at 48 hours. The positive control animals subjected to irradiation were all positive; 7/10 had eschar formation at 72 hours. The mean scores at the readings were, respectively, 2.4, 1.9, 1.6. The non-irradiated animals also showed some reaction, with mean scores of 0.2, 0.3 and 0.4; 1 animal at 72 hours had eschar formation.

The test was regarded as negative.

7. Toxicokinetics (incl. Percutaneous Absorption)

Human and hairless mouse skin *in vitro*.

A lotion of unspecified composition was used; it contained 0.2 % of a.i. Mouse skins were divided into two parts, and the experiments carried out in duplicate; the human skins, obtained in a frozen state, were divided into either 3 or 6 replicates, according to size. The skins were mounted in a Franz chamber of area 1 cm^2; the receptor fluid was distilled water and the epidermal surface was exposed to air. The integrity of each skin specimen was first assured by studying the permeation of ^3H_2O; less than 2 microlitres/cm^2/30 minutes was acceptable. The receptor fluid was supposed to be sampled at 2, 4, 8 and 24 hours; the graphical results suggest that the sampling intervals were 1, 3, 6 and 16 hours.

Lotion was applied at the beginning of the experiment, and the amount applied determined by difference. At the end of the period of exposure, the surface of the skin was washed twice with isopropanol, and the skin separated into dermis and epidermis with a scalpel. Estimation of the a.i. was by HPLC.

In the first series of experiments, 6 mouse skins were used, each divided into 2 parts, and 3 specimens of human skin, the first divided into 6 replicates, and each of the others into 3 replicates. About 10 mg of lotion was applied to each skin. From the results, the following values may be derived.

(a) In the mouse skin, the mean net amount applied (amount applied less amount recovered in washings at the end of the experiment) may be calculated to be 13.577 (all figures are in µg/cm^2). The amounts found in the receptor fluid over the period of the experiment totalled 14.195, and the amounts in the epidermis + dermis + receptor fluid amounted to 31.083.

(b) In human skin, the mean net amount applied was 4.04, and the amounts in the receptor fluid 2.87; the total in epidermis + dermis + receptor fluid amounted to 22.22.

It is clear, therefore, that there was probably a substantial endogenous contribution to the amounts of a.i. found.

In a second set of experiments, 2 samples were obtained from each of 3 mouse skins, and half of them were treated and the other half not. The skin from 1 human donor was divided into 6 parts, and one half of these treated and one half not. Unfortunately, the net amounts applied are not given for this experiment.

(a) In the mouse skins to which a.i. had been applied, the (mean) amount found in the receptor fluid was 14.59, and in the epidermis + dermis + receptor fluid 24.24. In those skins to which no a.i. had been applied, the corresponding figures were 5.58 and 18.50.
(b) In the human skin, when a.i. had been applied, the figure for the amount in the receptor fluid was 3.13, and in the epidermis + dermis + receptor fluid was 11.14. When no application of a.i. had been made, the corresponding figures were 3.62 and 9.66.

The time course of the appearance of the a.i. in the receptor fluid is given in the form of graphs. From these, the following figures may be derived.

(1) *First experiment* (a.i. applied to all specimens):
(a) Mouse skins. A maximum of 0.65 µg/cm^2/hr was found at 3 hours. This fell to about 0.1 at 6 hours, and then rose slowly to 0.3 at 16 hours.
(b) Human skins: Maximum at 1 hour to 0.22, followed by a slow fall to near zero at 16 hours.

(2) *Second experiment* (a.i. applied in half of the tests):
(a) Mouse. Following application of a.i., there was a peak of about 0.4 at 1 hour, followed by a fall to about 0.25, after which the level remained more or less constant up to 16 hours. This is somewhat different from the findings in the first experiment, but the number of experiments is smaller.

In the absence of any application, the initial rise to 0.35 is about the same, after which the level falls to near zero at 6 hours, and stays there.

(b) In human skin, the pattern is about the same whether or not an application has been made. There is a peak of about 0.45 at 1 hour, followed by a fall to near zero at 4 hours, which remains up to 16 hours. This is again somewhat different from the pattern in the first experiment.

It may be concluded that in mouse skin about half the a.i. found in the receptor fluid and in the skin is endogenous; in experiments with human skin, perhaps 75 % of the amount in the skin is endogenous, and all the amount found in the receptor fluid. From these experiments, it may perhaps be supposed that percutaneous absorption of the a.i. in man would be small. It should be noted that there seems to have been no attempt to estimate the isomers separately in these experiments.

Hairless mouse *in vitro*. Urocanic acid (chiral status not specified) was made up in an o/w emulsion at concentrations of a.i. of 2%, 0.2 % and 0.02 %. Skins were mounted in Franz cells and treated with 2 mg/cm^2 of the formulations in duplicate. There were 2 untreated cells as controls. The receptor fluid was water; 2 ml of this was collected at 4 hours and replaced with fresh water; the receiving fluid was then collected *in toto* at 24 hours. At this time the exposed surface of the skin was wiped and the skin stripped 10 times; the strippings were analysed for a.i. in groups of 5. The skin was then homogenised and the content of a.i. determined. Analyses were by HPLC.

At 4 hours, the amounts in the reservoir (µg/ml) were: 0, 0.276, 0.21, 0.188 (control, 2 %, 0.2%, 0.02 %, respectively). At 24 hours, the corresponding values were 0.785, 1.154, 0.604 and 0.781. Strippings 1 to 5 yielded (µg/cm^2) 0.2718, 17.355, 1.550, 0.5805; strippings 6 to 10 yielded 0.6058, 1.9545, 0.9945 and 0.5755. In the skin homogenates, no a.i. was found; the authors term these samples “subepidermal murine skin”. In summary, it may perhaps be

concluded that the amount in the reservoir with the 0.02 % concentration derives from endogenous sources; with higher concentrations, absorption may be proportional to concentration of a.i. The strippings seem to show that the amount in the stratum corneum of endogenous origin is about 0.44 μg/cm^2; the values increase with increasing concentration of a.i. The failure to find any a.i. in the skin following stripping is puzzling.

Human skin *in vitro*. Full thickness human cadaveric abdominal skin was stripped of muscle and fat and mounted in a Franz cell of diameter 1 cm^2. The a.i. was prepared as 3 concentrations of the potassium salt in an o/w emulsion; the concentrations of a.i. were 2 %, 0.2 % and 0.02 %, probably expressed in terms of acid. The receptor fluid was water; the epidermal surface was exposed to the ambient environment in the laboratory, but kept moist a wick. Each concentration was tested in duplicate, and two untreated preparations were set up as controls. Sampling was at 4 hours and 24 hours. At the end of the experiment the epidermal surface was wiped. The skin was stripped with adhesive tape 20 times; the content of a.i. in the strips was estimated in groups of 5 strips. The epidermis was then separated from the dermis by immersion in sodium bromide solution at 40° for 90 minutes. The experiments were repeated twice, and the results pooled. Estimation of the a.i. was by HPLC.

Results: (a) No a.i. was found in the receptor fluid at any time (less than 0.1 μg/ml). There was no significant difference between the strippings in the treated and untreated groups: the mean figures were (μg/cm^2): 2 %, 7.51; 0.2 %, 6.07; 0.02 % 4.52; control 4.67. On inspection of the individual figures there did not seem to be any definite gradient of concentrations with number of strippings.

In the dermis and epidermis, there was no detectable level of a.i. (less than 0.1 μg/cm^2).

In this investigation, there does not seem to have been any attempt to separate the *cis*- and trans-isomers. In contradistinction to a previous investigation there was no evidence of production of endogenous a.i.

Man. A study to determine the amount of deposition of a.i. in the stratum corneum was carried out in 30 healthy female volunteers. Groups of 15 subjects were randomly allotted to apply 1 gram of o/w emulsions of a.i. twice daily to the volar surface of both forearms for 16 weeks. The preparations applied by members of each group were identical except that one preparation contained 0.2 % of a.i. and the other 1 %. The upper arms of each subject served as controls. A template 3.61 cm^2 in area was used to delineate skin areas which were stripped 20 times, using adhesive tape, at the following time intervals: before treatment, and at weeks 1, 2, 4, 8, 12 and 16 after beginning treatment. For the first 2 samples, the amounts of a.i. in the strips were estimated in groups of 5 strips, but thereafter all the strips from a given area were analysed together. Estimation of a.i. was carried out in a blinded fashion using HPLC. It is mentioned that *trans*-urocanic acid was among the reagents used for the HPLC, but the ciral status of the a.i. used in the emulsion is not specified.

There was no significant difference in the amount of urocanic acid between test sites and control sites at any sampling, with the exception of 2 of the sampling times using the 1 % emulsion, and in these cases, the difference between the control and the test areas showed a greater amount of urocanic acid in the control strippings than in the strippings from the sites of application. In general, with a few exceptions, the amount of urocanic acid was greater in the

control than in the test strippings. The authors conclude that under the conditions of the experiment there was no deposition of a.i. in the stratum corneum.

Man. An investigation was carried out to determine the levels of urocanic acid in human skin.

(a) Samples of "stratum corneum/callus" (presumably thickened stratum corneum) were obtained from 10 volunteers and the amount of urocanic acid in each estimated by HPLC.

(b) Five cadaver skins were used. These were stripped 20 times using adhesive tape over an area of 3.24 cm^2. This procedure was carried out in triplicate in each skin. The strippings were extracted in groups of 5 successive strips, and the amounts of urocanic acid estimated by HPLC.

Results: The mean amount of urocanic acid in the samples of stratum corneum/callus was 0.263 % (w/w). The individual values were reasonably uniform (SD = 0.065). The mean amount of urocanic acid in the strippings was 6.06 µg/cm^2, but there was considerable variation in the amount from skin to skin. The range was 0.99 to 15.30; the SE is given as 2.75, which indicates a standard deviation of 6.15 and a coefficient of variation of 101.5 %.

The content of urocanic acid in human skin *in vivo* was determined as follows: A funnel containing 4 ml of ethanol/water, 1/1, was pressed firmly on the volar surface of the forearm for 2 minutes. The fluid was then analysed for urocanic acid (both isomers) and histidine by HPLC. The quantities found varied with the time of year: in healthy skin, levels of *trans*-urocanic acid were highest in winter, and levels of *cis*-urocanic acid were highest in summer. In patients with atopic skin, and in those with nickel contact hypersensitivity, the amounts of both isomers were reduced.

The reflection spectrum of urocanic acid in the skin *in vitro* was studied after treatment of the skin with an o/w emulsion containing 1 % urocanic acid. It was found that the maximum shifted from 268 nm in solution to about 310 nm in the skin; the difference was attributed to the adsorption of urocanic acid to protein.

8. Mutagenicity

A standard Ames test was carried out according to GLP; in addition, *E. coli* WP2 uvrA was also tested. The a.i. was provided as 2 powders: *Cis*- and *trans*- urocanic acid. Equal quantities of these were mixed extemporaneously and the mixture dissolved in (probably) 0.5 N sodium hydroxide. Further 1 N alkali was added drop by drop until complete dissolution of the a.i. had occurred. The stock solution so produced (presumably consisting of the sodium salts of the isomers, together with some free alkali) was then further diluted with water. Following preliminary range finding experiments, the concentrations chosen for the tests were (µg/plate): 33, 100, 333, 1000, 3333, 10 000. There was no evidence of toxicity or precipitation at these concentrations. Suitable controls were used, and activation was by "Aroclor"-induced rat liver microsomal preparations. The criteria for a positive result included the finding of at least a three fold increase in revertants in a dose related manner for strains TA1535, 1537 and 1538; for TA98 and 100, and for *E. coli* WP2 uvrA a two fold increase was required.

The study appears to have been a well conducted one. There was no evidence of any increase whatever in revertants in any experiment; the positive controls gave satisfactory responses.

In an appendix, a method for estimation of the a.i. by HPLC is given. The distinction between the *cis*- and *trans*-isomers is mentioned in the notations written on the records, but does not seem to have been taken into account in the accompanying text.

A test for chromosomal aberration *in vitro* was carried out in Chinese hamster lung cells, according to the Japanese guidelines for toxicity studies of drugs (1989). In the preparation of the stock solutions of the *cis*- and *trans*-isomers of the a.i. in physiological saline, it was noted that the *cis*-isomer was somewhat more soluble than the *trans*-isomer. The concentrations used for the test were 1.25, 2.5, 5 and 10 mmoles/l (approximately 173, 346, 691 and 1382 µg/ml). Careful initial cytotoxicity tests were carried out: these were negative up to 10 mmole with the *trans*-isomer, but positive at 5 and 10 mmole with the *cis*-isomer. This was probably due to the difference in solubility, noted above. Metabolic activation was carried out with S9 mix induced by phenobarbitone + 5,6-benzflavone. Suitable negative and positive controls were used. Incubation with the a.i. was for 2 days without activation, and with activation for 6 hours (in a 4 day culture of cells) followed by washing and a further 18 hours incubation with a.i. but without activation mix.

The experiments seem to have properly carried out. There was no evidence of chromosomal aberration at any concentration of a.i. The positive controls gave marked aberrations.

Two tests were carried out in cultures of human fibroblasts.

(a) Earlier work had shown that a combination of UV irradiation + a.i. led to the formation of thymidine-acrylic acid (and perhaps cytidine-acrylic acid) adducts in calf thymus; however, the UV fluxes used in those experiments were much too high to allow of DNA repair afterwards. It had also been shown that irradiation of the a.i. before mixing with DNA had no effect: it was necessary to irradiate the a.i. in the presence of the DNA to bring about the formation of adducts. The present experiments were designed to use a flux which would permit survival of the cells and possible DNA repair. They were carried out in accordance with the Code of Federal Regulations (USA).

Calf thymus DNA was exposed to 2 mmolar a.i. (= 276 µg/ml). A mixture of equal parts of the *cis*- and *trans*-isomers was used. Irradiation was with UVB from "an FS20 sunlamp". This equipment produced a peak output at about 313 nm; the output was measured at the culture level to ensure that 100 kJ/m^2 was administered. Untreated DNA was used as a control. Following enzymatic digestion, the DNA was end-labelled with ^{32}P-ATP and the products run on TLC; the radioactive spots were identified by autoradiography and were eluted. The results are given as follows:

Treatment	Relative adduct labelling
a.i. + UVB	9.4×10^{-9}
a.i. alone	8.9×10^{-9}
neither a.i. or radiation	2.3×10^{-9}

If the a.i. had formed a specific adduct with DNA, the migration on the tlc plate would have been expected to be different; it was not. The author does not regard the approximate 4-fold increase in RAL following a.i. as significant; it was thought due to purification and handling of the material.

(b) Unscheduled DNA synthesis (UDS) was studied. The experiments were so arranged that the cultures were either protected from radiation or exposed to UVB at 500 J/m^2. The dose of radiation was measured by a meter at the level of the culture. If a.i. was to be incorporated in the culture, the radiation was first passed through an 0.5 cm layer of 2 % a.i., with a consequent increase of exposure time to ensure the dose of UVB was standard. After irradiation, the cultures were incubated with ^{3}H-thymidine for 4 hours. After preparation of slides and autoradiography, the nuclear grains in "up to 25" lightly labelled cells were counted.

In all, 5 experiments are reported.

(i) Cultures with and without 2 % a.i. were compared; unexpectedly, the number of grains was significantly reduced (17 %) in the preparations from cultures containing the a.i.

(ii) The experiment was repeated with a.i. at 1 % and 0.1 %, and no reduction in grains was found; yet a 10 % reduction was found at 0.01 %. The author notes that although the reductions found were significant, they were small in absolute terms.

(iii) A comparison was made between cultures containing a.i., one set being irradiated and one set not. A reduction of 18 % in grain count was found in the presence of the a.i. The filter interposed in the radiation path in this case was an 0.5 cm layer of a 2 % solution of the *trans*-isomer of the a.i. A reduction of 18 % in grain count was found.

(iv) A similar experiment to (iii) using *cis*-a.i. instead of *trans*-a.i. in the filter gave a fall of 33 %.

(v) If the irradiation was filtered through *trans*-a.i. and incubation carried out with and without 2 % *trans*-a.i., there was an increase in UDS in the culture lacking a.i. This finding excluded the possibility that it was photolysis of *trans*-a.i. which produced the suppression noted in the previous experiments.

The conclusion of the author was that adduct formation and UDS did not occur under the circumstances of the experiment.

9. Carcinogenicity

Tests for effect on tumour production.

Man. The authors observe that the percentage of urocanic acid in dried skin from black African subjects is more than three times that in white skin, and that the African is exposed to more ultraviolet radiation than the Northern European. Yet the incidence of cancer induced by ultraviolet radiation is less in the African.

Hairless mouse. This is part of the paper describing the suppression of the contact hypersensitivity to oxazolone in the hairless mouse (see "Special investigations"). It is recognised that UV irradiation induces immunosuppression, and that *trans*-urocanic acid is a strong candidate for the cutaneous chromophore involved. The *trans*-isomer is isomerised by UV irradiation.

In the investigation, groups of 15 mice were subjected to irradiation of 2.7 x 10^{-4} w/cm^2 of UVB (280-315 nm) and 5.2 x 10^{-3} w/cm^2 of UVA (315-400 nm). One group had applications of an o/w emulsion containing 0.2 % *trans*-urocanic acid; a second group had emulsion only, and a

third group had the emulsion with a.i. but no irradiation. The sequence of the experiment was as follows:

Day 1: 100 µl of the emulsion (with or without the urocanic acid) was applied to the dorsal skin. Thirty minutes later, irradiation was carried out. A third group had the application of emulsion containing urocanic acid, but no irradiation. These applications were continued (probably daily) for 10 weeks.

Days 70 to 229 monitoring for tumour production.

Day 230: Application of the tumour promoter croton oil, 100 µl of a 0.1 % solution, to dorsal skin daily for 4 weeks, to reveal latent tumours. Days 258 to day 314: observation, and final classification of tumours produced.

The results show that tumours were not produced in the absence of UV irradiation. Before and after the application of croton oil, the numbers of tumour bearing mice were much the same whether or not urocanic acid had been applied. However, there was a highly significant increase in the numbers of tumours per animal in those given urocanic acid even before the applications of croton oil (1.94 times as many) and the effect was even more marked after the croton oil (3.6 times as many). In addition, the animals treated with urocanic acid showed, as well as an increase in the number of tumours, a decidedly higher incidence of malignant tumours among them, compared with the animals irradiated but treated with the emulsion without the urocanic acid. This paper also describes the study of immunosuppression by urocanic acid, which is briefly summarised above.

Since ultra-violet radiation is known to produce melanoma in certain strains of mice, and since this is associated with, and may by due to immunosuppression, an investigation was carried out in which immunosuppression due to ultra-violet radiation was investigated, and also the effect of this on the transfer of melanomatous tumours between syngeneic mice. The protocol was a very elaborate one, but may perhaps be briefly summarised as follows:

SPF C3H/HeN/cr-(MTV$^-$) mice were used.

(a) Mice were treated with an amount of ultra-violet radiation known to be sufficient to reduce the numbers of Langerhans cells and impair contact hypersensitivity. The radiation used was 5 W/cm^2 over the wavelength 280 to 320 nm (measured by a spectroradiometer). Because of screening by the cage the doserate received by the mice was 3 J/m^2/second. The mice were irradiated for 27 minutes (4.8 kJ/m^2) twice weekly for 4 weeks. The mice in this part of the experiment were not shaved, so that only the tails and ears were exposed to the full dose of radiation; one ear of each animal was protected from irradiation. Twenty-four hours after the last irradiation, K1735 melanoma cells were injected into the pinna, but in some animals, apparently, the injection was delayed until 8 days after the last irradiation. The melanoma cells had been induced in the same strain of mouse by UV irradiation, and maintained in tissue culture. If the tumour cells were injected within 24 hours of the last irradiation, the percentage tumour incidence was increased over the first 3 to 4 weeks; however, the total increase in tumours was the same in both groups after 5 weeks. A control group also gave the same percentage increase in tumour growth; it is not clear from the test what the treatment protocol for this group was: probably irradiation was omitted. If the growths of the melanoma cells had been accentuated by an effect of the ultra-violet radiation on skin immunity, it would have been

expected that the dendritic cells in those areas of skin would have been affected. While the numbers of $ATPase^{+}$, Ia^{K+} and $Thyl.2^{+}$ cells in the pinna were reduced, the time course of these reductions was not related to that of melanoma cell growth. In addition, a test for reduction of contact hypersensitivity in the unexposed pinna, using dinitrofluorobenzene induction on the irradiated pinna, was carried out. The degree of contact hypersensitivity was the same in the treated mice and in the control animals. Thus suppression of contact hypersensitivity did not seem to play a part in the growth of the melanoma cells.

(b) In this part of the experiment, mice were shaved on the ventral skin, and this area was exposed to 400 J/cm^2; the head and ears were protected. Control animals were identically treated without irradiation. This treatment was carried out on 4 consecutive days. Some of the animals were sacrificed and the exposed skin was removed for enumeration of dendritic cells; other animals were sensitised on the exposed skin with fluorescein isothiocyanate; and others were injected with melanoma cells in the irradiated area. In this part of the experiment, firstly, immune cells were reduced in the irradiated area; secondly, contact hypersensitivity induced with fluorescein isothiocyanate was considerably reduced compared with control animals; thirdly, however, the growths of the melanoma cells was unimpaired, whether measured by incidence or time of appearance of tumours.

(c) To test the effect of ultra-violet radiation on immunity to melanoma cells, fragments of such tumours were injected into one pinna and allowed to grow for 3 weeks; they were then removed by excising the ear. The mice used were treated with ultra-violet radiation (probably on the treated pinna); a control group was (probably) not irradiated, and it may be inferred that some mice were not treated with melanoma cells to induce immunity. Three weeks after removal of the pinna, mice were challenged with melanoma cells injected into the opposite pinna. There was no difference between the groups immunised with melanoma cells, although both showed less growth than controls not pretreated with melanoma cells. The results suggest that irradiation had no effect on immunity to melanoma. Overall, the authors feel that the enhancement of tumour growth by ultra-violet radiation is not due to immunological causes.

Hairless mouse. This investigation was designed to repeat “the photocarcinogenesis portion of a study in which the conclusion was reached that ultraviolet photoproducts of urocanic acid augmented ultraviolet photocarcinogenesis”.The protocol of this experiment was, however, somewhat different from that of the earlier study.

The study was carried out according to GLP guidelines of the USFDA. Five groups, each of 20 female albino hairless mice of the strain Crl SKH1 (hr/hr) BR were used (groups of a, b, c, d, e). Daily applications of 100 µl of an o/w emulsion of urocanic acid were made 5 days a week, for 10 consecutive weeks. The concentrations of a.i. used were: 0, 0.2, 2 and 20 mg/ml. Animals of group e had the same applications as those of group d, but these animals were not irradiated. Animals of groups a to d were irradiated 5 days a week, shortly after the applications of urocanic acid, as follows. In the first week, a minimal inflammatory dose was administered daily from an SSR source (W/cm^2 UVA 2.7×10^{-3}; UVB 5.4×10^{-5}). This was increased by 20 % in the second week, and similarly in subsequent weeks, so that by the tenth week the dose of ultra-violet radiation was 2.8 times greater than at first. From weeks 33 to 36 inclusive, each mouse had applied 100 µl of an acetone solution of 12-*O*-tetradecanoyl-phorbol-13-acetate 3 days a week, initially at 32 µg/ml, and increasing in subsequent weeks to 64, 128 and

256 µg/ml. Mice were examined daily; tumours were looked for and recorded weekly. Any mice dying or sacrificed before the end of the experiment were subject to full macroscopic necropsy. At the end of the experiment all remaining animals were sacrificed and subjected to necropsy.

Tumours were classified macroscopically at week 45. They were allotted to the groups: papillomas, squamous cell carcinomas, or tumours other than these. Representative tumours were sectioned and examined histologically by an independent pathologist. There was a good correlation between the clinical diagnoses and the histological ones.

Among the groups treated with ultra-violet radiation, there were no significant differences; the incidence of tumours, the intervals before their appearance, and so on, were the same. There was only one tumour bearing animal in group e (in which the animals were not irradiated). There was a suggestion from the histological evidence that the high dose of urocanic acid might have some protective effect, in that the percentage of carcinomas showing deep penetration was less in these groups than in the others.

The study was carefully carried out to a good protocol, and fully reported; it showed no evidence of a photocarcinogenic activity of urocanic acid.

In a paper by Forbes, from the same institute as that which carried out the negative carcinogenic study, there is a detailed examination of the discrepancies between the report of positive carcinogenicity by Reeves *et al* and the negative report by Sambuco *et al*. I believe his paper may fairly be summarised as follows.

(a) The incidence of tumours in the study by Reeves *et al* did not differ significantly between the two treatments (ultra-violet radiation only and ultra-violet radiation + urocanic acid). Incidence is defined as the percentage of mice bearing at least 1 tumour greater than 1 mm in diameter.

On the other hand, the tumour yield (defined as the number of tumours per mouse) was much higher in the group treated with urocanic acid and ultra-violet radiation, compared with the group treated with ultra-violet radiation only. (The figures: respectively, urocanic acid only, ultra-violet radiation only, and urocanic acid + ultra-violet radiation: all tumours, 0, 51, 141; papilloma, 0, 82.4, 61; carcinoma in situ, 0, 15.7, 30.5; squamous cell carcinoma, 0, 2, 8.5).

(b) The authors states that Reeve *et al* determine tumour yield by the total number of tumours/number of affected animals. Most workers prefer to record the total number of tumours/all surviving animals. The former method gives higher values than the latter, since in the latter the denominator must be higher (unless, indeed, all animals bear tumours). He suggests that the figures for tumours in the animals treated with ultra-violet radiation + urocanic acid in the Reeves study are what would be expected historically with ultra-violet radiation with or without treatment with urocanic acid, and considers the "control" figures an aberration. "Panel 8", from Forbes is included for inspection.

(c) The author claims several advantages for the study by Sambuco *et al* : it used SPF mice, individual housing, and 3 concentrations of urocanic acid (instead of one). In addition, he claims that the published figures for ultra-violet radiation in the report by Reeves *et al* are less than the amount of ultraviolet radiation actually used, on the basis of correspondence with the latter authors.

(d) The negative findings in the study by Sambuco *et al* correspond to historical controls. Forbes concludes his analysis with a theoretical justification for preferring the methods employed by Sambuco *et al* to analyse tumour production.

On the whole, the protocol of the experiments of Sambuco *et al* seems superior to that of the experiments of Reeves *et al*; but I do not think that the analysis of Forbes satisfactorily accounts for the differences between the groups with and without urocanic acid in the latter study.

10. Special investigations

Study of amounts of urocanic acid in skin.

Man. (a) The ratio of *cis/trans*-urocanic acid is greater in summer than in winter.

(b) The ratio of *cis/trans*-urocanic acid is greater in the forearm and cheek than in the skin of the back.

(c) UV irradiation of the skin of the back increased the *cis/trans*-urocanic acid ratio; this reverted to normal in 18 days, but the total level of urocanic acid remained elevated at that time.

(d) Both *cis*- and *trans*-urocanic acid, sodium salt, had about the same protective effect as each other against ultra-violet radiation. The compound monosodium 4-(5)-imidazolylmethyl-idenemalonate, an analogue which is incapable of isomerisation, also had about the same protective effect.

Effects on immune function.

Immune function in the skin is known to be reduced by UV radiation, and it is suggested that urocanic acid may be a photoreceptor for this effect, the *cis*-form produced by the radiation then influencing the Langerhans cells.

(a) The *cis*-isomer of urocanic acid inhibits the delayed type hypersensitivity induced by experimental herpes simplex virus infection in the mouse.

(b) The contact hypersensitivity produced in hairless mice by oxazolone is suppressed. (In this investigation, the tumour production induced by urocanic acid was also studied; this part of the investigation is summarised below.)

(c) High levels of histidine (the precursor of urocanic acid) in the diet produced a much increased level of urocanic acid in the skin of mice. Following this, the reduction in contact sensitivity to DNCB in the skin following UVB irradiation was studied. It was found that the effect of the feeding with histidine was to cause much greater inhibition of contact sensitivity compared with controls.

(d) Ultraviolet radiation can produce activation of herpes virus infections. Urocanic acid is plausibly postulated to be the intermediate in this reaction.

(e) In the rat, heart transplants showed less rejection if the recipients were treated with injected urocanic acid daily for 7 days. In 40 % of the treated animals, rejection seemed to have been prevented permanently.

(f) Urocanic acid binds covalently to thymus DNA under the influence of ultraviolet radiation. These adducts have been identified.

(g) There is evidence that UVB irradiation at the relatively high level of 50 kJ/m^2 suppresses contact hypersensitivity of the skin at a distant non-irradiated site. Whether urocanic acid plays a part in this reaction is not known. It has been shown that the time course of this reaction is identical with that of local suppression.

(h) It has been reported that stripping of the skin, which removes most of the Langerhans cells, prevents the reduction of contact hypersensitivity induced by UV irradiation. Another investigation, however, contradicts this report.

(i) In cultures of human monocytes, which contained *Staphylococcus epidermidis* to promote IL-1 production, *cis*- but not *trans*-urocanic acid depressed its production, and the proportion of DR-positive monocytes. In cultured lymphocytes, the proportions of helper and suppressor T-cells was altered by *cis*- but not *trans*-urocanic acid.

(j) In a study of the nature of the chromophore responsible for the immunosuppression associated with ultraviolet irradiation, the authors suggested that the cyclobutylpyrimidine dimers, which are known to be produced under such conditions, are probable chromophores. They availed of the fact that in the South American opossum, *Monodelphis domesticus*, there is an enzyme in the skin, activated by visible light, which repairs DNA by breaking down the cyclobutylpyrimidine dimers and restoring the integrity of the DNA. By using this species, and studying the effect of ultraviolet and white light on the contact hypersensitivity induced by 1-fluoro-2,4-dinitrobenzene, they concluded that urocanic acid was an unlikely candidate for the chromophore, and that the dimer was a more probable one.

(k) In an investigation of the mechanism of the reduction of contact hypersensitivity by UVB irradiation, the authors point out that not all strains of mice are equally sensitive to this effect. They showed that in sensitive strains, compared with relatively insensitive ones, there was a greater reduction in the hypersensitivity to dinitrofluorobenzene brought about by injection of *cis*-urocanic acid. (By this term the authors mean *trans*-urocanic acid, irradiated with UVB; in their laboratory, this gives just over 50 % *cis*-urocanic acid in the racemic mixture). However, although *cis*-urocanic acid would induce this lack of sensitivity, the authors had earlier shown that tumour necrosis factor-alpha (TNF-alpha) had a similar effect. In the present investigation, the authors were able to show that TNF-alpha had a similar effect to *cis*-urocanic acid on the Ia antibody in the Langerhans cells, and also on the histological changes in these cells, and on the effect on contact hypersensitivity. Furthermore, they were able to show that prior injection of an anti-TNF-alpha preparation inhibited these effects. They therefore postulate: in sensitive strains of mice, UVB induces isomeration of *trans*-urocanic acid; this in turn combines with a receptor, possibly in the Langerhans cell, but more likely in cells in the stratum spinosum, to produce TNF-alpha, which in turn is responsible for the changes in the Langerhans cells and the immunosuppression.

(l) Since it was recognised that immunosuppression was associated with ultraviolet radiation, and since the lymphocyte proliferation induced by phytoheamagglutinin (or concanavalin A) was inhibited by ultra-violet radiation, the authors tested, by two methods, the hypothesis that *cis*-urocanic acid might be the chromophore.

(i) Normal human lymphocytes from 6 healthy volunteers were cultured, and incubated for 4 days with either phytoheamagglutinin or concanavalin A; tritiated thymidine was added for the last 6 hours of culture. In addition to control tests, *cis*-urocanic acid and *trans*-urocanic acid were added to the cultures in concentrations from 10^{-10} molar upwards. The results showed that *trans*-urocanic acid had no effect, but that *cis*-urocanic acid inhibited the incorporation of thymidine at concentrations of 10^{-2} molar (1.4 mg/ml) and above. It was noted that normal human skin contains about 0.4 % of *trans*-urocanic acid (wet weight) (this may be calculated to be roughly 2.9×10^{-2} molar or 4 mg/g); ultra-violet radiation *in vitro* converts about half the amount of *trans*-urocanic acid exposed to irradiation to the *cis*-isomer).

(ii) Six human volunteers were subjected to prick tests 4 months apart. Seven antigens were given (tetanus toxoid, diphteria, tuberculin, etc.: this was a ready-made preparation, "Multitest Merieux"). A cream containing 5 % of finely-divided powdered crystals of *cis*-urocanic acid or a dummy cream was applied to either forearm in a double-blind manner; the first application was 3 hours before the first prick test, and was repeated 3 times a day for 2 days. A second prick test was applied 4 weeks later; each subject served as his own control. The application had no effect on the delayed hypersensitivity.

(m) Phototoxicity is associated with PUVA treatment. A new bifunctional psoralen, which does not have this effect, is 4,4'5-trimethylazapsoralen (TMAP). This compound was investigated in mice.

It was known from earlier investigations that TMAP with low dosage UVA had induced such changes as reduced numbers of Langerhans cells and Thy-I^+ cells in BALB/c mice. In the present work, SPF female C3H/HeN (MTV^-) mice were used. The radiation used was 320-400 nm, controlled with a spectroradiometer.

TMAP in 70 % alcohol was applied to the shaved dorsal skin 3 times a week; 45-60 minutes after each application, 10 kJ/m^2 of ultra-violet radiation was applied to the dorsal skin. This schedule was continued for 4 weeks. The ears were shielded from irradiation. Controls were non-irradiated animals; in addition, some animals received drugs alone; others alcohol applications alone; others alcohol + ultra-violet radiation; others ultra-violet radiation alone. In yet another set of animals, 8-methoxypsoralen (8-mop) replaced the TMAP.

(i) Skin in the irradiated area was removed and examined for immune cells by staining and counting the numbers of dendritic cells.

(ii) Dorsal skin of irradiated mice was treated with dinitrofluorobenzene (DNFB); 6 days later a challenge with DNFB was made on each ear. These mice were then killed, and single cell suspensions were made from the spleen, which were injected intravenously into normal syngeneic mice. The recipient mice were then sensitised by DNFB and challenged 6 days later, as above.

(iii) "Twenty-four hours after the last treatment" i.e., probably after the last ultra-violet irradiation, the dorsal skin of the animals was painted with DNFB; 18 hours later, a single cell suspension was prepared from inguinal, axillary and subscapular lymph nodes. This suspension was injected into each hind foot pad of syngeneic mice. These latter mice were then challenged 8 days later with DNFB on the ears.

The results may be summarised as follows.

All animals treated with 8-mop + ultra-violet radiation showed severe phototoxicity; this was absent in those animals treated with TMAP + UVA, UVA alone, or drugs alone.

The number of immune cells in the skin was reduced by ultra-violet radiation alone and by alcohol + ultra-violet radiation; the addition of 8-mop or TMAP reduced the number of cells still further. The reduction in $ATPase^+$ cells and Ia^+ cells was significantly greater in the skin from animals treated with TMAP + ultra-violet radiation; the numbers of Thy-1^+ cells was reduced to the same extent in both groups.

Contact hypersensitivity. No change was found in skin from animals treated with drugs alone; despite changes in numbers of immune cells, ultra-violet radiation alone had no effect; but the addition of TMAP or 8-mop to the ultra-violet radiation produced marked decrease in contact hypersensitivity.

(iv) Transfer of reduction of contact hypersensitivity responses. Those animals receiving suspensions of spleen cells taken from animals treated with either 8-mop or TMAP followed by ultra-violet radiation showed reduced hypersensitivity. Thus it was concluded that lymphoid suppressor cells were present in the spleen following such treatment.

Cell suspensions from lymph nodes. Contact hypersensitivity was produced when DNFB challenge was administered, 6 days later, to recipient mice. This hypersensitivity was much reduced if the donor mice had previously been treated with 8-mop or TMAP + ultra-violet radiation. Thus, antigen presenting cells are functionally altered by such treatment.

(v) It is possible that the 50 % reduction of immune cells produced by ultra-violet radiation might be insufficient to cause decreased overall immune function; or, morphological changes may not correlate with impaired function. Doses of ultra-violet radiation alone and of TMAP + ultra-violet radiation were chosen so as to give about the same degree of reduction (about 50 %) of the numbers of cutaneous immune cells. The ability of cell suspensions from lymphatic glands after such treatment to induce hypersensitivity was not affected by ultra-violet radiation alone, but was much reduced by ultra-violet radiation + TMAP. Thus there is a qualitative difference between the effects on hypersensitivity produced by ultra-violet radiation and that produced by ultra-violet radiation + TMAP.

(n) It is known that contact hypersensitivity (CHS) is depressed by psoralen + UVA treatment. This rather resembles the effect of UVB by itself, which is also known to be associated with systemic immunosuppression. In the present investigation, both monofunctional and bifunctional psoralens were investigated.

The animals used were C3H/HeNCr(MTV$^-$) and BALB/c AnNCr mice. UVB and UVA were produced from tubes which had outputs of 270-390 nm and 320-400 nm (wavelengths checked by spectroradiometer). The outputs at 20 cm were 4.1 and 22 J/m^2/second respectively.

(i) A keratinocyte cell culture line was used. It was exposed to UVB without psoralen, or to UVA with appropriate doses of the psoralen under test; after 12 hours, supernatant was taken for use in testing.

(ii) C3H mice were injected with 15 µg of supernatant protein; after 5 days, the mice were immunised with allogeneic BALB/c mouse splenic cells; after 6 days, the animals were challenged with the same cells by injection into each hind footpad. Suitable negative and positive controls were used. This procedure demonstrated that delayed type hypersensitivity was suppressed by supernatant protein from cultures that had been exposed to UVB and also when the cultures had been irradiated with 200 to 500 J/m^2 of UVA + 400 ng/ml of 8-methoxypsoralen. Higher doses of UVA were cytotoxic.

(iii) The cultures were irradiated with UVA at 500 J/m^2; 8-methoxypsoralen was added in concentrations from 0 to 1000 ng/ml. Concentrations greater than about 200 ng/ml gave rise to a supernatant which reduced contact hypersensitivity.

(iv) The irradiation of the cultures with UVA was maintained constant at 500 J/m^2, and equimolar doses (1.85 nmoles/ml) of the following compounds added to the incubation: trimethylazapsoralen; 8-methoxypsoralen; 5-methoxypsoralen; angelicin; 4,4',6'-trimethyl-angelicin. All the agents had much the same effect in producing a supernatant which would inhibit delayed type hypersensitivity.

(v) Inhibition of contact hypersensitivity.

Mice which had been injected with supernatant protein as above were tested for inhibition of contact hypersensitivity. After 5 days, dinitrofluorobenzene (DNFB) was applied to the abdominal skin. After 6 more days, a DNFB challenge was applied to each ear. Suitable positive and negative controls were used. Exposure of cultured keratinocytes to 200 J/m^2 of UVA alone caused the release of a factor into the supernatant which reduced contact hypersensitivity induced by DNFB. A dose of 50 J/m^2 was subthreshold, but at this dose the addition of 200 ng/ml of 8-methoxypsoralen caused release of a factor which suppressed contact hypersensitivity to DNFB.

The authors conclude that the dose relationships support the hypothesis that different mechanisms are involved in the suppression of contact hypersensitivity and delayed type hypersensitivity under the conditions of these experiments. The type of psoralen used does not seem to make much difference.

(o) In a similar investigation, the effect of UVA on immunosuppression was investigated. Mice of the C3H/HeN(MTV^-) and BALB/c strains were used. UVA was produced at wavelengths from 320 to 400 nm, measured with a spectroradiometer. An established mouse keratinocyte culture was used. The C3H mice were shaved on the back and subjected to 10 kJ/m^2 of UVA 3 times a week for 4 weeks. Some animals had 122 µg of 8-methoxypsoralen in 300 µl of alcohol applied to the area 45 minutes beforehand (about 400 µg/ml). After 24 hours the animals were killed and the epidermal sheets stained for immune cells, which were counted.

Contact hypersensitivity (CHS) was induced by shielding the ears of the irradiated animals from the UVA. Twenty-four hours after the last treatment, the skin in the treated area was painted with dinitrofluorobenzene (DNFB). Both ears were challenged with DNFB 6 days later.

Delayed type hypersensitivity was induced by first immunising the animals with BALB/c spleen cells, 24 hours after the last ultra-violet irradiation. After 6 days, the same cells were injected into each footpad.

Induction of immunosuppressant material. A culture of keratinocytes was exposed to UVA, with or without the addition of 8-methoxypsoralen, followed by incubation for 12 hours. This was given IV to C3H mice. Contact or delayed hypersensitivity was induced after 5 days, as described above.

The results were as follows: UVA irradiation with or without alcohol pretreatment gave a 50 % reduction in immune cells in the exposed skin; there was also altered morphology. These changes were more marked if topical 8-methoxypsoralen were used 45 minutes before the ultra-violet irradiation.

Contact hypersensitivity was not impaired after UVA treatment or 8-methoxypsoralen treatment individually. Thus the changes in the cutaneous immune cells (above) did not affect the response. However, the addition of a psoralen (probably 8-methoxypsoralen) to the culture as well as irradiation produced factors which suppressed delayed type hypersensitivity as well.

(p) In view of the known immunosuppressive effect of ultra-violet radiation below 340 nm, and the animal evidence that ultra-violet radiation at 340 to 440 nm may enhance immunity, in animal experiments, the authors decided to investigate human volunteers. The radiation was provided by a commercial sunbed device, which emitted radiation very carefully filtered to remove radiation below 340 nm, and also to remove radiation from 440 to 800 nm, and infrared radiation from 800 to 3000 nm. The radiation produced was checked by metering. The doses used were: 1,130,000 J/m^2 of UV-A1 and 1,290,000 J/m^2 of UV-A1-light (the distinction between these categories is not further commented upon). The respective values in W/m^2 were 750 and 860. In all, 14 irradiations were carried out.

Twenty-seven healthy volunteers were recruited; the test groups comprised 7 females and 6 males, and the non-irradiated control group 7 females and 7 males. Subjects giving a marked erythematous reaction to a test exposure were excluded. The whole body was irradiated for 50 minutes at each session. The experiments were commenced in November, to minimise any effects of natural insolation.

Tests for immunity were as follows:

(i) A “Merieux multitest” applied to the left forearm.

(ii) Counting of lymphocytes: total lymphocytes, and lymphocytes in the following categories:

pan-T, T-helper (T4), T-suppressor (T8); and the T4/T8 ratio.

The left forearm is said to have been protected from radiation. The timetable of the investigation was as follows:

Before commencement: lymphocyte counts, multitest application.

Day 2: reading and scoring of multitest.
Day 5: begin phototherapy.
Day 23: end phototherapy: 14 irradiations in all, weekends excluded.

Day 33: lymphocytes counted, multitest applied.

Day 35: multitest read and scored.

Day 57: lymphocytes counted, multitest applied.

Day 59: multitest read and scored.

The results are considered for day 35 and day 59. On day 35, the reaction to the multitest was significantly reduced compared with the control on the "irradiated left forearm". The protocol, however, calls for the left forearm to be protected from radiation. No other differences were found. On day 57, no differences between the control and irradiated groups could be found. The authors conclude that they had failed to show any effect of exposure to these wavelengths on the immune status of the subjects. They review 2 other studies in which such differences were found, but the irradiation used in those investigations was not identical with that used in the present investigation.

(q) Ultra-violet radiation is known to stimulate cultured human keratinocytes to generate products which block spleen cell proliferation in the mixed lymphocyte reaction to antigenic stimulation. Cytokines are also produced which cause immunosupression in the intact animal. Human fibroblasts in culture which carry the chloramphenicol acetyltransferase gene (under the control of the HIV long terminal repeat promoter) are caused to express the gene by exposure to ultra-violet radiation.

The present investigation examines whether *cis*-urocanic acid produces these effects.

Human keratinocytes in culture were exposed to 200 J/m^2 of UVB or exposed for 1 hour to *cis*- or *trans*- urocanic acid. The cells were then cultured for 18 to 24 hours and the super-natant removed. Twenty µg of protein from the supernatant was injected iv into the tail veins of 2 or 3 C3H/HeN mice. After 5 days, subcutaneous injections of spleen cells from BALB/c mice were given to these animals. Seven days later, spleen cells were taken and mixed with gamma-irradiated BALB/c stimulator cells. The spleen cells were cultered for 4 days, and for the last 18 hours, 3H-thymidine was added. The incorporation of the thymidine into the DNA was measured.

Human fibroblasts in culture were transfected by using a plasmid containing pHIVcatSVneo (the chloramphenicol acetyltransferase gene and the long terminal repeat chain of the HIV virus). The fibroblasts were incubated for 18 hours with *cis*- or *trans*-urocanic acid or (as positive control) exposed to 5 J/m^2 of UVC (about 254 nm). Expression of the cat gene was measured by exposure to labelled chloramphenicol, followed by ethyl acetate extraction and TLC.

Results. The factors released by keratinocytes subjected to ultra-violet radiation significantly suppressed the ability of the C3H mouse spleen cells to proliferate. No effect of *trans*- or *cis*-urocanic acid at 10 µg/ml was found.

Ultra-violet radiation powerfully stimulated the expression of the cat gene by the transfected fibroblasts, but *cis*-urocanic acid gave the following results: 0.01 % (100 µg/ml) no effect; 0.1 % (1 mg/ml) a non-significant increase in cat activity of about 12 %; 1 % (10 mg/ml) a significant increase of about 28 %. The last concentration was highly cytotoxic.

In this investigation, urocanic acid does not seem to have had the same effects as ultra-violet radiation on the tests used; however, it should be noted that UVC was used in the fibroblast experiment.

(r) Mouse. This communication gives a short account of an investigation into a hypothesis that DNA damage initiates the immunological changes which follow ultra-violet radiation to the skin.

The excision repair of DNA damage in the mouse skin following ultra-violet radiation can be accelerated by the application of T4N5 liposomes (containing T4 endonuclease V) to the skin after exposure. In these experiments, the liposomal preparation was applied to mice immediately after ultra-violet radiation. The effect sought was prevention of suppression of delayed type hypersensitivity to *Candida albicans*. The hypersensitivity was unaffected by ultra-violet irradiation if the liposomes were applied; and inactivation of the T4N5 by heat treatment removed its ability to prevent the delayed type hypersensitivity associated with ultra-violet radiation. The authors therefore suggest that it is DNA which is the primary photoreceptor, and not urocanic acid.

In an abstract which seems to reproduce the same data, the authors again suggest that DNA is the primary photoreceptor in the skin for the suppression of immunity by ultra-violet radiation.

(s) Since it is known that exposure to UVB (280 to 320 nm) causes a dose related suppression of systemic cell-mediated immunity, it has been postulated (by the authors and others) that the *trans-cis* isomerism of urocanic acid in the skin in response to ultra-violet radiation is the photoreceptor for this effect.

Since the absorption spectrum of urocanic acid lies partly in the UVA, the authors investigated the possibility that UVA might also cause immunosuppression.

Shaved mice were exposed to banks of fluorescent tubes consisting of either BlackLightBlue (Sylvania), Blue (F40B, Philips) or PUVA (Sylvania).

Following irradiation, skin was removed from the treatment site and a non-irradiated site, extracted, and analysed for urocanic acid content by HPLC. A dose dependent isomerisation of urocanic acid was found at the irradiated site with all three tubes. Their efficacy in this regard, in descending order, was: PUVA, BLB, Blue. No further details are given.

(t) In experiments in female mice of the strain C3HBu/Kam(H-2^{K}), it was found that migration of dendritic cells to draining lymph nodes was produced by UVB. It is probable that this effect plays an essential part in the inhibition of contact hypersensitivity in the skin under these circumstances. This effect was enhanced if the skin was first sensitised with fluorescein isothiocyanate. The mediator of this response was possibly tumour necrosis factor-alpha. Neither the *cis*- nor the *trans*-isomers of urocanic acid had any effect on dendritic cell numbers in the skin, whether there had been previous sensitization or not. The authors conclude that the immunosuppressant action of urocanic acid acts by a different mechanism to that described in this work, and may not play a part in the suppression of hypersensitivity induced by UVB.

(u) In an important review article on urocanic acid and immunosuppression, Norval *et al* make the following points relevant to the present summary. Firstly, urocanic acid is the major absorber of ultra-violet radiation in the skin, and it may be the chief naturally occurring

photoprotective agent in man. Secondly, it is formed by the deanimation of histidine, and the ratio of urocanic acid to histamine in skin may be important. Thirdly, it seems to be the chemical mediator of the transient alteration in immune surveillance following ultra-violet radiation. Fourthly, while the equilibrium ratio of *cis/trans* urocanic acid *in vitro* is 74 %, it is about 40 % in the superficial layers of human skin, following 32 mJ/cm^2 (= 2 MED) of ultra-violet radiation, falling to 15 % in the deeper layers. Fifthly, the absorption spectrum of urocanic acid is the only one which produce immunosuppression.

(v) In another article from the same laboratory, the authors studied the suppression of the delayed type hypersensitivity response (DTH) to *Herpes simplex* virus (HSV) in a mouse model. The isomers of urocanic acid, and various analogues, were tested. It was known from previous work that prior painting of the skin with urocanic acid suppressed the subsequent DTH reaction to HSV. The results showed that the *cis*-isomer of urocanic acid was much more powerful than the *trans*- isomer in suppressing the DTH. However, several analogues were also nearly as powerful. For instance, the *cis*- and *trans*- isomers of 2-pyrrole-acetic acid (which lacks the N_3) were so; replacement of the N function in the latter compound with S (2-thiophene-acrylic acid) also yielded a potent inhibitor, and so on. Hydrogenation of the side chain (dihydro-urocanic acid) also gave a compound which was potent; and histamine itself was not very much less potent than urocanic acid.

It may be wondered whether the activity of such a variety of analogues does not shed some doubt on the specifity of urocanic acid in suppressing DTH *in vivo*.

(w) Pane (1992) draws attention to the formation of a cyclobutane dimer of urocanic acid in the guinea pig skin *in vitro*, following irradiation. Its significance is uncertain, but it may be relevant to the matters discussed in this section.

(x) An investigation of the possible effects of urocanic acid on the reaction of human skin to DNCB was carried out. A group of 40 healthy subjects was recruited (32 female and 8 male), and tested in four groups each of 10 subjects. Members of a group were asked to apply a preparation of *cis*-urocanic acid to the lower half of the body, amounting to about half the surface area of the skin. The amount applied may be calculated to be about 0.8 mg/cm^2. The concentrations of urocanic acid applied by members of each group were, respectively, 0, 0.02 %, 0.2 % and 2.0 %. These applications were made daily for 17 days; the applications for the last 3 days were supervised by nurses. On day 18 a challenge dose of 40 µg of urocanic acid was applied (the author states that he recognises this to be a low dose, but says it might serve to "...maximise the change that a subtle difference might be detected.") These applications were made to treated skin.

After a rest period of 21 or more days, subjects were challenged with four doses of DNCB applied to the inner surface of untreated skin of the upper arm; the inner aspect was chosen as an area with little exposure to sunlight. The doses of DNCB (µg) were 0, 3.125, 6.25 and 12.5. The reactions produced were graded clinically on a scale from 0 to 3, and the area of induration and the skin thickness were also measured. Spontaneous reactions to DNCB were commonly found after 10 to 20 days; there was no significant difference between the groups, despite different pretreatment with urocanic acid. Thus, there was no evidence that sensitivity to DNCB was affected by urocanic acid. In addition, a "subset of 20 patients" (how selected is not stated) was subjected to extensive haematological investigation, including determination

of lymphocytes, T cell count, B cell count, T helper cell count, T suppressor cell count, and the response of lymphocytes to various mitogens. Skin biopsies for counting Langerhans cells were taken before treatment with urocanic acid began, and daily during the first 14 days of treatment. It is not clear whether these biopsies were taken from the "subject of 20" or from all subjects. The results of the haematological investigations showed no significant difference whether or not urocanic acid had been used; there was also no definite trend with increasing concentration of urocanic acid in the treated groups. The Langerhans cell counts showed that skin treated with placebo had a significantly lower count than control untreated skin; this difference disappeared when untreated sites were compared with sites which had been treated with placebo at the end of the experiment. The finding in the early part of the experiment appears to be an aberration. It is difficult to understand the author's interpretations of the haematological findings. Overall, urocanic acid appears to have had little if any effect on immune function or the response to DNCB, under the circumstances of the experiment.

(y) In a further experiment an attempt is made to see whether the elicitation of skin reactions to DNCB could be affected by prior topical urocanic acid. From internal evidence, it seems likely that the 20 subjects were the same as that "subset of 20" investigated in the previous study, but this is not stated. All had been sensitised to DNCB; the concentrations of DNCB used were lower than those used in the earlier work. From the individual records provided it is possible to determine that six subjects were tested with the dose of DNCB that had previously elicited a reaction; four subjects were tested with half of the dose which had previously elicited a positive reaction, and 10 subjects were tested with 10 % of the dose of DNCB that had previously elicited a positive reaction. The DNCB was applied after the application of 4 different concentrations of *cis*-urocanic acid to four different skin sites, on the previous day. Reading was carried out 2 to 4 days later. All subjects reacted to DNCB; although the intensity of the reactions varied with the subjects and the dose of DNCB, there was no suggestion that prior application of *cis*-urocanic acid had any effect.

(z) A very extensive set of preliminary experiments was carried out to determine: the best strain of mouse; the best method of measurement of ear swelling; the best induction/challenge agent and its optimal concentrations; the optimum period for induction; and the optimal concentration and mode of administration of cyclosporin for the suppression of the contact hypersensitivity reaction.

As a result of these tests, male mice of a CDF1 CSH/HeN strain were used. The number of mice in the groups in which the effect of urocanic acid was studied is apparently not stated; in the preliminary testing groups of 3 mice were used. The induction agent was picryl chloride applied once to the skin of the back in a concentration of 4 %; challenge was by dermal application of an 0.5 % solution to the ear 3 days later; thickness of the ear was measured 24 hours later with an automatic gauge. In tests of the capacity of cyclosporin to inhibit the contact hypersensitivity reaction, the immunosuppressant was applied daily for 6 days to the skin of the back in a dose of (probably) 20 mg/kg b.w./day; on the third day the induction with picryl chloride was carried out and the challenge as before. This treatment schedule reduced the contact hypersensitivity by 90 to 100 %.

Solutions of *cis*- and *trans*- urocanic acid dissolved in DMSO (concentration not stated) were applied to the skin of the back (in the same manner as that used for cyclosporin) in doses

(mg/kg b.w./day) of 0.05, 0.5, 5, 50 and 500. *Trans*-urocanic acid inhibited the contact hypersensitivity reaction by 4.46 to 27.54 %, but there was no dose relationship: both 0.5 and 50 mg/kg b.w./day gave approximately equal inhibition, and more than that produced by 5 and 500 mg/kg b.w./day. *Cis*-urocanic acid caused some inhibition at 5 mg/kg b.w./day, but at no other dose.

The author concludes that neither isomer of urocanic acid had any effect on contact hypersensitivity reaction in this model.

(aa) Since UVB suppresses immunity, and since it also converts *trans*-urocanic acid to *cis*-urocanic acid, the authors studied the effects of these agents on the ability of Langerhans cells to stimulate the growth of allogeneic T-cells in culture.

Human Langerhans cells were obtained from skin from patients undergoing plastic surgery; following trypsinisation, two methods of density gradient centrifugation were used, producing concentrations of Langerhans cells ranging, with one method, from 8 % to 25 %, and with the other, from 70 % to 90 %. The Langerhans cells were suspended in Hank's solution.

Mononuclear cells were isolated from human blood from donors unrelated to the skin donors. The T-cells were concentrated by density centrifugation. The two cell types were cultured together for 5 days, at which time ^{3}H-thymidine was added and a final 18 hours of incubation carried out.

There was stimulation of T cell growth both with the less concentrated and the more highly concentrated suspensions of Langerhans cells, more intense with the latter. However, the addition of *cis*-urocanic acid or *trans*-urocanic acid (6.5 to 400 µg/ml), or of *trans*-urocanic acid irradiated with UVB, had no effect on the stimulation of T cell growth by Langerhans cells.

Prior irradiation of the suspensions of Langerhans cells reduced the degree of stimulation of T cells, but again the results were not affected by *cis*-urocanic acid, *trans*-urocanic acid, or previously irradiated *cis*-urocanic acid. The authors conclude that the isomers of urocanic acid have no direct effect on the antigen presenting functions of the human Langerhans cells.

11. Conclusions

Acute toxicity was low.

Dermal absorption studies suggest a low absorption, but studies are complicated by the physiological presence and synthesis of urocanic acid in the skin. Tests for absorption in skin of hairless mouse *in vitro*, in some experiments, showed greater amounts in skin + receptor fluid than had been applied; in other experiments there was perhaps a small degree of absorption. In cadaveric human skin *in vitro* there did not seem to be any absorption.

Application to the skin of volunteers did not lead to any increase in the amount of urocanic acid found in strippings, when compared with controls. Percutaneous absorption therefore seems slight, but there is a considerable variation.

Dermal irritation was not produced by any application containing 2 % of urocanic acid, but this is the use concentration. In other animals tests, no irritation was produced, but the concentrations used are not stated.

Up to 10 % of urocanic acid is reported to be non-irritant in the eye.

Tests for phototoxicity and photosensitization in guinea pigs were negative.

Tests for mutagenic activity in *S. typhimurium* and in *E. coli* were negative, as were tests for chromosomal aberration *in vitro* in Chinese hamster lung cells. Unscheduled DNA synthesis in human fibroblasts *in vitro* was not induced.

A test for unscheduled DNA synthesis in human fibroblasts *in vitro* following exposure to UVB at 500 J/m^2 was negative.

Urocanic acid forms an adduct with calf thymus DNA *in vitro*; this was not increased by exposure to UVB at 100 kJ/m^2.

One test for photocarcinogenicity in mice was negative. In a different set of experiments in mice an increase in tumours was found, but these findings have been criticised as being analysed by methods which would exaggerate the apparent incidence of tumours.

Most of those reported experiments, which were designed to study the effects of urocanic acid on the reduction of immunity by exposure of the skin to ultra-violet radiation, showed that urocanic acid enhanced the effects of ultra-violet radiation on reducing immunity.

Despite some contradictions, the evidence is strong that this compound, when applied to the skin, enhances the effect of ultra-violet radiation in reducing immunity. Under these circumstances, the committee feels that it cannot conclude that the compound is suitable for use in cosmetics.

Classification: D.

S 72: *HOMOPOLYMER OF (+)-N-(2- AND (+)-N- (4-((2-OXOBORN-3-YLIDENE)METHYL)BENZYL)ACRYLAMIDE*

1. General

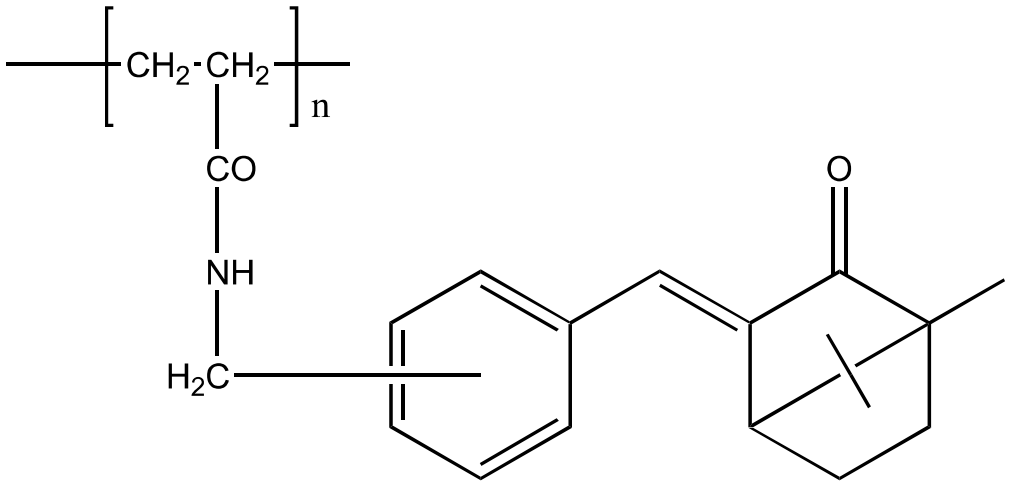
1.1 Primary name

Homopolymer of (+)-N-(2- and (+)-N- (4-((2-oxoborn-3-ylidene)methyl)benzyl)acrylamide

1.2 Chemical names

Homopolymer of (.+.)-N-((4-((4,7,7,-trimethyl-3-oxobicyclo(2.2.1)hept-2-ylidene)methyl))-phenyl))-methyl)-2-propenamide.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $(C_{21}H_{25}NO_2)_X$

Mol weight: indeterminate: highest value between 17000 and 40000; about one third has a MW less than 4000.

1.7 Purity, composition and substance codes

It is a mixture of isomers on positions 4' and 2' of the phenyl ring.

1.8 Physical properties

Appearance: The substance is a light brown powder in appearance.

Maximum absorbance is at 295 nm.

Test for photostability *in vitro.*

A 4 % o/w emulsion was studied. This was exposed in a layer 1µm thick to SSR from a xenon arc, filtered and refracted to give UV wavelengths only. The intensity of irradiation was 0.42 mW cm^{-2} in UVB and 15 mW cm^{-2} in UVA, estimated to be about 3 times the intensity to be expected in the Mediterranean. The results showed the compound to be stable, losing only 1.35 % in 1 hour.

1.9 Solubility

Insoluble in water; soluble in organic solvents.

2. Function and uses

Proposed for use as a sunscreen at levels up to 6 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Mouse. A limit test was carried out on a group of 6 male and 6 female albino CFLP mice. The a.i. was administered once by gavage in a dose of 5000 mg/kg b.w. as a suspension in CMC 0.5 %. No abnormality was found over 14 days or at necropsy. The LD_{50} was greater than 5000 mg/kg b.w.

Rat. A similar experiment gave the same result; the LD_{50} was greater than 5000 mg/kg b.w.

3.7 Subchronic oral toxicity

Rat. A 3 month oral study was carried out in 4 groups of SD rats with 10 male and 10 female animals in each group. The a.i. was made up in 2 % polysorbate + 0.01 % dimethicone in water, and administered by gavage in doses of 0, 150, 450 and 1350 mg/kg b.w./day. The study was carried out in conformity with OECD guidelines. There were no deaths, and no significant abnormal findings. The NOAEL is put at 1350 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Six male NZW animals were used; testing was carried out according to the J.O. de la République Française of 2/2/82. Sites on either side of the dorso-lumbar spine were prepared, one abraded and one not. A 20 % suspension in carbitol was applied, in a volume of 0.5 ml, to either side, with occlusion for 24 hours. At first, slight to well defined erythema, with or without slight oedema, was noted at 4/6 intact sites and 6/6 abraded sites. At 72 hours, slight erythema was found in two animals only. The index of irritation was 1.2 (maximum 8). The a.i. at 20 % in carbitol was deemed to be “slightly irritant”.

Three NZW animals were tested according to OECD guidelines. Areas were prepared on either side of the dorso-lumbar spine; one side was used for testing and the other as a control. On a pad moistened with 0.5 ml of water, 500 mg of a.i. was applied to the test site, and held in position with a semiocclusive dressing for 4 hours. Over the first 72 hours, there were slight erythematous changes in 2 animals, and moderate erythema in the third. All changes had disappeared by the sixth day. In accordance with 83/467/CEE, the substance was classified as "non-irritant".

4.2 Irritation (mucous membranes)

Rabbit. A Draize test was carried out in 6 NZW animals, according to the protocol of J.O. de la République Française 21/10/84. A 20 % solution of a.i. in castor oil was used in one eye, with the opposite eye acting as control. Observation was at 1 hour and then daily for 7 days. Although there was marked red coloration of the conjunctivae of 5/6 animals after 1 hour, the overall score indicated that the substance should be classified as "very slightly irritant."

Three male NZW animals were tested according to OECD guidelines, using the a.i. as a powder. There was slight redness and chemosis initially, but the overall score over 72 hours indicated that the material was "non-irritant".

5. Sensitization

Test for capacity to cause delayed contact hypersensitivity.

Guinea pig. Thirty female Dunkin-Hartley albino animals were used: 20 test and 10 control. The a.i. was made up at 40 % and 20 % in carbitol; further dilutions were made by adding liquid paraffin to these suspensions. Areas of 4 x 6 cm were prepared on the upper back. Control animals were treated identically with test animals throughout, except that a.i. was omitted. In the test animals, the usual intradermal injections of Freund´s complete adjuvant with or without a.i. were given. One week later, a patch saturated with a 20 % suspension of a.i. was applied to the same site for 48 hours with occlusion. Challenge applications were made 2 weeks later to 2 sites on the flank: 8 % and 4 % applications were made and occluded for 24 hours. No differences were seen between the control and test animals; the test was negative.

Test for capacity to produce contact hypersensitivity.

Man. A preliminary and a main study were carried out. In the preliminary test, the a.i. was applied as 0.5 ml of a series of dilutions in castor oil; the concentrations tested were 2.5 %, 5 %, 10 %, 15 %, and 20 %, for 48 hours with occlusion. Since no adverse reaction was seen, the concentration of 20 % was used in the main test in 30 volunteers. A dose of 0.5 ml of the solution of a.i. in castor oil was applied to a strip of material 2 x 2 cm. This was placed on the forearm and occluded for 48 hours. This application was repeated to the same site 5 times in all, over a period of 3 weeks. Following a 2 week rest period, a challenge application of the same strength was applied to 2 different area on either forearm, again for 48 hours with occlusion. There was no evidence of hypersensitivity. (There is a slight difficulty in being sure that the concentrations given above were those actually used, but this was almost certainly the case).

Test for capacity to produce photosensitization.

Guinea pig. Forty-two female animals were used: 10 test, 10 negative control, 20 positive control, and 2 for range finding studies. The a.i. was made up as a 20 % suspension in carbitol. The light sources were (a) a lamp emitting from 285 to 400 nm and (b) a lamp emitting from 320 to 400 nm. Dosage was measured with Osram "Centra" radiometers. Following a range finding test, a concentration of 20 % a.i. was chosen for testing.

Induction. 0.025 ml of the test solution was applied to a circular depilated area of 2.5 cm^2 on the back of each animal. This was allowed to remain in place for 30 minutes. The animals were then placed in a restraining cage and exposed to UVA + UVB radiation for 10 minutes: 485 and 185 mJ cm^{-2} respectively. This procedure was repeated every 48 hours, 5 times in all. Control animals were treated similarly, except that no chemical was applied to the skin. For a positive control, 0.1 % dibromosalicylanilide was used.

Challenge. After a 12 day rest, applications were made in the same manner as before, and 30 minutes later, animals were exposed to UVA only, at 10 J cm^{-2}.

There was no reaction in any group, nor any evidence of irritation. As a result, the positive control was repeated in a further 10 animals, this time using tetrabromosalicylamide in petrolatum. This gave positive reactions. It was concluded that there was no evidence for the production of photosensitization by the a.i.

6. Teratogenicity

Rat. Groups of about 20 pregnant animals were given doses of a.i. by gavage during days 6 to 15 of pregnancy. The doses used were 0, 100 and 1000 mg/kg b.w./day. There was no evidence of teratogenic activity.

7. Toxicokinetics (incl. Percutaneous Absorption)

Test for percutaneous penetration.

Hairless rat *in vivo*. Six female rats were anaesthetised and an application of 2 mg of an ointment containing 5 % a.i. was made over an area of skin of 1 cm^2, delineated by a silicon ring. Occlusion was not used. The a.i. was labelled with 14-C in the aromatic ring. After 4 hours, the area of application was cleaned, and the animals were transferred to individual metabolism cages which permitted the separate collection of urine and faeces. After 96 hours the animals were sacrificed and the area of application was stripped 6 times with adhesive tape. The gastrointestinal tract and the area of application were removed. Radioactivity was estimated in the urine and faeces (24 hours collections), in the gastrointestinal tract, in the carcass, in the skin in the area of application, and in the strippings. The amounts found were (in percentages of the amount applied): urine over 96 hours, 0.052; faeces over 96 hours, 1.521; GIT at sacrifice, 0.015; skin in area of application, 0.053; stratum corneum in area of application, 0.095. Total, 1.829 % of amount applied (100 µg). If the amount in the skin and strippings at the site of application be excluded, the percentage of a.i. absorbed was 1.681 % of the applied amount. It was also shown in this experiment that the excretion half-life of the absorbed a.i. was about 24 hours.

8. Mutagenicity

A standard Ames test was carried out. There was no evidence for an increase in revertants, with or without activation.

A Chinese hamster ovary cell line was used to test for chromosomal aberrations *in vitro*. There was no evidence of clastogenic activity.

Mouse. A micronucleus test was carried out in accordance with GLP. The test was negative.

Tests for photomutagenic activity.

A test for photomutagenic activity was carried out according to GLP, using the tryptophan-requiring organism *E. coli* WP2 for the test. The active ingredient was dissolved in DMSO. Range finding tests showed slight precipitation at 5000 µg/plate, and this was used as the top dose. Suitable positive and negative controls were used; the positive controls were 8-methoxypsoralen with irradiation, and 4-nitro-quinoline-1-oxide in the absence of radiation. At least 3 replicates were used at each dose level.

Ultraviolet radiation was derived from Osram "Vitalux" lamps. The following doses of ultraviolet radiation were used (mJ/cm^2): UVA 5.6 and 11.2; UVB 1.8 and 3.6. Using glass filtering, UVA 230 and 460. The actual doses were measured by a meter. For a positive result, the protocol required a dose related increase in the number of revertants, significant at less than 0.01. Various dose intervals were used in the experiments. There was no statistically significant increase in revertants at any concentration of active ingredient, although there was a slight increase with high levels of UVB radiation at the lower doses of active ingredient; there was no increase with UVA. The test was negative.

A test for the production of chromosomal aberrations *in vitro* was carried out using CHO cells, according to GLP. After range finding experiments, the concentrations of active ingredient used were 31.25, 62.5 and 125 µg/ml. There was negligible inhibition of the mitotic index at any dose of active ingredient. The controls were as in the previous experiment. The doses of ultraviolet radiation (mJ/cm^2) were: UVA 200; UVB 33, and UVA with glass filter 700. Tests were carried out in duplicate. Significance was set at p less than 0.05. Harvesting was at 22 hours.

No significant increase of aberrations was found with exposure to the active ingredient in the presence of ultraviolet radiation, and the results were consonant with historical controls in the laboratory. The positive controls were strongly positive. The test was negative.

10. Special investigations

Test for capacity to produce phototoxicity following cutaneous application.

Guinea pig. The a.i. was used as a suspension in castor oil at 20 % w/v, and was applied under patches, with occlusion, for 90 minutes. Following preliminary testing, 5 animals were used as negative controls, being treated with a.i. but not irradiated. The test group comprised 11 male and 11 female animals. In each, 2 areas were treated with a.i., but after removal of the patches, only one of the areas was irradiated. The irradiation was from 2 lamps, with outputs at 285 to

350 and 310 to 400 nm respectively. Both lamps were used at a distance of 10 cm from the skin for 5 minutes, and then the longer wavelength lamp was placed 5 cm from the skin for 90 minutes. The total energy was 12.5 J cm^{-2}, comprising 99 % UVA and 1 % UVB. This amount of irradiation equalled 1 med under the conditions of the experiment. No contemporaneous positive control was used, but the authors give earlier results from their laboratory, using the same technique, with known phototoxic substances such as methoxypsoralens, angelica and rue extracts, etc., which yielded the expected positive results. It was concluded that the a.i. did not induce phototoxicity in this experiment.

11. Conclusions

Acute toxicity was low, and the substance has been shown to be stable in ultraviolet light. Tests for capacity to irritate mucous membranes and skin were negative. Tests for production of hypersensitivity were negative. Tests for photosensitization and phototoxicity were accepted as being negative, despite to the lack of some contemporaneous controls. Subchronic oral toxicity testing gave a NOAEL of at least 1350 mg/kg b.w./day. Percutaneous penetration was low. Tests for mutagenicity and chromosomal aberration (*in vitro* and *in vivo*) were negative. Tests for photomutagenicity were carried out with a "Vitalux" lamp, which was not ideal; however, the results of the test were negative, and the active ingredient was shown to be stable to intense ultraviolet radiation. A test for teratogenesis in the rat was negative.

Classification: A.

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

HOMOPOLYMER OF (+)-N-(2- AND (+)-N- (4-((2-OXOBORN-3-YLIDENE)METHYL)BENZYL)ACRYLAMIDE
S 72

Based on a usage volume of 18000 mg, containing at maximum 6 %

Maximum amount of ingredient applied:	**I (mg)= 18000 x 6/100 = 1080 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 1.7 %**
Dermal absorption per treatment:	**I (mg) x A (%)= 18.36 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg = 1080 mg x 1.7/100 / 60 kg = 0.306 mg/kg b.w.**
No observed adverse effect level (mg/kg): (species, route of application)	**NOAEL = 1350 mg/kg b.w.**
Margin of Safety:	**NOAEL / SED = 1350/0.306 = 4412**

OPINIONS ADOPTED DURING THE 65TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 24 Mai 1996

P 70: *BENZETHONIUM CHLORIDE*

1. General

1.1 Primary name

Hyamine 1622, benzethonium chloride, phemerol chloride

1.2 Chemical names

4'-(1,1,3,3-tetramethylbutyl) phenoxy-ethoxyethylene-dimethyl-benzylammonium chloride

1.3 Trade names and abbreviations

Colipa No. : P 70
EEC No. : Annex II. No. 415

1.5 Structural formula

1.9 Solubility

Soluble in water, alcohols and other organic solvents.

2. Function and uses

Use expected as a preservative at levels of 0.1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

- Oral, Rat, LD_{50} = 420 mg/kg
- I. P., Rat, LD_{50} = 33 mg/kg
- I.V., Rat, LD_{50} = 19 mg/kg

3.4 Repeated dose oral toxicity

Subacute study

- In a 28-day feeding study, rats received diets with 0, 20, 100, 500 or 2500 ppm, providing intake levels of 0, 1.7, 8, 40 or 200 mg/kg b.w./day. The changes in the top-dose group included growth retardation, caecum enlargement, signs of liver damage and decreased serum levels of inorganic phosphorus in males. The last finding was the only effect considered treatment-related in males fed 500 ppm. The diet with 100 ppm (8 mg/kg b.w./day) was a clear NOAEL.

- A supplementary 28-day study in rats with the same feeding levels was conducted to verify and extend certain findings in the previous study. The results confirmed most of the changes

seen at the top dose, including caecal enlargement. The latter finding was not accompanied by histopathological changes. Decreased levels of serum-P seen at the two higher levels in the previous study did not occur in the present study. Therefore, 500 ppm (or 40 mg/kg b.w.) was the NOAEL in the supplementary study.

3.5 Repeated dose dermal toxicity

Subacute study

In a dermal application of 2 ml 0.1 % solution to the skin of rabbits daily, 5 days/week for 4 weeks no systemic effects were observed (Summary Report).

Subacute study

In a 16-day study, F 344/N rats (5 males + 5 females/group) received topical applications of a fixed 250 ml volume of ethanol solutions corresponding to 0, 6.3, 12.5, 25, 50 or 100 mg BTC/kg b.w./day. Animals were treated 5 days per week for a total of 12 doses. No mortality was observed. The body weight development was reduced at 50 or 100 mg/kg b.w., probably related to the stress produced by the skin lesions. There were no other signs of predictive systemic toxicity; absolute and relative weights of thymus were also reduced at these dose levels. Skin alterations were found in most animals; thickening or hardening in rats at 25, 50 and 100 mg/kg b.w.; histopathological skin lesions – ranging from epithelial hyperplasia with minimal inflammation, which were found at all doses, and the intensity of which were dose related – were observed at all dose levels. The no systemic effect level was then at least 25 mg/kg b.w./day.

In another 16 day study B6C3F1 mice (5 males + 5 females/group) received the same BTC dose levels in a fixed 100 µl volume of ethanol solution, 5 days per week for a total of 12 doses. One male died at 100 mg/kg b.w.; a relation with the treatment cannot be excluded. The body weight development was higher than in the controls. The absolute and relative weights of thymus were decreased in 100 mg/kg b.w. females. There were no other signs predicting systemic toxicity.

The skin lesions were more or less similar to those observed in rats. The no systemic effect level was then at least 50 mg/kg b.w./day.

3.8 Subchronic dermal toxicity

In a 13 week study, F344/N rats (10 males + 10 females/group) received topically in an ethanol vehicle (volume not exceeding 300 µl), 0, 1.56, 3.13, 6.25, 12.5, 25.0 mg BTC/kg b.w./day). Animals were treated 5 days per week. Clinical findings were recorded weekly, body weights at the beginning of treatment, weekly thereafter and at the end of the study. Necropsy was performed on all animals and brain, heart, right kidney, liver, lungs, right testis and thymus weighed.

A complete histopathological examination of tissues was performed in control and high dose groups. The skin at the site of application and untreated skin areas were examined histopathologically in all animals. No mortality was observed. At the higher dose, the final mean body weight and body weight gain of males was significantly lower than those of the controls. At the

same dose level, a decrease in the weight of the thymus was noted, postulated to be due to the stress produced by the skin lesions. An increase of the myeloid cells in the bone marrow related to skin inflammation was found at 25 mg/kg b.w. No other direct or potentially systemic alterations were found.

Clinical skin reactions were observed at the site of applications in animals receiving 3.13 mg/kg b.w. or more. Histopathological skin lesions, ranging from epithelial hyperplasia and inflammation to necrotizing ulceration involving the underlying and subcutaneous tissues. These changes were observed in all dosed animals, and their severity was dose related. The no systemic effect level was then at least 12.5 mg/kg b.w./day.

In another 13 week study B6C3F1, mice (10 males and 10 females/group) were topically treated with the same dose levels in an ethanol vehicle not exceeding 100µl, 5 days per week. Clinical findings were recorded weekly, body weights at the beginning of treatment, weekly thereafter and at the end of the study. Necropsy was performed on all animals and brain, heart, right kidney, liver, lungs, right testis and thymus weighed. A complete histopathological examination of tissues was performed in control and high dose groups. The skin at the site of application and untreated skin areas were examined histopathologically in all animals. No mortality was observed. All mice survived to the end of the study. The final mean body weights of all dosed groups of males and females were similar to those of the controls, although the mean weight gain of 25 mg/kg b.w./day males was reduced. Marginal increases of the relative weights of liver and kidney noted in 12.5 and 25 mg/kg b.w./day males were due to the lower body weights and not considered of toxicological significance. No other direct or potentially systemic alterations were found.

Clinical findings included crusting, scales, thickening and reddening of the skin at the site of application in animals receiving 6.25 (males only), 12.5 or 25 mg/kg b.w./day, histopathological lesions from 6.25 mg/kg b.w./day upwards included minimal epithelial hyperplasia, chronic inflammation and focal necrosis of the epithelium involving the underlying dermis and subcutaneous tissues. At 1.56 or 3.13 mg/kg b.w./day minimal epithelial hyperplasia with or without chronic inflammation were present.

The no systemic effect level was there at least 12.5 mg/kg b.w./day.

3.10 Chronic toxicity

In a one year study, groups of 3 dogs were fed 0, 5, 100 or 500 ppm (providing intake levels of 0, 0.4, 8 or 40 mg/kg b.w./day) in the diet. No changes were observed in growth rate, haematology or in gross or microscopic pathology. The NOAEL can be considered greater than 40 mg/kg b.w.

A two-year study has been conducted with groups of 5 rats/sex, fed diets containing 0, 50, 200, 1000, 2500 or 5000 ppm (providing 0, 4, 16, 80, 200 or 400 mg/kg b.w./day). The top dose induced mortality. With 2500 and 5000 ppm testicular atrophy and caecal enlargement occurred. With 1000 ppm there was only caecal enlargement. The NOAEL can be considered at least 80 mg/kg b.w.

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin irritation in rabbits did not occur when 2 ml of a 0.1 % dilution were applied daily 5 days a week for 4 weeks.

In humans, 0.1 ml of a 5 % aqueous solution applied under patches for 48 hours, was irritating.

4.2 Irritation (mucous membranes)

Very slight irritation to the eye of rabbits was produced at concentrations as low as 0.01 and 0.03 %.

It has been considered that an eye irritation study has to be provided at the in-use concentration.

The test was performed on New Zealand White rabbits according to OECD guideline n° 405 (3 rabbits). A 0.1 % aqueous solution of BTC was minimally irritant to the rabbit eye without rinsing after instillation of the solution.

5. Sensitization

A sensitization test in humans with 0.12 % in formulations applied to the skin under closed patches was negative.

It has been considered that a sensitization test must be provided according to the present requirements.

A maximisation test according to the Magnusson and Kligman method was performed according to OECD guideline n° 406 (20 tests and 10 controls DH Guinea Pig). BTC concentrations were selected on the basis of the results of a screening test performed to detect the concentration giving a very slight erythema at 24 hours observation. Filter paper patches saturated with 0.2 or 0.5 % (w/w) aqueous solutions of BTC were applied under occlusion for a period of 24 hours. No skin reactions were noted at the challenge sites of the tested or control group animals at the 24 or 48 hours observation.

6. Teratogenicity

Fertility and reproductive performance were examined in rats treated orally with 1.1, 3.6 and 35.6 mg/kg b.w./day prior to and during mating and during the gestation and lactation period. The high-dose produced growth depression, increased irritability, respiratory signs in the parents and decreased viability and body weight of pups at birth. Fertility and general reproductive performance were not affected. The NOAEL has to be considered higher than 35.6 mg/kg b.w./day for the fertility and reproductive performance.

An oral teratogenicity study in New Zealand white rabbits (15/group) with 1, 3 and 10 mg/kg b.w./day on gestational days 7 to 19 revealed signs of maternal toxicity with 3 and 10 mg, increased mortality of mothers and pups with 10 mg, and an increased incidence of supernumerary ribs with 3 and 10 mg. Supernumerary ribs are known to occur secondary to

maternal toxicity (Khera 1985). No teratogenic effects have been observed; the NOAEL for maternal toxicity and embryotoxicity was 1 mg/kg b.w./day.

In a second teratogenicity study in New Zealand white rabbits (15 to 27/group) with oral dosing of 1.1, 3.6 and 35.6 mg/kg b.w./day, on gestational days 7 to 19, the high dose induced maternal and foetal mortality. A dose-related increase in foetal resorptions occurred in all treatment groups although the change was statistically significant only in the high-dose group. No substance related malformations were found at any dose level. The NOAEL for maternal toxicity and embryotoxicity in this study was 3.6 mg/kg b.w./day.

- In a teratogenicity study in Long Evans (20 per group) rats with oral dosing of 1.1, 3.6 or 35.6 mg/kg b.w./day on gestational days 6 to 15 the high-dose group showed decreased maternal body weight and an increased number of smaller pups. An increased incidence of skeletal variants (ossification effects) occurred in all treated groups.

Skeletal malformation was increased in the high-dose group. Slight hydrocephalus was seen in one pup of the mid-dose group and in 5 pups (in 2 litters) of the high-dose group; workers assume that the delays of ossification, according to their low incidence (almost in one litter) “are secondary to the maternal toxicity and do not represent a primary action of the substance on the embryo”. Nevertheless, the mid dose was not clearly without effect on maternal toxicity.

The study has been renewed by the same workers in Long Evans rats (18 to 20/group) with oral dosing of 0, 0.06, 1.1, 3.6 or 35.6 mg/kg b.w./day, on gestational days 6 to 15; this second teratogenicity study showed lower maternal body weights, increased variation of skeletal ossification and increased incidence of skeletal malformations (wavy ribs) in the top-dose group only. The last finding was considered to be within the limits for historical controls. Under the conditions of this study no teratogenic potential was found. The NOAEL for maternal toxicity and embryotoxicity was 3.6 mg/kg b.w./day.

Peri-and postnatal effects were examined in rats dosed orally with 1.1, 3.6 and 35.6 mg/kg b.w./day from day 15 of gestation through day 20 of lactation. A slight decrease in foetal viability occurred in all dosed groups and in postnatal survival in the mid- and top-dose group. Those findings may be related to the maternal toxicity.

An additional oral teratogenicity study has been carried out (1995). Sprague Dawley CD pregnant rats (24/group) were treated by oral gavage on gestational days 6 to 15. BTC doses were 0, 10, 30, 100 and 170 mg/kg b.w./day in water vehicle, 10 ml/kg volume). All animals were autopsied on day 20 of gestation. Maternal examination included mortality clinical signs, body weight, food consumption and gross pathology; at cesarian section, corpora lutea, implantation sites, resorptions, fetal viability and fetal body weight were recorded. Gross external visceral and skeletal examinations were done on foetuses.

The high dose induced maternal mortality, reduced body weight development, body weight loss, reduction of the food consumption, together with other clinical signs, mainly alopecia, hypersalivation, fur staining, hypothermia, ptosis and abnormal faeces. Necropsy in rats that died during the study presented gastrointestinal lesions e.g. black spots on the mucosal surface of the stomach and gaseous distension of intestine or caecum, possibly corresponding to postmortem alterations.

The treatment did not have any effect on the number of resorptions, litter size, fetal viability or fetal body weights at any dose level.

External visceral and skeletal examinations of the litters did not reveal variations or malformations attributable to the treatment. No significant differences in ossification were found among the five groups.

Under the conditions of this study, maternal toxicity was not evidenced up to 100 mg/kg b.w./day, BTC is not teratogenic nor embryotoxic in CD rats up to the maternal toxic and lethal dose of 170 mg/kg b.w./day.

7. Toxicokinetics (incl. Percutaneous Absorption)

Dermal absorption was examined by applying 10 ml of a 10 % aqueous solution of the ^{14}C-labelled compound under occluded patches to the skin of two rabbits on 4 consecutive days. One rabbit had the skin abraded. Blood samples taken on each day showed an average concentration of 0.2 ppm, which corresponds to 0.003 % of the amount applied. No mention is made of analyses in urine, faeces or carcasses and it is impossible to make any assessment of the total amount absorbed.

Maternal and foetal absorption of the ^{14}C-labelled compound was examined in pregnant rats treated orally with 1.1 and 3.6 mg/kg/day on days 6 through 15 of gestation. Average blood levels in the two groups were 1.5 and 0.97 ng/g respectively. In urine, the maximum levels were 52 and 149 ng/ml after a single oral dose. Virtually all radioactivity was found in the maternal faeces and carcass. Results of foetal analyses varied between not-detectable and 6.8 ng/g foetus.

The percutaneous absorption of a 0.5 % aqueous emulsion has also been investigated in human volunteers by measuring the rate of deposition in the stratum corneum and calculation of the permeability constant.

In a first study using 16 volunteers and a surface recovery method (at 0.5 hour then at hourly intervals from 1-6 hours) two penetration rates were noted. First a rapid transfer to the stratum corneum from the start to one hour, with 4.56 $\mu g/cm^2$ in 30 minutes and 5.19 g/cm^2 in 1 hour. The further penetration rate into the stratum corneum from 1 h to 6 h was calculated as 0.25 $\mu g\ cm^{-2}h^{-1}$. After reaching of the steady flow a percutaneous penetration constant of 50 $\mu g\ cm^{-2}h^{-1}$ was calculated.

In a second experiment using 6 volunteers the amount stored in the stratum corneum after 30 minutes application was determined in another way using an abrasion technique to remove surface layers. The result indicated a similar percutaneous penetration constant, namely about 50 $\mu g/cm^{-2}\ h^{-1}$. According to the rationale of the recovery method, the first experiment recorded both the amount that entered the stratum corneum and stayed there over the entire duration of the experiment, and the amount that remained there the stratum corneum and was transferred to the viable tissue (circa 0.25 $\mu g\ cm^{-2}\ h^{-1}$). The second experiment only confirmed the first figure. The value of this method for measuring skin absorption is controversial, but the data indicated appreciable absorption through the skin can occur with a 0.5 % formulation.

Data from *in vitro* studies using an aqueous emulsion of 0.5 % compound and excised abdominal skin did not, however, indicate any significant absorption. The concentration of benzethonium chloride in the receptor fluid remained below the detection limit (0.1 µg/ml) during the 72 hours exposure.

The additional data provided in Submission VII were essentially a detailed justification of the surface recovery technique which has been used by Pr Agache to determine the BTC skin absorption (see 10.).

8. Mutagenicity

An Ames test with *S. typhimurium* exposed to 100 nmoles/plate and up to 7500 µg BTC/plate was negative.

It was stated that in an *in vitro* assay with CHO cells no evidence was found of sister chromatid exchange or chromosome aberrations, but a report was not available.

More data concerning mutagenicity have been requested.

An Ames test with *S. thyphimurium* strains TA98, TA100, TA 1535, TA 1537 was negative in the absence (0,01 to 1.0 µg BTC/plate) or presence (1.0 to 100.0 µg BTC/plate) of metabolic activation. Activation was brought about by the addition of S9 mix from male rat liver induced by „Aroclor 1254".

A chromosome aberration test in Chinese hamster ovary cells negative in the absence (0.96 to 9.6 µg BTC/ml) or the presence (3.0 to 30 µg BTC/ml) of metabolic activation. Activation was brought about by the addition of S9 mix from male SD rat liver induced with „Aroclor 1254": no statistical significant or dose related increase in chromosomal aberrations, no cell cycle delay were noted.

A sister chromatid exchange assay without activation (0.96 to 9.6 µg BTC/ml) and with activation (3.0 to 30 µg BTC/ml) was negative. Activation was brought about by the addition of S9 mix from male SD rat liver cells induced with „Aroclor 1254". No cell cycle delay was noted.

9. Carcinogenicity

Groups of 60 males and 60 females B6C3F1 mice aged of 5 to 6 weeks were topically treated with 0, 0.15, 0.5 or 1.5 mg BTC kg b.w./day in ethanol vehicle (volume 50-131µl) for males and females 5 days per week for 103 weeks. An interim examination was performed after 15 months. All animals were observed twice daily for moribundity and mortality. Clinical signs were recorded monthly and body weights were recorded weekly through week 10, once during week 12 and monthly thereafter. Necropsy was performed on all animals. At the 15-month interim sacrifice, the left kidney, right kidney and liver were weighted. A complete histopathological examination of tissues was performed in control and high dose animals. The skin at the site of application and untreated skin areas were examined histopathologically in all animals. Survival of dosed mice was similar to that of the controls throughout the study. Mean body weights of all dosed groups were similar to those of the corresponding controls.

Reddening of the skin was observed at the site of application in all dosed male groups and in 0.15 mg/kg b.w./day females. Crusts were observed in 0.5 mg/kg b.w./day females. There were no other clinical findings considered to be treatment related.

There were no increased incidences of neoplasms, in particular, of those associated with the skin, that were attributed to the treatment with benzethonium chloride. Treatment related non-neoplastic lesions at the site of application were epithelial hyperplasia of minimal to mild severity. Epithelial hyperplasia was commonly observed in 1.5 mg/kg b.w./day males and females at the 15-months interim evaluation. At the end of the study, a dose related increase in the incidence of epithelial hyperplasia was observed in males and females.

Under the conditions of this dermal carcinogenicity study there was no evidence of carcinogenic activity of benzethonium chloride in male or female B6C3F1 mice up to the highest dose applied, 1.5 mg/kg b.w./day.

10. Special investigations

Complementary studies:

Several subcutaneous injection studies have been reported in rats and mice.

In one study in rats, a dose-related increase in the incidence of granulomatous reactions (mainly fibrosarcomas) occurred at the injection site.

Concentrations as low as 0.002 % inhibited the motility of the isolated ileum of rats and rabbits. Blood pressure measurements in the dog indicated nearly complete blockage of sympathetic ganglia at an i.v. dose of 2 mg/kg.

11. Conclusions

Classification 1 can be accepted if

- use as preservative is limited at the maximum concentration of 0.1 %;

- use is restricted to the rinse off products.

12. Safety evaluation

Discussion

Determination of the NOAEL dose

In summary, benzethonium chloride has moderate acute toxicity by the oral route and high toxicity following parenteral exposure.

It produced very slight irritation at the maximum “in use” concentration (0.1 %) and significant irritation when applied at a concentration of 5 %. It was not sensitizer to guinea pig or human skin in spite of the use of maximisation in the experiment.

The acceptable NOAEL in a 28 day repeated oral studies in rats was 40 mg/kg b.w./day. The NOAEL in the same animal species was at least 80 mg/kg b.w./day in a 2 years study. The NOAEL was 40 mg/kg b.w./day in a one year toxicity study on dogs.

Benzethonium chloride was administrated dermally to rats and mice in subacute (16 days), subchronic (13 weeks) and carcinogenicity studies (2 years). In all studies, the maximum applied dose was limited by local skin effects of various degrees of severity. In spite of those local reactions, some adverse effects, for which a relation to the treatments cannot be excluded, were noted.

The non systemic observable effects were then respectively 50 mg/kg b.w./day for the 16 day study on mice, 25 mg/kg b.w./day for the 16 day study on rats, 12.5 mg/kg b.w./day for the 13 week studies on mice and rats.

The initial data from teratogenicity studies on Long Evans rats allow 3.6 mg/kg b.w./day to be considered as a no effect level for maternal toxicity and embryotoxicity. In a study reviewed on Sprague Dawley CD Rats, the no effect level for maternal toxicity and embryotoxicity increased to 100 mg/kg b.w./day.

In rabbits, according to a first study the non effect level by oral route was 1 mg/kg b.w./day. However, as the results were considered as questionable, the teratogenicity study has been renewed in the same conditions. The no effect level was then of 3.6 mg/kg b.w./day.

There was no evidence of BTC effect on fertility and reproductive performance, nor in peri or postnatal studies in rats at the highest level used (35.6 mg/kg b.w./day).

There was no evidence of mutagenicity according to results obtained in the *in vitro* Ames tests, chromosome aberration test and sister chromatid exchange assay.

There was as well no evidence of carcinogenicity according to the results obtained in a 103 week mice study (upper dose rate: 1.5 mg/kg b.w./day).

Experimental safety margin

The results obtained concerning the skin absorption of BTC are controversal:

- Experimental data on living animals (rabbits and rats) have shown very light absorption of BTC.

- Absorption has also been investigated in human volunteers according to two methods which have not been entirely validated; nevertheless, according to the authors, they have shown deposition of BTC on the stratum corneum and its transfer to the viable tissues circa 0.25 $\mu g\ cm^{-2}\ h^{-1}$.

- An *in vitro* test using an 0.5 % aqueous emulsion of BTC on excised abdominal skin did not indicated any significant absorption.

According to the authors, “absorption studies with human skin have dealt with three phenomena: the amount that penetrated the stratum corneum *in vivo* and remained within it (circa 5 $\mu g/cm^2$), the amount that entered the viable epidermis *in vivo* (circa 0.25 $\mu g\ cm^{-2}h^{-1}$) and the amount that over 72 hours crossed the full thickness skin *in vitro* (none of the

compound was found to have crossed in these circumstances). The amount stored in the stratum corneum had almost no chance to be transferred later to the viable tissue because of the desquamation of superficial layers where concentration is maximal. Furthermore, surfactants are known to absorb strongly on stratum corneum.

On the other hand, the absorption rate was not found in the experiment *in vitro* which was done under the same environmental conditions (overhydrated stratum corneum) and with a detection limit of 0.9 µg (0.1 µg/ml for a 9 ml receptor tor chamber). By contrast with the *in vivo* experiment which assessed the transfer rate through the stratum corneum only, the *in vitro* experiment assessed the transfer through the whole skin layers. A strong binding of ionized molecules in epidermal and dermal tissues is well known. The surfactant nature of BTC could enhance this phenomenon. Accordingly it is reasonable to conclude that the amount of BTC that penetrated the viable epidermis was almost totally adsorbed by the skin tissue. Therefore, one should conclude that the maximum amount of BTC available to internal organs is below the *in vitro* detection limit, that is 0.9 µg/ 72h/ 3.14 cm^2 for a 0.5 % concentration, which makes about 0.8 ng cm^{-2} h^{-1} for the 0.1 % limit concentration".

It was noted that in Submission V, Industry proposed to limit the use of BTC to areas where there is limited skin contact namely hair care products, deodorants and after shaves.

In such a case, the skin area exposure can be estimated as follows: scalp: 900 cm^2 (and more than 30 000 cm^2 on hair shafts); antiperspirants (axillas) : 900 cm^2 after shave products (face) 900 cm^2, corresponding to a total of 2 700 cm^2.

Applying the indicated penetration rate (0.8 ng cm^{-2} h^{-1}) on the 2 700 cm^2 skin area for 24 hours brings a maximal exposure of 52 µg which would be less than 1µg/kg b.w./day.

In such a case, the safety margin would be, according to a NOAEL = 3.6 mg/kg b.w./day and a systemic exposure dose: 1 µg/kg b.w./day:

$$\mathbf{SM = \frac{3600}{1} = 3600}$$

Results obtained from absorption studies are not convincing as they are quite different according to the method undertaken: no penetration has been demonstrated in a passive form study (*in vitro*), since a light penetration has been obtained by a more active but controversal system in an *in vivo* assay.

Competition may exist between the local reaction induced on skin by BTC and its absorption through the skin. Therefore, an acceptable proper result has no chance to be obtained by additional assays.

Calculated safety margin

According to the all given data, the NOAEL in oral repeated dose studies may be considered as being 3.6 mg/kg b.w./day which corresponds to the lowest oral dose presenting no adverse effect from all acceptable given studies.

The best way to reduce the potential risk resulting from penetration would be to insure that the product is used at a limited concentration (0.1 %) for reduced time of contact with the skin (rinse off products).

According to the SCC general scheme for determining the safety margin of preservatives used in cosmetics (SPC 1247/93 Rev 02.94), the maximum daily use of such type of product is 16.6 g/day ; for rinse off products, it is accepted to assume a rinse off coefficient of 10 % i.e. 10 % retention (and thus assumed as available for absorption through skin). The total exposure to rinse off product is then 1.66 g/day.

Due to the existing experimental data this coefficient can be assumed as highly exaggerated; nevertheless, if we consider such a figure, the maximum daily exposure to BTC is 1.66 mg/day (supposing that a consumer uses all products simultaneously and containing the maximum concentration of 0.1 % at the extensive frequence use), i.e. 27.7 µg/kg b.w./day.

In such case, the safety margin would be

$$\mathbf{SM} = \frac{\mathbf{3600}}{\mathbf{27.7}} = \mathbf{130}$$

P 71: *BENZALKONIUM CHLORIDE, BROMIDE AND SACCHARINATE*

1. General

1.1 Primary name

Benzalkonium chloride, bromide and saccharinate.

1.2 Chemical names

Alkyl (C8-C18) dimethyl benzylammonium chloride, bromide and saccharinate.

1.3 Trade names and abbreviations

Colipa No. P 71
EEC No. Annex VI part 2 No. 16

1.5 Structural formula

1.9 Solubility

Soluble in water and alcohols, poorly soluble in hydrocarbons, oils and fats.

2. Function and uses

Used as a preservative at levels of 0.1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

- Oral LD_{50} - values for rats and mice obtained for commercial products with different alkyl groups usually vary between 0.5 and 1.0 g/kg b.w.
  According to another worker, the oral LD_{50} of benzalkonium chloride in rats (7 dose levels; 8 rats/sex/group) was 234.3 ± 26.5 mg/kg b.w. Signs of gastrointestinal irritation were observed.
- Intravenous LD_{50} - values in mice of 12.8 - 26 mg kg b.w. have been reported.

3.6 Repeated dose oral toxicity

Short-term oral administration to several animal species in the diet or the drinking water containing concentrations of 0.02 % or more induced toxic effects.

3.7 Subchronic oral toxicity

In a 15 weeks study (1951) dogs were fed benzalkoniumchloride at dietary levels of 0.031 %, 0.062 %, 0.125 %, 0.25 % or 0.5 % (single dog) or 1 % (two dogs) corresponding to 15, 31, 62, 125 or 250 or 500 mg/kg/b.w. Pathological changes in the gastrointestinal tract and mortality occurred at 0.5 % and 1 %. Growth rate showed a dose-related decrease at 0.25 % and above. These results indicate that benzalkoniumchloride is toxic to dogs at dietary levels above 0.125 % (250 mg/kg/b.w.) but the number of dogs is too limited for proper evaluation.

In a 90 day study, (1969), a commercial mixture of n alkyl dimethyl benzyl ammonium chlorides and n alkyl dimethyl ethyl benzyl ammonium chlorides was given by gavage to groups of 15 male and 15 female rats at levels of 5, 12.5 and 25 mg/kg b.w., diluted in distilled water (5 ml of test solution/kg b.w.), six days per week.

No significant differences were observed between control and treated groups regarding survival rate, body weight, haematology, autopsy or histology. According to these data, the NOAEL in rats was at least 25 mg/kg b.w. However, the study does not meet present requirements.

In an another 90 day study (1969), the same commercial mixture was given by capsule to groups of 2 male and 2 female dogs at levels of 5 - 12.5 - 25 mg/kg b.w. six days per week. No differences between control and treated groups regarding body weight, haematology, biochemistry, autopsy, organ weight, histology. According to the authors, the NOAEL in dogs was at least 25 mg/kg b.w. However, this study does not meet present requirements. Moreover, because of the incidence of spontaneous pathological findings in controls as well as untreated animals, it is difficult to decide on the no effect level.

3.8 Subchronic dermal toxicity

A dermal 90-day study (1978) was conducted on rats with a formulation containing 1 % stearyldimethylbenzylammonium chloride and 0.2 % benzalkonium chloride 50 %. Once daily, 5 days/week for 13 weeks the rats received topically 2.4 ml/kg (2.4 mg benzalkonium chloride/kg). It is stated that no significant local or systemic effects occurred. However, the report is confusing and incomplete.

3.10 Chronic toxicity

Chronic oral toxicity

In a two-year study (1948) benzalkonium chloride was fed to groups of 12 male and 12 female rats at dietary levels of 0 % (controls), 0.015 %, 0.031 %, 0.062 %, 0.125 %, 0.25 % and 0.5 % (corresponding to approximately 7.5, 15, 31, 62, 125 and 250 mg/kg b.w.). Survival, growth rate and food intake were decreased at 0.5 %. Rats of this group showed pathological changes in the gastrointestinal tract but not in other tissues. No treatment related changes were observed on the other groups; haematology (Hb, RBC, WBC, differential counts) after 13 and 17 months was not affected, and no significant differences in pathology (c.14 tissues) between test and control rats were observed. It seems that no evidence of toxicity was demonstrated at dietary levels of 0.25 % (125 mg/kg b.w.) or below. However, the study does not meet present requirements. Moreover, because of the high incidence of spontaneous pathological findings it is difficult to draw any definate conclusions.

In an another two-year study (1951), benzalkonium chloride was fed to groups of 12 male rats at dietary levels of 0 % (controls), 0.063 %, 0.125 %, 0.25 % and 0.5 %. Rats fed 0.5 % showed extreme diarrhoea and bloated abdomen. All rats of this group died within ten weeks, showing pathological changes in the gastrointestinal tract. Mortality was not affected in the other groups, but occasionally pathological gastrointestinal changes were also found in a few rats fed 0.063 % or 0.25 %. Growth was slightly reduced at 0.063 % and showed a dose-related decrease with increasing dose levels. Despite the limited number of animals in this chronic study, it may be concluded that benzalkonium chloride was toxic to male rats at dietary levels of 0.063 % (31 mg/kg b.w.) or more.

In a poorly documented two-year study on dogs (1959), dogs given 50 mg/kg b.w./day by gavage (at a concentration of 5 %) showed changes in the intestinal tract after one year.

Chronic dermal toxicity

Dermal life-time studies: Mice were treated topically with 0.02 ml of a solution containing 8.5 % or 17 % benzalkonium chloride on the shaved dorsal skin (1-inch area) of groups of 50 females twice a week during life-span (until the animals died spontaneously). The same volume and concentrations were applied to the interior ear of groups of 5 rabbits during life-span. In both species, benzalkonium chloride caused ulceration, inflammation and fibrosis at the applications sites, but no skin tumours. The systemic tumour incidence (e.g. tumours of liver, lungs, lymphatic system, etc.) was similar to that of control animals but the significance of the latter finding is unclear because of methodological deficiencies.

4. Irritation & corrosivity

4.1. Irritation (skin)

Skin irritation tests in rabbits with 0.1 % solutions, and in humans with 1.0 % solutions were negative.

Skin irritation by repeated application:

- With extended contact period in the rabbit, or repeated application in humans, these concentrations produce distinct irritation.
- In rabbits, repeated application of 0.3 % induced only mild erythema.

4.2 Irritation (mucous membranes)

Eye irritation in rabbits may occur upon a single application of 0.01 % solution and above and upon repeated application of 0.004 %.

Concentrations of 0.01 % and above caused eye irritation in guinea pigs when applied repeatedly on the same day.

Single treatment of human eyes with 0.1 %, or daily treatment with 0.03 - 0.04 % caused irritation.

Soft contact lenses disinfected daily with 0.0025 % benzalkoniumchloride + 0.01 % EDTA induced severe irritation when brought into contact with the rabbit eye for 6 hrs/day.

5. Sensitization

A sensitization test in 100 male and 100 female volunteers with 0.1 %, applied daily for 5 days, followed by a challenge treatment with 1 % after 3 weeks, was negative. In the literature only a few cases of sensitization in humans have been reported.

6. Teratogenicity

In an oral teratogenicity study, groups of 15 pregnant rabbits were treated by gavage with 0, 10, 30 or 100 mg/kg/day (in aqueous solutions of 0.5, 1.5 and 5.0 % respectively) from day 7 through day 19 of gestation. All rabbits of the high dose group died. The intermediate dose caused maternal and embryotoxicity. Signs of maternal toxicity occurred also in the low-dose group (10 mg/kg b.w.) but appear related to the gastrointestinal irritation due to the tested ingredient. There were no indications of teratogenic properties.

A dermal teratogenicity study was conducted in rats treated topically with 0.5 ml aqueous solutions of 1.6, 3.3 and 6.6 % (estimated to be about 30, 60 and 120 mg/kg) once daily from day 6 to day 15 of pregnancy. No teratogenic nor maternal adverse effects were observed.

7. Toxicokinetics (incl. Percutaneous Absorption)

Skin penetration tests *in vitro* with human skin were conducted in aqueous solutions of 0.005 M to 0.1 M benzalkoniumchloride (i.e. 0.17 to 3.4 %). No penetration into the dermis was detected when the solution was unbuffered or acid. Measurable penetration occurred when the epidermal barrier was damaged or with intact skin in solutions of pH 11.

No penetration was found *in vitro* with skin from hairless rats exposed to 2.5 % ^{14}C-dimethyl-benzylammoniumchloride for 4.5 hours.

In a similar *in vitro* test with human epidermis the mean penetration was 1.47 % of the dose applied.

However, results from an *in vivo* study to measure percutaneous absorption in rats indicate much higher absorption than indicated from the *in vitro* data. C^{14}-radiolabelled compound (0.4 ml) was applied to shaved skin of groups of 6 male and 6 female rats under occlusive dressing for 72 hours and the amount of material excreted in the urine and faeces during that time measured; the amount remaining in the carcass of the animals was also determined at that time. In the female animals values of 0.7 ± 0.4 %, 6.1 ± 3.4 % and 7.0 ± 2.2 % were obtained for urine and faecal elimination and remaining in the carcass respectively. The corresponding values in the male animals were 0.8 ± 0.3 %, 9.9 ± 2.6 % and 5.3 ± 1.6 % respectively. The bulk of the applied dose remained on the treated skin. These data indicate that 14 % of the applied dose was absorbed in the females and 16 % in the males, giving an overall value of 15 %.

The distribution of the compound was studied after oral, rectal and intramuscular administration of 10 times lethal dose to rabbits, dogs and cats. Most of the dose remained at the application site. After oral and rectal administration, small amounts were detected in blood and liver. Upon rectal administration, a small amount was found also in the kidneys.

8. Mutagenicity

- AMES test with *S. typhimurium* strains TA 98, TA 1538, TA 1537, TA 100 exposed to 10-100 µg/plate was negative.

- A micronucleus test in mice treated i.p. with 20 mg/kg b.w., twice, with an interval of 24 hours did not reveal increased numbers of micronuclei.

- The substance was found to induce repairable DNA damage in the *E.coli* DNA polymerase A assay with 20 µl/disc of a 0.01 to 1 % aqueous solution, but no mutagenic properties were observed.

- No forward mutations were induced in *Schizosaccharomyces pombe* P_1 with or without metabolic activation.

- A chromosome aberration test with CHO-cells *in vitro* was negative.

10. Special investigations

Further to the SCC request, Colipa has provided a submission VI including:

- The report of a 2 weeks preliminary toxicity study on benzalkonium chloride by oral route (gavage versus dietary admixture) in rats.

- A calculation of cosmetic exposure to benzalkonium, based on the figure of the Colipa document 93/067 dated February 1993, discussed with SCC on May 1993.

- An evaluation of the safety factor, based on a 100 mg/kg/day NOAEL.

- Comments and conclusions indicating industry believes that:

  - a further 90 days study to support cosmetic uses of P71 is not justified,
  - concentration should not be reduced, and proposing to permanent allow benzalkonium chloride, bromide and saccharinate in cosmetics products in the following way:

Annex VI part 1	0.1 %
Annex Ill part 1	3 % rinse off hair products
	0.5 % other products

2 weeks toxicity by oral route in rats

Aim of the study:

The objective of this study was to define the mode of administration and the doses of the test substance benzalkonium chloride (Myristalkonium, Pharmascience) when administrated to Sprague Dawley rats during a 90 days toxicity study.

The study was designed in an attempt to minimize the known direct effects of the test substance on the gastrointestinal tract and to determine a suitable method of administration for a chronic toxicity study.

Therefore, test substance was administered orally at different levels over a period of 17 days, in parallel to groups of rats by gavage and by dietary admixture, and the effects were compared.

General protocol:

7 groups of Sprague Dawley rats, each of 5 male and 5 female animals were constituted:

Group	Mode of administration	Animals per group	Nominal dose (mg/kg/day)
MALES			
1	GAVAGE	5	0
2	GAVAGE	5	3
3	GAVAGE	5	10
4	GAVAGE	5	30
5	ADMIXTURE DIETARY	5	10
6	ADMIXTURE DIETARY	5	30
7	ADMIXTURE DIETARY	5	100
FEMALES			
1	GAVAGE	5	0
2	GAVAGE	5	3
3	GAVAGE	5	10
4	GAVAGE	5	30
5	ADMIXTURE DIETARY	5	10
6	ADMIXTURE DIETARY	5	30
7	ADMIXTURE DIETARY	5	100

- The vehicle for groups 1, 2, 3, 4 was water for injection; a constant dose volume of 10 ml/kg/day was used.

- The vehicle for groups 5, 6, 7 was AO4C powder diet supplied ad libitum.

Clinical signs and mortality were checked at least once a day, food consumption and body weight were determined twice a week. Haematological and blood biochemical examinations were carried out and at the end of week 2 in all animals. At the end of the study, all animals were subjected to macroscopic examination; stomach, duodenum, liver, kidneys and all macroscopic lesions of all animals in the control and high dose group (groups 1, 4 and 7) were subjected to histopathological examination.

This study was conducted in compliance with the Good Laboratory Practice Regulation.

Results:

Preliminary observation:

An analysis mistake occurred in the report regarding justification of the use of the oral route recourse in the study of long term toxicity of benzalkonium chloride: in fact, if a 1 % concentration was administered daily to the rat at a volume of 5 ml per rat, according to an estimated weight of 250 g/rat, this would lead to an applied dose of 200 mg/kg/day and not 50 mg/kg/day as indicated in the report.

This error does not influence the study itself.

Clinical signs:
Ptyalism and loud breathing were observed in the groups treated by gavage at 10 or 30 mg/kg/day.

No treatment-related clinical signs were observed in the groups given the test substance by dietary admixture.

Mortality:
The only mortality observed during the treatment period was considered to be unrelated to administration of the test substance.

Food consumption:
Slightly lower mean food consumption was observed in the males treated by gavage at 10 or 30 mg/kg/day when compared to that of controls. No effects on food consumption were noted in the animals treated by dietary admixture.

Body weight:
A trend for lower mean body weight gain was observed in animals of both sexes treated by gavage at 10 and 30 mg/kg/day when compared to that of respective controls. No effects on body weight gain were noted in the animals treated by dietary admixture.

Haematology and blood biochemistry:
No differences of toxicological significance were observed between control and treated animals at the end of the treatment period.

Macroscopic and microscopic examinations:
No macroscopic or microscopic findings attributed to the administration of the test substance (by gavage or dietary admixture) were noted at the end of the treatment period.

A slight local coagulative hepatocellular necrosis was noted in the liver of a male given 100 mg/kg/day in the diet, considered to be of no toxicological importance.

In view of the results as a whole, a reasonable NOAEL may be considered is 30 mg/kg b.w.

Cosmetic exposure calculation

Colipa has presented the following table indicating the cosmetic exposure P 71 and calculated a safety margin of 709 according to its different uses:

P 71 - Cosmetic Exposure and Safety Factor

	UNITS	PRESER-VATIVE	INTIMATE HYGIENE	SKIN PROD. NON-RINSE	HAIR PROD. NON-RINSE	HAIR PRODUCT RINSE OFF	ALL USES
Daily exposure/ product	mg/kg/d	$21.44.10^3$	3.10^3	2.10^3	1.10^3	$0.16.10^3$	$27.6.10^3$
Daily cosmetic use	mg/d	$21.44.10^3$	3.10^3	2.10^3	10.10^3	16.10^3	
Max concentration	%	0.1	0.5	0.5	0.5	3	
Daily exposure/P 71	mg/kg/d	0.357	0.250	0.167	0.083	0.080	0.937
Daily bioavailability	mg/kg/d	0.0536	0.0375	0.025	0.0125	0.012	0.141
Safety Factor		1866	2670	4000	8000	8330	709

The parameters considered were the following:

- Limitations as preservative to 0.1 %
- 10 % scalp/hair repartition coefficient
- 10 % rinse-off coefficient
- Body weight 60 kg
- Cutaneous penetration 15 %
- NOAEL: 100 mg/kg/day

} NOAEL

According to the table, it is suggested that P 71 is used:

- as preservative in cosmetics products corresponding to only 21.44 gr total daily use,
- in non rinse products, only in those applied to the face. In that case, a 2 g/day cosmetic use, indicated as a realistic figure, is more than the quantity indicated in the general scheme SPC/1247/93 rev. 02 94 (1.6g/day in case of twice a day application).
- It is also suggested that the daily cosmetic use of intimate hygiene products is 3 g/day.

11. Conclusions

In the absence of long term studies able to define a clear non effect level by the oral route, it is suggested :

- to limit the concentration of P 71 at 0.1 % as preservative,
- to reduce its concentration for “other uses” to 0.1 % w/w for intimate hygiene, skin non rinse (face only), hair non rinse products,
- to maintain an allowed maximum 3 % concentration for “other uses” in rinse off hair products,

- to clearly identify products or sites where the "other uses" employment of P 71 can be accepted,

- because of the irritancy of the compound at concentration below the in use levels, to carry the label "avoid contact with eyes" on all products containing P 71.

If so, classification 1 can be accepted.

Classification: 1

12. Safety evaluation

Discussion: Determination of the NOAEL dose

According to the provisional data submitted

- Benzalkonium possesses considerable irritant properties for the eyes and the gastrointestinal tract and was considered highly toxic, under certain conditions of acute exposure which seem not relevant to exposure in use.

- In the previous subchronic and chronic toxicity studies by gavage in rats, some evidence of irritation to the gastrointestinal tract was seen at the lowest dose used (12.5 mg/kg/b.w.) but no other effects were seen; more marked toxicity was observed at 25 mg/kg b.w. and above. In long term studies, when benzalkonium chloride is delivered in diet, systemic toxicity appears at higher doses (31 to 125 mg/kg b.w.).

Changes of the intestinal tract were also reported in long term studies when dogs are fed at dietary levels of 50 mg/kg b.w.

- In an oral teratogenicity study in rabbits some effects were reported on the maternal animals at the lowest dose investigated (10 mg/kg b.w.). There was no effect on the developing fetuses.

- There was no evidence of mutagenic and sensitising potentials.

According to the new data submitted (submission VI)

- As might have been foreseen, considering previous studies, in the new 14 days study, administration by gavage disturbs animals from the dose of 10 mg/kg/b.w./day. In the absence of clinical signs and dose related effect from 10 to 30 mg/kg/b.w./day, it seems realistic to consider the 30 mg/kg/b.w. dose as not toxic.

However, this result is obtained on a limited duration test, inconsistent with teratogenicity test in rabbits which suggests emergence of a gastrointestinal maternal irritation at 10 mg/kg/b.w. day and above.

The addition of benzalkonium chloride in the food verifies a lack of reaction, particularly gastrointestinal, until a dose of 100 mg/kg/b.w. day has been achieved.

Gavage seems therefore to have an influence on the gastrointestinal tolerance.

- Limited explorations made in the complementary short-time study do not allow the prediction of what could occur after a 90 days study at same doses. Nevertheless, the results obtained with

the previous longer term toxicological studies, have shown no real toxic effect other than the irritation of the intestinal tract, related to gavage, at similar doses.

The study duration seems to have no influence in the potential toxic and irritating effects of benzalkonium chloride.

Taking everything into consideration, 30 mg/kg b.w. should be retained as the NOAEL.

Calculation of the safety margin:

The daily product exposure indicated by Colipa (21.44 g/kg b.w.) is correct as it considered the maximum exposure indicated in the SCC general scheme (27.6 g/kg b.w.) minus applications where benzalkonium chloride is also employed for "other uses" (e.g. intimate hygiene: 3 g; skin non rinse products: 2 g; hair products non rinse: 1 g; hair products rinse off: 0.16 g).

In such conditions, the daily bioavailability of P 71 is effectively 0.141 mg/kg b.w./day.

and the safety margin $SM = \frac{30}{0.141} = 212$

According to the above mentioned comments, regarding the gastrointestinal irritancy occurring by gavage at levels upper than 30 mg/kg b.w. in most species, a further 90 days study at higher dose levels is not justified. In this case, however, the given safety margin (212) in the lack of reliable NOAEL appears rather low; it is then suggested to reduce the concentration of Preservative P 71 to:

3 % for the hair rinse off products

0.1 % for all "other uses".

The maximum daily bioavailability in cosmetics products would then be 0.0806 mg/kg b.w./day as explained in the table:

	UNITS	PRESER-VATIVE	INTIMATE HYGIENE	SKIN PROD. NON-RINSE	HAIR PROD. NON-RINSE	HAIR PRODUCT RINSE OFF	ALL USES
Daily cosmetic use	mg/d	$21.44.10^3$	3.10^3	2.10^3	10.10^3	16.10^3	
Repartition and/or rinse off coefficient	%				10 %	100 %	
Daily exposure/ products	mg/kg/d	$21.44.10^3$	3.10^3	2.10^3	1.10^3	$0.16.10^3$	$27.6.10^3$
Max concentration		%	0.1	0.1	0.1	0.1	3
Daily exposure/P71	mg/kg/d	0.357	0,05	0.03	0.017	0.080	0.534
Cutaneous penetration	%	15 %	15 %	15 %	15 %	15 %	
Daily bioavaila-bility	mg/kg/d	0.0536	0.0075	0.005	0.0025	0.012	0.0806

The safety margin would then be:

$$SM = \frac{30}{0.0806} = 372$$

- in all cases, cases for which “other uses” are accepted must be clearly defined.

P 91: *3-IODO-2-PROPYNYL BUTYLCARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butylcarbamate

1.2 Chemical names

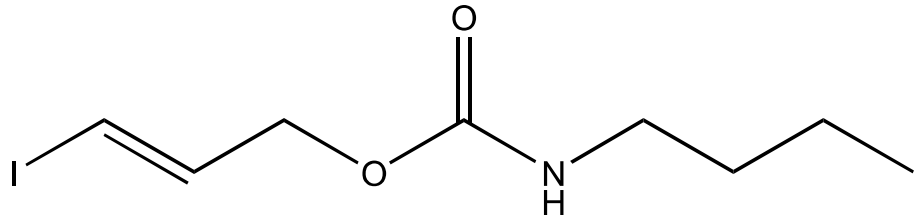
3-iodo-2-propynyl butylcarbamate

Iodopropynyl butylcarbamate

1.4 CAS no.

55406 53 6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}NO_2I$

1.9 Solubility

Low solubility in water (156 ppm at 20°C) and is soluble in organic solvents.

2. Function and uses

It is proposed for use as a preservative in all types of cosmetic products at levels up to 0.05 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal signs of toxicity were seen at 500 mg/kg or below.

3.7 Subchronic oral toxicity

In a subchronic study, rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg. Histological examination showed hepatocyte enlargement at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The no-effect level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritation study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe edema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours.

The potential of P 91 to produce primary skin irritation in humans following a single topical application was examined. Amounts of 0.2 ml corn oil containing l % and 3 % P 91 were applied onto the upper back, nonabraded skin of six subjects using occlusive patches. The patches were removed 24 hours after application and skin readings were performed 30 minutes, 24 hours and 48 hours after patch removal.

The l % solution of P 91 was slightly irritating to the majority of the study pannel (one subject showed no irritation, four subjects showed faint erythema and one subject showed moderate erythema 30 minutes after patch removal). The 3 % solution was moderately irritating to the majority of the pannel (two subjects showed faint erythema and four subjects showed moderate erythema 30 minutes after patch removal). There was no evidence of edema in any of the subjects, and erythema was generally less intense or no longer apparent 24 and 48 hours after patch removal.

The potential of concentrations of 0.0, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 % P 91 in corn oil to produce primary skin irritation in humans following a repeated topical application (three times over a five day period) was examined. Amounts of 0.2 ml P 91 in corn oil were applied onto the upper back, nonabraded skin of 7 subjects using semi-occlusive patches (1 x 1 inch). The patches were removed 24 hours after application. Skin readings were performed 24 hours (first two applications) or 48 hours (final application) after patch removal. In all subjects, observation of all treated areas remained negative throughout the test.

The potential of P 91 to produce primary skin irritation or sensitization in humans following repeated topical application was examined. Amounts of 0.2 ml corn oil containing 1 % P 91 were applied onto the upper back, nonabraded skin of 170 subjects using semi-occlusive patches (1 x 1 inch). Treatment was conducted three times a week for a total of ten applications (24 hours contact per application); skin readings were made 24 or 48 hours after patch removal.

Challenge (1 % P 91 in corn oil) was conducted on both the application site and a virgin site 14 days after the tenth application; each site was evaluated 24 and 48 hours after application.

No significant irritation was observed during induction. Upon challenge, all test areas remained negative in all subjects.

The potential of a formulation containing P 91 to produce primary skin irritation or sensitization in humans following repeated topical application was examined. The study was conducted with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission). Amounts of 0.2 ml corn oil containing 1:50 and 1:100 aqueous dilutions were applied 10 times (3 times/week) onto the upper back, nonabraded skin (1 inch by 3/4 inch) of 51 subjects using semi-occlusive patches. The patches were removed 24 hours after application. Challenge application (1:50 and 1:100 aqueous dilutions) were made to the induction site and to a virgin site, 14 days after the last application, and skin readings were performed 24 hours and 48 hours after patch removal. The dilutions of a 2.6 % formulation of P 91 (viz. 0.026 % and 0.05 % P 91) did not induce any skin reactions throughout the study. The significance of this study for assessment of the sensitization potential of P 91 is doubtful.

4.2 Irritation (mucous membranes)

Severe effects were noted in an eye irritation study in rabbits. The substance (0.1 g) produced moderate to severe hyperaemia, chemosis, discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. If the compound was washed out of the eye 20-30 seconds post instillation, only transient irritant effects were seen.

The eye irritancy of a 0.5 % solution of P 91 in corn oil as well as the effects of 0.5 % P 91 in a baby shampoo have been tested in rabbits. Groups of 6 animals were used in each case. No signs of any irritant effects were seen with the corn oil formulation. In the case of the baby shampoo, slight irritant effects were seen for about 24-48 hours, but similar effects were seen in ‘control’ baby shampoo that did not contain P 91. Thus 0.5 % P 91 in corn oil or in a baby shampoo formulation did not produce eye irritation.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximisation test. Induction concentrations were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson & Cligman maximisation tests have been carried out on formulations containing 0.5 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical) were used. In the second study the concentrations were 0.1 % and 0.5 %, respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation. There was no evidence of sensitization in either test. These studies suggest that P 91 does not have potential for sensitization.

The potential of P 91 to produce photosensitization was examined in guinea pigs (adapted Buehler method). Induction was conducted with a 5 % (w/v) formulation in PEG400 (0.3 ml over a 25 mm area, occluded during four hours, three times a week for three consecutive weeks). Thirty minutes after removal of the occlusive dressing, animals were irradiated with UVA and UVB for two hours. Appropriate control groups were included. Challenge and rechallenge of treated and naive animals was conducted with 5 % (w/v) in PEG400 (+UV), 12 and 19 days after induction, respectively.

Equivocal evidence of photosensitization was obtained. After primary challenge positive results were obtained (3/10 animals showed grade 1 (slight but definate) erythema whereas all other animals in the naive and induction group showed a $\pm$ (barely perceptible) reaction). However, the three grade 1 responses were not re-elicited after rechallenge (maximum grades of $\pm$ were observed in both the induction and the naive group). Clear conclusions can not be drawn, the more so as the dose used for induction may have been relatively low.

A guinea pig photosensitization study was conducted with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission).

Induction was conducted with a 25 % solution (0.1 ml over the nuchal area, non-occluded, five times a week for two consecutive weeks). The animals were irradiated with UVB (4.5 J/cm^2; a unusually high dose) and UVA (10J/cm^2). Control animals were not irradiated during induction. Challenge of treated and naive animals was conducted with 2.5 %, 5 % and 25 % aqueous solutions (+ UVA), 17 days after induction.

Irritation was not observed at the start of the induction phase but reached maximum severity (erythema and edema) after four treatments. Following challenge with the 25 % solution, clear skin effects were observed in all animals of the induction group but not in the naive animals. No skin effects were observed at the 2.5 % or 5 % test sites following challenge. It was concluded that the test article was a photoallergen at 25 %. The significance of this study for assessment of the photosensitization potential of P 91 is not clear.

The potential for P 91 to absorb light in the ultraviolet and visible spectrum (in the range of 190 to 800 nm) was determined. Maximum absorbance occurred at 191 nm (extinction coefficients were 6570 L/mol-cm at pH 5 and approx. 6000 L/mol-cm at pHs 7 and 9). A smaller absorbance peak was detected at 227 nm (extinction coefficents were approx. 500 L/mol-cm at pHs 5, 7 and 9). No other wavelength maxima were detected.

6. Teratogenicity

Teratogenicity studies have been carried out in both the rat and the mouse. In the study in rats compound was given on day 6-15 of gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The no-effect level was 50 mg/kg. A similar dosing regime was used in the study in mice. No compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The no-effect level was 125 mg/kg.

6.2 Two-generation reproduction toxicity

A two-generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week pre-mating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound-related effects were seen at any dose level on clinical chemistry or at necropsy. Slightly reduced weight gain was seen in the males at 750 ppm during the premating period in both the initial generation and the F1 generation. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index (= no. of pups alive at day 1/ total number of pups) at 750 ppm in either generation, while a marginal effect was also noted at 300 ppm in the F1 generation. Postnatal growth of the offspring however was not affected and no effects were seen on the development of the offspring. The no-effect-level in this study was 120 ppm test compound in the diet (roughly equivalent to a dose of 10 mg/kg b.w./day).

7. Toxicokinetics (incl. Percutaneous Absorption)

In a percutaneous toxicity study in rabbits, a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing did not result in deaths. The only signs of toxicity seen were slight irritant effects at the application site.

The potential skin absorption of P 91 was examined in an *in vitro* skin penetration study with previously frozen, excised human cadaver skin (thickness 400-800 µm; epidermis + papillary dermis). Five µl of a 0.1 % solution of ^{14}C-labelled P 91 in acetone (approx. 6 µg P 91/cm^2 skin) were applied to six samples from each of four donors (total 24 skin samples). Since a constant air flow was maintained through the evaporation cell (10 cell volumes/min) it may be assumed that the acetone (5 µl) instantly evaporated. The amount of radiolabel in the receptor fluid bathing the visceral side of the dermis was determined periodically during the 24-hour experimental period. Excess radiolabel on the epidermal surface of the skin was removed (by two successive tape strips) 24 hours after application. Radiolabel evaporating from the epidermal surface was trapped in vapor traps and quantified periodically during the 24-hour experimental period.

The mean ($\pm$ SD; n = 24) skin penetration (the sum of radioactivity recovered in the dermis and receptor fluid) was 54 $\pm$ 12 % (38 $\pm$ 5 %, 54 $\pm$ 10 %, 55 $\pm$ 3 % and 68 $\pm$ 6 % for the respective individual donors). Peak penetration into the receptor fluid occurred within 2-8 hours of application for all donors. 14 $\pm$ 3 % of the applied radioactivity evaporated from the skin surface during the 24-hour experimental period. Overall recovery of radioactivity was 87 $\pm$ 10 % of the applied radioactivity.

Under the conditions of this study 54 % of a 6 µg/cm^2 dose of P 91 penetrated excised human skin during a contact period of 24 hours. It may be noted that P 91 was delivered to the skin as a thin film during a full 24 hour contact period. Vehicles used to formulate P 91 as well as the time they remain on the skin will likely influence skin absorption of P 91.

8. Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies.

Negative results were obtained in the Salmonella assay versus strains TA1535, 1537, 1538, 98 and 100 but this study was limited by investigating only 3 concentrations (6.2 - 55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537, 98 and 100 and concentrations of 1-1000 µg/plate against TA 1535. In all cases the top concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 % - 25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

A phototoxicity study was conducted in guinea pigs with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission). Aliquots (0.1 ml) of 2.5 %, 12.5 %, 18.75 % and 25 % aqueous formulations were applied topically on the depilated dorsal skin of 10 guinea pigs. Thirty minutes after application the animals were irradiated with UVA. No irritation was observed with 2.5 % and 12.5 % concentrations, with 18.75 % questionable erythema was noted in a number of animals while questionable to minimal erythema was noted with 25 % in a number of animals. Based on these results the formulation was not considered to be phototoxic.

The significance of this study for assessment of the phototoxicity of P 91 is not clear.

The comodogenic potential of P 91 was examined in 12 human subjects with a history of acne. The test substance (0.1 % P 91 in white cream) and the controls (blank patch, vehicle control and positive control Acetulan) were applied to 4 x 4 cm areas on the upper back (non-abraded skin) using occlusive tape for three times a week for four weeks, resulting in a 28-day continuous period of exposure. Folicular biopsy samples were taken at the end of the exposure period and comodeone density was determined stereomicroscopically. Several subjects showed moderate erythema at the 0.1 % P 91 site during treatment.

Acetulan increased the production of comodones in this study. It was stated that 0.1 % P 91 in white cream was not comedogenic, but the scoring scale was not clearly presented.

A quantitative structure activity relation (QSAR) analysis was conducted to evaluate the potential of P 91 or structurally related chemicals to produce sensitization in humans. The

Contact Allergens Database Evaluation System (CADES), the national Library of Medicine (NLM) and the STN International online databases were searched.

No references indicative of skin sensitization were found for P 91 and structurally related chemicals (e.g. those containing carbamic acid H_2NCOOH, butyl carbamate, propynyl-iodide moiety R-CH_2CCI, or propynyl-halogen moiety). The closest structurally related compounds with potential skin sensitization are the dithiocarbamates ($H_2NC(S)_2$) such as the fungicides Maneb, Zineb and Ziram.

A QSAR analysis, focused on the aliphatic carbamate and iodoacetylene moieties of P 91, was conducted to assess the potential to produce sensitization in humans.

No evidence was found indicating that P 91 is sufficiently protein reactive to initiate a sensitization reaction. Also no reference to either allergy or irritation by P 91 was found in a MEDLINE search.

In a literature search no evidence was found for cross-reactions of 3-iodo-2-Propynyl butyl carbamate with dithiocarbamates used in the rubber industry. This information is, however, considered unsatisfactory proof for the absence of cross sensitivity.

A human cross sensitization study was conducted to determine the potential for P 91 to elicit skin contact cross sensitization reaction in humans with an existing allergy to dithiocarbamate compounds. Ten volunteers with a history of sensitivity to Thiuram Mix (European Standard Patch Test battery No. 3, consisting of 0.25 % w/w of each of tetramethylthiuram monosulfide, tetramethylthiuram disulfide and dipentamethylene thiuram disulfide), received a single application of a 0.1 % concentration of P 91 in soft yellow petrolatum (0.2 ml; 3 cm^2 area; occluded; 24-hr period of contact), as well as a patch containing petrolatum alone (vehicle control). The sites were examined 48 and 96 hours after application. No noticeable skin reactions were observed in any of the 10 volunteers. It was concluded that P 91 does not cause cross sensitization reactions in humans with a known sensitivity to dithiocarbamate compounds. It should be noted, however, that the challenge concentration of P 91 (0.1 % in petrolatum) was rather low, exposure lasted for only 24 instead of 48 hours and sensitivity to dithiocarbamates was not confirmed prior to the start of the study.

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C-radiolabelled material. Following iv administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the level and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicate that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

The compound is a carbamate and studies have been carried out to investigate whether significant cholinesterase (ChE) activity inhibition occurs in rats following intravenous administration. P 91 was given in PEG400: water vehicle at 2-16 mg/kg and blood samples

were taken and analysed for erythrocyte ChE activity at 15, 30 and 60 minutes and 5 hours post dosing. No effects on blood cholinesterase activity were observed.

Data on minimum inhibitory concentrations of 3-iodo-2-propynyl butyl carbamate demonstrated the efficacy of this compound at levels ≤ 0.1%.

11. Conclusions

The test substance has relatively low acute oral toxicity ('harmful if swallowed' according to EEC criteria) and is not harmful following acute dermal exposure. It is a mild to moderate skin irritant in rabbits. In humans, exaggerated exposure conditions (1 % - 3 % P 91, 24 hour occlusion) resulted in transient, slight to moderate irritation. Repeated semi-occluded application of formulations containing levels up to 1 % P 91 did not induce any skin reactions in humans.

The pure substance is a severe (corrosive) eye irritant; however, formulations containing 0.5 % did not produce any eye irritation.

No evidence of sensitization was obtained in a Magnusson & Kligman maximization test. A human repeated insult patch test with 1.0 % P 91 in corn oil did not reveal any sensitizing potential. In a literature search, no evidence was found for cross-reaction of P 91 with dithiocarbamates used in the rubber industry. P 91 did not cause cross sensitization reactions in humans with a known sensitivity to Thiuram Mix. The significance of the data provided on cross sensitization may, however, be doubtful.

Equivocal evidence of photosensitization was obtained with pure substance and with a formulation containing 2.6 % P 91 besides other active ingredient(s). QSAR analysis did not reveal evidence of sensitizing potential for P 91 or closely related compounds. P 91 (0.1 % in white cream) was not found to be comedogenic in humans. The ultraviolet-visible absorption spectrum of P 91 showed two absorbency peaks (at 191 and 227 nM).

Mutagenic potential has been investigated in Salmonella assays for gene mutation, in a study to investigate unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro,* and in an *in vivo* micronucleus test. Negative results were consistently obtained. There was no evidence of teratogenic potential in studies in two species (rats and mice). In a two-generation reproductive toxicity study in rats a reduction in life birth index was observed. The no-effect-level in this study was 120 ppm in the diet (c. 10 mg/kg b.w./day). An *in vitro* skin penetration study indicated considerable (ca. 54 %) dermal absorption. The compound is well absorbed orally but is rapidly metabolised and excreted.

Conclusion

- With the below restrictions, the safety margin of P 91 would be acceptable if used in cosmetic products alone. However, concern was raised about application of P 91 in other products. Therefore industry should provide a realistic estimate of the exposure to P 91 from sources other than cosmetics.

- The provided human cross sensitization study was unfit to determine the potential for P 91 to elicit skin contact cross sensitization reaction in humans with an existing allergy to dithiocarbamate compounds. A proper cross sensitization study should still be provided.

- As this compound has been under consideration for a very long period, the information requested should be available within half a year.

Classification: 2

12. Safety evaluation

CALCULATION MARGIN OF SAFETY

In Submission VII (April 1996) industry proposed to restrict the fields of application as well as the concentration of P 91 in the following way:

- the concentration will be reduced to 0.05 % (instead of the 0.5 % originally requested),
- P 91 would not be allowed for use in oral hygiene products and lip products.

With these restrictions the calculation of the Margin of Safety is as follows:

Exposure

- Total oral hygiene products: not applicable
- eye products: 0.06 g
- non-rinse products: 20.3 g
- rinse-off products: 1.7 g
- Total systemic exposure (SED): 22.06 g x 0.05 %[(1)] x 54 %[(2)] = 5.956 mg/human/day
  = 0.0993 mg/kg b.w [(3)]/day

Margin of Safety =	**NOAEL/SED = 10/0.0993 = 101**

[(1)] Maximum level of use (0.05 %) is assumed.

[(2)] 54 % skin absorption is assumed.

[(3)] 60 kg/human is assumed.

S 28: *2-ETHYLHEXYL-4-METHOXYCINNAMATE*

1. General

1.1 Primary name

2-ethylhexyl-4-methoxycinnamate

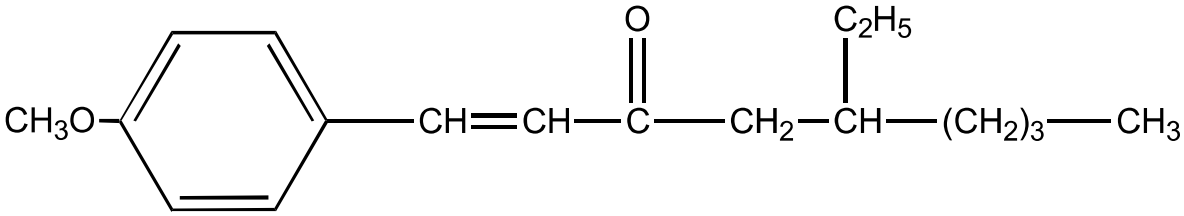
1.2 Chemical names

2-ethylhexyl-4-methoxycinnamate

1.3 Trade names and abbreviations

Parsol MCX

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{18}H_{26}O_2$
Mol weight: 290.

1.8 Physical properties

Appearance: Colourless pale yellow slightly oily liquid.

1.9 Solubility

Miscible with alcohols, propylene glycol, etc.
Immiscible with water.

2. Function and uses

Use level up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}: Mouse, greater than 8 g/kg b.w. Rat, greater than 20 ml/kg b.w.

3.4 Repeated dose oral toxicity

Rat. Three week oral study. Groups of 5 male and 5 female animals were given 0, 0.3, 0.9 and 2.7 ml/kg b.w./day by gavage for 3 weeks. All animals of the top dose groups exhibited loss of body weight and a reduced relative and absolute weight of the thymus. Male rats showed a decrease in absolute weight of the left kidney and female rats showed a decrease in the absolute weight of the heart. At the two lower doses, the only significant alteration observed was an increased absolute weight of the pituitary gland in male rats receiving the lowest dose. As the number of animals was small, the investigators considered this not to be biologically significant. The NOAEL was put at 0.9 ml/kg b.w./day.

3.7 Subchronic oral toxicity

Rat. Thirteen week oral study. Four groups of 12 male and 12 female SPF rats received the compound in the diet at levels of 0, 200, 450 and 1000 mg/kg b.w./day. During the experiment the usual clinical observations were carried out, as well as extensive haematological and biochemical studies. Full gross necropsy was carried out on all survivors. Histological investigations were carried out in half the animals of the control and top dose groups. The organs studied included the heart, lungs, liver, stomach, kidneys, spleen, thyroid and retina. In the remaining animals histological examination of the liver only was carried out. Six control animals and six top dose animals were allowed to recover over 5 weeks, and then examined.

The results of the experiment showed no dose related mortality. The kidney weights of top dose animals were increased, but were normal in the recovery animals; the increase was attributed to a physiological response to an increased excretion load. There was a diminution of glycogen in the liver, and a slight increase in iron in the Kupfer cells in the high dose animals. Two of these also showed minimal centrilobular necrosis of the liver with some infiltration; similar less marked findings were made in 2 of the control animals as well. These findings were attributed to infection. High dose females had increased GLDH which reversed during the recovery period. The NOAEL was put at 450 mg/kg b.w./day.

Rat. Thirteen week dermal study. Four groups of 10 male and 10 female SD rats were treated by an application of various concentrations of a.i. in light mineral oil. The doses were 0, 55.5, 277 and 555 mg/kg b.w./day applied to shaved skin 5 days a week for 13 weeks (The top dose is believed to be about 135 times the amount which would be used daily by the average consumer). Various laboratory and clinical tests were carried out during the experiment.

All animals survived. All animals showed a slight scaliness at the site of application, which was attributed to the vehicle. Body weight gain was greatest at the low dose. Haematological investigations showed no significant change. SAP was elevated in high dose animals, but not

significantly. The relative liver weight in high dose animals was elevated, but appeared normal on microscopical examination. The NOAEL is 555 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Test for capacity to cause irritation of the skin.

Guinea pig. The a.i. was applied undiluted twice daily to 20 animals for 16 days. There were no signs of irritation.

Man. Occlusive applications of undiluted a.i. were made to 60 subjects, of whom 20 had sensitive skin. The applications were made for 24 hours. Observations at removal of the patches, and 24 and 48 hours later, showed no evidence of a reaction.

In 51 male and female subjects, similar patch tests were carried out. The dilution of the a.i. (if any) was not stated. There was no irritation.

A formulation (concentration not stated) tested on the skin of 50 subjects caused no adverse effect.

In 53 subjects, a Draize repeated insult patch test at a concentration of 2 % caused no irritation.

In 54 subjects, a Draize repeated insult patch test of a 7.5 % dilution of a.i. in petrolatum caused no irritation.

A 10 % solution of a.i. in dimethylphtalate was used. A total of 58 subjects was recruited, 12 males and 46 females, aged 18-63. Of these, 6 subjects failed to complete the test for reasons unconnected with the experimental procedure.

Induction applications were made on the skin of the back, for 24 hours with occlusion, 3 times a week for 9 applications. Following a rest period of 2 weeks, a further patch was now applied to a new site on the back for 24 hours with occlusion. The area was inspected at 0, 24 and 48 hours after removal of the patch. No adverse reaction was noted at any stage of the experiment.

4.2 Irritation (mucous membranes)

Rabbit. Groups of 4 animals had 0.1 ml of a test preparation instilled into the conjunctival sac (concentration not stated). No further treatment in one group; in the other, the instillation was followed by washing out. There were no signs of irritation.

A Draize test carried out with undiluted a.i. was found to be practically non-irritant.

5. Sensitization

Tests for capacity to cause sensitization.

Guinea pig. Twenty animals received applications of undiluted a.i. twice daily for 16 days. After a 3 day interval without treatment, a daily challenge application was made for 3 days. There was no evidence of sensitization.

Two groups of 4 animals were used. Animals of one group were exposed to 0.05 ml injections of undiluted a.i. daily for 5 days. In the other group, 0.025 ml of a 50 % acetone solution of a.i. was applied to 2 cm^2 areas of shaved skin on either side. There was no evidence of sensitization.

Man. A Draize repeated insult patch test was carried out at a concentration of 2 % in 53 subjects. There was no sensitization.

In 54 subjects, a formulation of 7.5 % of a.i. in petrolatum was applied for 48 hours under occlusion for 11 applications. After a 14 day rest, a challenge application of a single dose was made. There was no adverse reaction.

In an extensive series of patch tests carried out in man, the a.i. was found to be very rarely responsible for allergic contact effects.

Test for capacity to produce photosensitization.

Tests which „showed that the product did not provoke photosensitization." No details supplied.

6. Teratogenicity

Tests for teratogenic activity.

Rabbit. Groups of 20 female animals were mated and given a.i. in doses of 0, 80, 200 and 500 mg/kg b.w./day by gavage during the period of organogenesis. Except for a slight reduction of maternal and foetal weight in the top dose animals, no abnormality was found.

Rat. Following a pilot study, groups of 36 rats were mated and treated with 0, 250, 500 and 1000 mg/kg b.w./day of a.i. (probably by gavage) during days 6-14 of pregnancy. Owing to an error, the preparation of the control foetuses led to their destruction, so this part of the test was repeated under identical conditions. Subgroups of each dose group were allowed to litter normally and rear the offspring. The percentage of resorptions in the high dose group was elevated by comparison with the other groups. The investigator records, however, that this relatively high rate is the usual one with this strain of rat in this laboratory, and he attributes the difference to an unusually low level of resorption in the other groups. No abnormality was found.

7. Toxicokinetics (incl. Percutaneous Absorption)

Tests for percutaneous absorption.

(a) *In vitro* tests.

Rat. Naked rat skin. This was studied in a chamber experiment. The investigators used a 1 % solution of a.i. in carbitol, and the amounts applied were 120, 360 and 1200 µg/cm^2. Most of the material was found in the stripped skin; there was less in the stratum corneum, and least in the chamber. The approximate amounts found in the chamber were: after 6 hrs, 1.13 %; after 16 hrs, 11.4 %; and at 24 hrs 17.9 %. The figures for the horny layer and strippings combined were, respectively, 31.4 %, 44.4 % and 45.7 % (percentages of applied doses). The amount of a.i. applied did not seem to affect the results.

In another set of experiments, various amounts of „Parsol 1789“ (4-tert-butyl-4'-methoxydibenzoylmethane) were added to the a.i. in the formulation. There seemed to be no effect on the absorption of the a.i.

Pig. A similar experiment using mini-pig skin was carried out in which „Parsol 1789“ was used as well as the a.i. Using 3 sorts of formulation, about 3 % of a.i. was found in the chamber in 6 hrs. Using the concentrations proposed for a particular commercial use (i.e., 2 % of „Parsol 1789“ and 7.5 % of a.i.) about 2.2 % of the amount of a.i. applied was found in the chamber. It is calculated by the authors that the total absorption for a 60 kg consumer would be about 56 mg, or 0.9 mg/kg b.w. This figure may be too high; a different calculation gives a value of 0.2 mg/kg b.w.

Man. A test on human abdominal skin in a chamber was carried out. With 7.5 % a.i., about 0.03 % is found in the chamber in 2 hours, 0.26 % in 6 hours, and 2.0 % in 18 hours. Various combinations of a.i. and „Parsol 1789“ were investigated. A calculation shows that these results might indicate an absorption of about 0.2 mg/kg b.w. in use.

(b) *In vivo* tests.

Man. Eight healthy volunteers had small amounts of radioactive a.i. applied to the interscapular region. One group of 4 had the material applied under a watch glass; the other 4 had it applied on gauze, with occlusion in one case. Tests for absorption of a.i. were negative except for about 0.2 % in urine. The concentrations used were not stated.

In a preliminary experiment, a capsule containing 100 mg of a.i. was taken orally. As a lipophilic substance, the a.i. is very likely to be metabolised; it is known in any case to be hydrolysed by plasma esterases, although slowly. The cumulative excretion of 4-methoxycinnamate in the urine over 24 hours was studied by GC/MS of the methyl ester derivative. (This method would also detect 4-hydroxycinnamic acid). Over 24 hours, an amount of cinnamate was found in the urine equivalent to about one-fifth of the amount that would have been expected if all the dose of a.i. had been absorbed. Nearly all of the metabolite was found in the first 6 hours.

In the main part of the experiment, an o/w cream containing 10 % a.i. was used. Applications of 2 grams of this material (= 200 mg a.i.) were made to the interscapular area of each of 5 male subjects, aged 29 to 46. The area of skin covered was 750 cm^2. After application, the area was covered with 3 layers of gauze, left in place for 12 hours. Blood was taken at times 0, 0.5, 1, 2, 3, 5, 7 and 24 hours. Urine was collected at 0, 2, 3, 4, 5, 6, 7, 12, 24, 48, 72 and 96 hours.

The control plasma samples showed a level equivalent to about 10 ng/ml before any application had been made. There was no evidence of any rise in plasma levels during the experiment. The urine showed a „physiological“ level of 100 to 300 ng/ml. No significant increase in this amount was found in any sample. The experiment seems to have been carefully conducted. The authors conclude that very little, if any, of the compound was absorbed after application to the skin, compared with the reasonably well marked absorption after ingestion.

8. Mutagenicity

Tests for mutagenic activity.

Salmonella mutagenesis assays were performed on the usual strains. There was a positive result with TA 1538 without metabolic activation. This was thought to have been a batch effect. From another laboratory, a very weak positive was found with TA 1538 without activation, at 10 µl/plate; it was not found in 2 replicates, nor in a second Ames test.

A test for mutagenesis and crossing over in *S. cerevisiae* was negative.

A test using Chinese hamster V 79 cells showed a very slight increase in mutant colonies with dose.

A test in human lymphocytes *in vitro* was negative.

A test for cell transformation in Balb/c 3T3 cells was negative.

A test for unscheduled DNA synthesis was negative.

Feeding tests in *Drosophila:*

There was an increase in the frequency of sex-linked recessive lethals; this was attributed with fair certainty of a batch effect.

There was no evidence of mutagenicity in feeding tests (adults and larvae).

Somatic mutation and combination tests using wing structure were negative.

Mouse. A standard micronucleus test was carried out. No effect was found up to 5000 mg/kg b.w.

Tests for photomutagenic activity.

A test was carried out in cells of *S. cerevisiae*, which had previously been shown not to be affected by a.i. *(supra)*. Evidence of mitotic gene conversion, gene mutation, and mitotic crossing-over was looked for. Doses of a.i., dissolved in DMSO, ranged from 0.05 to 625 µg/ml, and radiation up to 500000 J m^{-2} UVA and up to 12000 J m^{-2} UVB. Chlorpromazine was used as the positive control. Suitable negative controls were also employed. The experiment appears to have been well carried out. The results show that the a.i. is not photo-mutagenic under these conditions; that UVA and (more markedly) UVB are mutagenic; and that the a.i. protects against this effect in a dose dependent manner.

A test for the production of chromosomal aberrations was carried out in Chinese hamster ovary cells in culture. The test was carried out in accordance with GLP. The intensity of the ultraviolet radiation (mJ/cm^2) ranged from 200 to 2000 for UVA and from 4 to 25 for UVB. The positive control was chlorpromazine; the negative controls consisted of cultures irradiated but without the addition of active ingredient, and cultures not irradiated but with the addition of the active ingredient. The doses of active ingredient used ranged from 5 to 25 µg/ml. It was noted that the top dose of UV irradiation was clastogenic, but that there was a protective effect with the active ingredient. The positive control showed satisfactory activity. There was no evidence of a photoclastogenic effect.

10. Special investigations

Tests for capacity to produce phototoxicity.

Man. In 10 subjects, patches were applied for 24 hours and the areas then exposed to a suberythematous dose of UV irradiation. There was no evidence of phototoxicity.

Tests for inhibition of UV-induced tumours.

Hairless mouse. The animals were exposed to repeated doses of UV simulating the solar energy spectrum. After a rest period, 3 applications a week were made to an area of skin of 12-o-tetradecanoyl phorbol-13-acetate (at first at 10 µg/ml, but later at 2 µg/ml, as the higher concentration was found to be irritant). Suitable controls were used. The test group was completely protected by 50 % a.i., and 7.5 % gave an effect equivalent to reducing the insolation four-fold. It had been suggested that the a.i. could itself have been a promoter, but there was no evidence of this.

11. Conclusions

The compound appears to have low acute toxicity. A subchronic oral toxicity study showed a NOAEL of 450 mg/kg b.w./day. A subchronic dermal study showed a NOAEL of 550 mg/kg b.w./day, which was the highest dose tested. The a.i. does not irritate the mucous membranes in conventional animal tests. The data presented suggest that the compound is not a skin irritant or sensitiser in animals; however, tests for sensitization were carried out at levels below the proposed maximum use level. Clinical investigation shows that this compound is very rarely responsible for allergic contact dermatitis in man.

There is no carcinogenicity study, but an extensive range of mutagenicity studies has been carried out; these show no evidence of mutagenicity. A test for photomutagenicity in *S. cerevisiae* was negative. Photoclastogenicity tests in CHO cells *in vitro* were negative.

Animal studies for teratogenic activity showed a NOAEL of more than 500 mg/kg b.w./day (which was the highest dose tested). Percutaneous absorption was studied in naked rat, minipig, and human skin *in vitro*; and experiments show that there is a decreasing amount of absorption as one goes from rat skin to human skin; the last suggests that about 0.9 mg/kg b.w. might be absorbed. Experiments with radioactive a.i. in man indicate that only about 0.2 % of the applied amount appears in the urine. In a detailed study in man, which compared oral and percutaneous absorption, using GC/MS, although about one-fifth of 100 mg of ingested a.i. was found in the urine, none at all was found when 200 mg of active ingredient was applied to the skin in a concentration of 10 %.

Classification: 1.

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

2-ETHYLHEXYL-4-METHOXYCINNAMATE
S 28

Based on a usage volume of 18 ml, containing at maximum 10 %

Maximum amount of ingredient applied:	**I (mg) = 1800 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 2 %**(*)
Dermal absorption per treatment:	**I (mg) x A (%) = 36 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg = 1800 mg x 2 % / 60 kg= 0.6 mg/kg b.w.**
No observed adverse effect level (mg/kg): (rat, 13 week oral study)	**NOAEL = 450 mg/kg b.w./day**
Margin of Safety:	**NOAEL / SED = 750**

(*) This figure is derived from experiments in human and animal skin in vitro. A carefully carried out study in man showed absorption of about 20 % following oral ingestion, but none of the a.i. appeared in the plasma after dermal application.

OPINIONS ADOPTED DURING THE 66TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 18 July 1996

MUSK XYLENE

1. General

1.1 Primary name

Musk Xylene.

1.2 Chemical names

1-tert-Butyl-3,5-dimethyl-2,4,6-trinitro-benzene.
1-(1,1-dimethyl ethyl)-3,5-dimethyl-2,4,6-trinitro-benzene.

1.4 CAS no.

CAS no.: 81-15-2
EINECS no.: 201-329-4

1.5 Structural formula

1.6 Empirical formula

Emp. formula: $C_{12}H_{15}N_3O_6$
Mol weight: 297.27

1.8 Physical properties

Appearance: pale yellowish crystals or fine crystalline powder.
Melting point: 114 °C
Vapour Press.: < 0.1 mm Hg 20 °C
Flash point: > 100 °C

1.9 Solubility

Solubility in water: virtually insoluble.

2. Function and uses

Fragrance ingredient used for a wide variety of applications. May be used in fragrance compounds at concentrations of up to 5 %. RIFM found the average of the upper 90 th percentils to range between 0.5 % (fine fragrance) and 4.1 % (detergents).

Typical concentrations of fragrance compounds in cosmetic products and estimated upper concentrations are reported by Colipa to be:

	Fragrance in products	Musk Xylene in products
Toilet soap	1 %	0.04 %
Shampoo	0.5 %	0.01 %
Skin cream	0.5 %	0.0075 %
Deodorant	0.5 %	0.0075 %
After shave	2 %	0.03 %
Cologne/toilet water	5 %	0.075 %
Fine fragrance	10-20 %	0.05-0.1 %

Determination of normal and upper levels of exposure to Musk Xylene has been determined through a detailed RIFM survey of major fragrance manufacturers (see tables 1 and 2 in annex) -1990-.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}: According to the 1975 RIFM monograph, the LD_{50} in rat was greater than 10 g/kg b.w.

Full report is not available.

3.2 Acute dermal toxicity

Dermal LD_{50}: Similarly RIFM monograph reports the acute dermal LD_{50} in rabbit as above 15 g/kg b.w.

Full report is not available.

3.8 Subchronic dermal toxicity

Subchronic dermal toxicity and neurotoxicity

Results of a 90 day dermal toxicity in the rat - 1990 - are reported; Musk Xylene application (unoccluded) was renewed daily at levels of 7.5 - 24.0, 75 or 240 mg/kg b.w./day in phenyl ethanol alcohol; the treatment volume for the study was 2 ml/kg b.w./day. The test substance and/or the vehicle were applied on approximately 25 % of the body surface; the skin was clipped but not abraded; the animals were housed in individual cages with collars to prevent ingestion.

No deaths were observed.

The only change has been some liver weight increase at the higher doses. The changes were not associated with histopathological modifications.

According to the results, the NOAEL were 75 mg/kg b.w. for the males and 24 mg/kg b.w. for the females.

Musk Xylene clearly did not have the neurotoxic effects of Musk Ambrette - 1984 -. Detailed data are not available.

3.10 Chronic toxicity

Oral: In a 80 week feeding study - 1990 - with groups of 50 male and 50 female mice receiving 0, 0.075 and 0.15 % of Musk Xylene in the diet (corresponding to a total of 0, 1.61 and 2.82 g for the males and 0, 1.49 and 2.76 g for the females, of Musk Xylene intake during the 80 weeks), no adverse effects were observed in respect to body weight, haematology, organ weight and organ histopathology.

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin primary irritation.

The RIFM monograph reports:

- that Musk Xylene applied full strength for 24 hours under occlusion, to intact and abraded rabbit skin was not irritating.
- that a 5 % solution in petrolatum applied under occlusive patch for 48 hours produced mild irritation on human skin.

Full reports are not available.

Skin irritation by repeated application.

In a 90 days study, Musk Xylene was administered by dermal application (unoccluded) to groups of male and female rats on a daily basis at levels of 7.5, 24.0, 75.0 or 240.0 mg/kg b.w./day in phenyl ethyl alcohol; the treatment volume for the study was 2 ml/kg b.w./day; the test and/or the control materials were applied weekly on approximately 25 % of the body surface, to the skin shaved; the treatment site was examined and weekly scored (according to the method of Draize) for erythema and oedema.

No significant local effects were observed at the treatment area till up to 240 mg/kg b.w./day.

4.2 Irritation (mucous membranes)

None information available.

5. Sensitization

• Skin sensitization

The 1975 RIFM monograph reports a Human maximisation study on 25 volunteers. Musk Xylene was tested at concentration of 5 % in petrolatum. No reactions were observed.

6. Teratogenicity

None information available.

7. Toxicokinetics (incl. Percutaneous Absorption)

• *A percutaneous absorption study in vitro - 1996 - has been performed in fresh, full thickness F344 rat skin. Musk Xylene was poorly absorbed through unoccluded rat skin into the fluid receptor: 0.61 ± 0.16 % (n = 8) at 24 hours. However significant amount of radioactivity (approx 50 - 60 %) was recovered from within the skin at the end of the experiment; according to these results, Musk Xylene seems to be well absorbated into the skin although systemic absorption over 24 h is likely to be very low.*

• Absorption, distribution and excretion of Musk Xylene have been studied in rat and human after topical application, *in vivo* - 1984 -:

- In rat ^{14}C Musk Xylene was applied topically to 9 cm^2 of shaved skin at a total dose of 0.5 mg/kg b.w. and maintained under occlusion for 6 hours and then rinsed off. Approximately 8 % of the applied dose was absorbed; 14 % of the dose remained in the skin; after 48 hours a total of 20 % had been absorbed with 2 % remaining in the skin; after 5 days virtually all the 20 % absorbed had been eliminated.

- In 2 human volunteers, 1 mg ^{14}C Musk Xylene was applied over an area of 100 cm^2, corresponding to 0.01 mg cm^{-2} skin surface; after 5 days an average of 0.27 % of the applied dose was excreted in urine and less than 0.1 % in faeces; plasma levels never exceeded the limit of detection (0.2 mg/ml).

- In an autoradiographic study in the rat, authors compared the tissue distribution of radioactivity in rats 24 hours after a single dermal dose of 0.5 mg/kg ^{14}C Musk Xylene with the distribution 24 hours after the last of 14 daily doses (0.5 mg/kg day). Most radioactivity remained at the site of application with absorbed radioactivity being concentrated in the liver and gastrointestinal tract consistant with excretion via the bile. After 14 daily doses, tissue levels were only marginally greater than those after a single dose.

- Quantitative measurements of radioactivity in organs, blood and application skin site were performed 24 hours after 7 daily doses and 6, 24 and 48 hours after 14 doses of ^{14}C Musk Xylene; the greatest concentration was found in the application site; little was found in organs; the highest concentration being in fat, liver and thyroid; comparison of results between 1, 7, 14 daily applications showed no significant accumulation of radioactivity except at the site of applications.

- Following intravenous injections of ^{14}C Musk Xylene, it was shown that a single dose was only cleared slowly from the body with a half life of 40 hours; however, the repeated dose indicated that a steady state reached by about 7 days with relatively little accumulation.

- The same authors identified a major biliary metabolite in rat as an alcohol due to hydroxylation of Musk Xylene at the tertiary butyl group; the same hydroxylated Musk Xylene was found and faeces by another worker (1991) following oral administration.

- Urine was found to be the major route of excretion in Human with the major metabolite, although unidentified, being chromatographically different to that found in rat.

• In another study on rats by oral route – 1991 – after a dose of tritiated Musk Xylene of 70 mg/kg b.w., 75 % of the dose appeared in faeces after 7 days and 10 % in urine; less than 2 % remained in carcass.

• *Lehman-McKeeman (1995a, 1996) showed that the gastrointestinal flora of mice could convert Musk Xylene to an amine derivative in the para position to the tertiary butyl group, and that this metabolite was an extremely potent inactivator of the CYP2B enzyme that Musk Xylene had induced. Further, it was noted that, unlike the carcinogenic nitroaromatic compound, 2,6-dinitrotoluene, Musk Xylene was negative in the in-vivo-in-vitro unscheduled DNA synthesis assay, indicating that, despite nitroreduction by the gastrointestinal flora, the amine was not likely a liver carcinogen. In addition there are differences between gastrointestinal flora in rodents and humans that would make nitroreduction less likely to occur in humans. These pharmacokinetic and metabolic findings support the consideration that mouse hepatocarcinogenicity is not a significant risk factor human safety.*

8. Mutagenicity

• Ames test – 1981 – was performed with and without metabolic activation in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100; Musk Xylene was tested at concentrations up to 200 µg/plate in dimethyl sulfoxide; no evidence of mutagenicity occurred.

• In another Ames test – 1986 – no evidence of mutagenicity was found with and without metabolic activation in TA 98 and TA 100 at concentration up to 500 µg/plate.

• In a Mouse lymphoma assay – 1992 – a 10 concentrations ranging from 20 to 400 µg/ml and 10 to 125 µg/ml in absence or presence of activator (Rat liver S9) respectively it was found increased inhibition of total cell growth at the highest concentration but no increase in mutation frequency.

• Chromosomic aberrations were tested – 1992 – in CHO cells at 5 concentrations ranging from 2.5 to 40 µg/ml and 3.8 to 30 µg/ml in absence or presence of activator (Rat liver S9) respectively. Metaphase cells were harvested at 24 and 48 hours. There were no increase or aberration frequency.

• In a test for induction of UDS in rat primary hepatocytes – 1992 – at 5 concentrations ranging from 1 to 30 µg/ml no net increase in nuclear grain counts was observed.

• In an *in vivo*/*in vitro* rat hepatocyte unscheduled DNA synthesis assay, Musk Xylene did not induce a significant increase in the mean number of net nuclear grain counts in hepatocytes via oral gavage at doses of 500, 1500 and 5000 mg/kg b.w.

9. Carcinogenicity

• In the same 80 week study (see 3.10.) there was a significant increase in tumour incidence in both male and female mice at both dose levels; the main tumours seen were adenomas/carcinomas of the liver and Harderian gland tumours (males); the incidences of

tumours were not dose related. A non effect level was not established. Nevertheless, as stated by the authors of the study, tumours of the liver, haematopoietic organs, lung and Harderian gland are frequently observed in the B6C3F1 strain of the mouse which has been used.

As it is also well recognised that enzyme induction is associated with an increase incidence of such tumours at least in the liver, investigations were made to evidence a correlation between tumours and enzyme induction.

• Musk Xylene has been shown to induce cytochrome P450 when administrated by intraperitoneal route to rats – 1992 –.

• Assessment of the Enzyme inducing characteristics of Musk Xylene in B6C3F1 mice has been made - Submission III – 1994 – by means of 2 studies:

- In a pilot study, groups of 10 male mice received IP injections of 50, 100 or 200 mg Musk Xylene/kg b.w. for 7 days. 50 mg/kg b.w. gave rise to mild centrolobular hepatocellular hypertrophy. Hydropic changes, scattered mitoses and nuclear size variations were seen in the 100 mg/kg b.w. group.

In the high dose group, these effects were more marked and smooth and rough endoplasmic reticulum increased. Mitochondial fragments indicated toxic effects. The nuclei were normal with some margination of the chromatin.

The increase of liver weight (up to 132 % of normal) and protein content (up to 170 %) as well as the induction of P450 isozymes CYP IA1 and IA2 were seen in a dose dependant manner up to 1 320% for both isoenzymes and to 583 % for CYPIA2 alone.

- In a feeding study groups of 25 males were given 0.015, 0.045 and 0.15 Musk Xylene (approximately 22, 66 and 220 mg/kg b.w.) with the diet for 4 weeks. A recovery group had access to control diet for other 14 days. Labelling index was estimated using BrdU; liver slices were prepared for histology and electromicroscopy and P450 isoenzyme induction, studies were performed.

The histological and electromicroscopical pictures were more or less the same than those seen in the pilot study; there was an increase in the number or size of peroxiomes.

No effects were seen on hepatic parameters including enzyme induction at the 0.015 dose level. At the two upper dose levels the relative liver weight and protein content as well as induction of both isoenzymes (CYPIA1/IA2) were increased in a dose dependant manner. No differences from controls were seen in the recovery groups. The subacute feeding study suggests a NOAEL of about 20 mg/kg b.w. for the evaluated parameters in this strain of mice.

The authors consider it unlikely that the positive results seen in the carcinogenicity study can be associated to the results presented in this study.

• The effects of Musk Xylene on Mouse hepatic microsomal activities were characterised once more in 1994 - submission IV – 1995 –.

The purpose of this work was to characterise the effects of Musk Xylene on mouse hepatic microsomal enzyme activities. Male B6C3F1 mice were dosed for 7 days at 0 or 200 mg Musk Xylene/kg after which microsomes were prepared.

Musk Xylene treatment increased liver weight by 40 %, caused hepatocellular hypertrophy and increased total cytochrome P-450 2-fold over control. Microsomes from Musk Xylene-treated mice showed increased activity for the dealkylation of ethoxy- and methoxyresorufin, results consistent with increased CYP1A1 and 1A2 protein levels determined by Western blotting.

No increase in pentoxyresorufin-0-dealkylation activity was seen, but Musk Xylene treatment markedly increased CYP2B protein levels. Preliminary *in vitro* studies showed that Musk Xylene inhibited mouse CYP2B enzymes (IC_{50} = 1μM), but did not affect the activities of CYP1A1 or 1A2. This inhibition was not NADPH-dependent. These results indicate that, in mice, Musk Xylene causes generalised hepatic changes similar to classical CYP2B inducers. However, Musk Xylene is also a potent inhibitor of the CYP2 enzymes.

According to the authors, as the increase in liver tumours with Musk Xylene was not caused by a genotoxic mechanism it remained to be seen what was the cause. Further studies (Thatcher and Caldwell, 1994, Caudill et al., 1995 and Lehman-Mc Keeman et al., 1995) have shown that Musk Xylene is a weak inducer of CYP1A2 and a significant inducer of CYP2B enzymes in this strain of mouse, indicating that Musk Xylene acts in a manner similar to phenobarbital. Phenobarbital is considered to be a nongenotoxic chemical that causes liver tumours in rodents at doses causing enzyme induction, but which is not carcinogenic to humans based on extensive human use. Musk Xylene is considered to cause hepatic tumours in mice by mechanisms like those of phenobarbital and thus is not considered to be a carcinogen for humans. The no-effect level for enzyme induction by Musk Xylene was 10 mg/kg b. w.

"Since there has been no human carcinogenic hazard associated with chronic, high dose human exposure to Phenobarbital, then mouse liver tumours seen with Musk Xylene exposure are also likely to not represent any relevance to humans."

10. Special investigations

• Photoirritation

A definitive study on the potential for photoirritation by Musk Xylene has not been published.

As part of photosensitization study – 1988 – guinea pigs were treated topically with 10 % Musk Xylene and irradiated with 100 KJm^{-2} UV. No skin irritation or photoirritation were observed.

• Photoallergy

In a group of 12 guinea pigs using induction concentrations up to 10 % (0.1 ml applied topically over sites injected with Freund´s complete adjuvant) and irradiation with 100 KJm^{-2}, there was photoallergic response in one animal at 10 % and 1 % but not at 0.1 %.

A clinical study showed some evidence of cross reactivity when applied Musk Xylene on patients photoallergic to Musk Ambrette; this was considered as a cross reactivity.

• Concentration in tissues

Musk Xylene has been found in fish in Japan and more recently in Germany and Switzerland.

The Japanese study found 0.2 mg/kg in fresh fish. The German data indicate a maximum level of 0.023 mg/kg fresh fish in farmed trout.

The Swiss data indicate a maximum level of 0.07 mg/kg fresh fish with the average being around 0.03 mg/kg.

That indicates a second possible route of exposure to Musk Xylene through eating fish.

Musk Xylene has been analysed – 1993 – in human fat and breast milk; according to the authors, the quantity found in human fat varied between 0.02 and 0.22 mg/kg fat. Interestingly the quantity present did not vary with age as did the quantities of other substances investigated. Although the number of samples investigated were probably not sufficient to draw definitive conclusions, there is a strong indication that the quantity of Musk Xylene reaches a steady state, being eliminated as fast as it accumulates.

The quantity of human breast milk varied between 0.02 and 0.19 mg/kg fat in the milk; the average of content of the milk was 2.2 %.

11. Conclusions

• Information concerning eye irritation, teratogenicity/reproduction is not available.

• Information concerning the short term studies mostly comes from a RIFM monograph without data.

• Detailed information able to make the assessment of the NOAEL in the long term feeding study in mice are not available.

• Full data are also not available concerning the results concluding on a possible carcinogenic potential of Musk Xylene in mice. However, numerous detailed results showing that Musk Xylene induces and inhibits Mouse hepatic cytochrome P450 2B enzymes are given.

• It can be assumed from the given information and from the results of the subchronic dermal toxicity that Musk Xylene can be mildly irritating to the skin.

• According to the results Musk Xylene is not photoirritant or sensitizer to the skin (but no experimental data by maximalisating induction are available); it was weakly photoallergenic in one animal study and showed some evidence of cross reactivity in human sensitive to Musk Ambrette.

• In a subchronic dermal toxicity study, some increase of the liver weight were evidenciated at high dose of Musk Xylene. The NOAEL was 75 mg/kg b.w./day for the male and 24 mg/kg b.w. for the female rats.

• This liver sensitivity was confirmed in a long term feeding study in mice where hepatic adenoma/carcinomas and Harderian gland tumours were mainly observed.

• Those alterations observed at high level of test material were demonstrated to be related with cytochrome P450 induction.

• As this ingredient has no food use status, it make sense, as suggested by industry, not to use Musk Xylene in lip product or in flavours for the oral hygiene products.

• Musk Xylene showed no evidence of mutagenic potential in the absence or presence of metabolic activation in any of the *in vitro* tests which have been made (Ames test, CHO, MLM, UDS).

• Absorption, distribution and excretion of Musk Xylene have been widely investigated in animal and human:

- *According to a further study performed in vitro in fresh full thickness unoccluded rat skin only 0.61 % of the amount applied was absorbed during 24 hours follow up and more than 50 % remained in the skin.*
- In the rat 20 % of the amount applied on the shaved skin under occlusion for 6 hours is absorbed during 48 hours follow up and 2 % remained in the skin,
- in human volunteers, 0.5 % of the applied amount was absorbed during the 6 hours where Musk Xylene remained on the skin; after 5 days 0.27 % of the applied dose was excreted with urine and less than 0.1 % in faeces.

• Concerning the presence of Musk Xylene in human fat and breast milk the results obtained in 1993 and 1994 are questionable:

- No data are given concerning the methodology of sampling the breast milk to guaranty that quantities dosed were not due to environmental contamination.
- The extraction procedure for human milk fat seems inappropriate since the quantity of fat analysed in the breast milk is not at all related to the well-known daily needs of the infant (rarely below 2 %); according to the given results, if a mother was producing a milk that contained only 0.1 % fat (as reported by the authors), she would need to transfer 20-30 l of milk to her infant on a daily basis to ensure adequate fat for growth and development.

According to these results, it can be at least suspected that Musk Xylene was present in fat and breast milk, from which origin is not demonstrated (cosmetic and/or food consumption?); however quantitative values, as defined above, cannot be retained from such studies.

To solve the problem, additional studies performed with adequate methods and according to good laboratory and clinical practices should be undertaken.

Proposition:

According to the potential risk of migration of Musk Xylene in the breast milk, complementary studies should be undertaken to evidenciate such possible issue and if so, to assess the possible maternal and embryotoxicity of the test material.

Proposed classification: 2.

12. Safety evaluation

• According to complementary data received from Colipa to assess the safety margin, the calculated maximum exposure to Musk Xylene before percutaneous absorption of 1.286 mg/kg b.w./day can be considered as an exaggerated figure, as it assumes a consumer uses all products simultaneously and containing the maximum concentration at the extensive frequence use:

Product type	Typical quantity per application expressed in grams (note 1)	Frequency of application per day	Retention Factor (note 2)	Exposures expressed in g/day		Max. conc. (%) of Musk Xylene (note 3)	Consumer exposure (assuming 60 kg body weight and extensive use) in mg/kg/day
				Normal use	Extensive use		
Body lotion (note 5)	8	1 to 2	100%	8	16	0.1	0.267
Hand cream	0.8	1 to 2	100 %	0.8	1.6	0.1	0.027
Face cream	0.8	1	100 %	0.8	0.8	0.1	0.013
Cologne (note 4)	0.75	1 to 5	100 %	0.75	3.75	1.5	0.937
Antiperspirant/ deodorant	0.5	1	100 %	0.5	0.5	0.4	0.033
Hairspray	5	1 to 2	10 %	0.5	1	0.0026	0.0004
Shampoo	12	2 to 7 per week	1 %	0.034	0.12	0.01	0.0002
Shower gel	5	1 to 2	10 %	0.5	1	0.025	0.0042
Foam bath	17	1 to 2 per week	1 %	0.024	0.049	0.1	0.0008
Toilet soap	0.8	3 to 6	10 %	0.24	0.48	0.046	0.0037
						TOTAL:	1.2863

Note 1: Cosmetic exposure data from Colipa.

Note 2: Proportion of product remaining on skin.

Note 3: These maximum levels are rarely encountered. Typical use levels range from about 10 % of these values down to less than 1 ppm.

Note 4: Includes use of aftershaves or fine fragrances as alternatives to colognes. These deliver an equivalent quantity of perfume compound and are unlikely to be used together.

Note 5: General purpose body lotions contain up to 0.1 % nitromusks. However, some expensive lotions sold in small quantities as part of a perfume range and designed to perfume rather than act as emollients, may contain up to 0.4 %. Because of less frequent use over a smaller body area, exposure will be reduced to that of general body lotions.

The main significant reported adverse effect was the carcinogenicity in mice; in the given study there is not a NOAEL, the lowest dose being closed to 100 mg/kg b.w./day.

Another, related, significant and adequately reported adverse effect concerned the increase of the rat liver weight in the subchronic dermal toxicity; a NOAEL was established at 24 mg/kg b.w./day.

According to the maximum exposure indicated by Colipa, and assuming 100 % percutaneous absorption the simple comparison of that NOAEL with calculated total human exposure of 1.286 mg/kg b.w./day would give a safety factor of:

24/1.286 = **19**

This safety factor however does neither take into account percutaneous absorption nor differences between skin absorption in rat or human. Based on experiment showing the percent dose absorbed under forced conditions to the rat 90-day study (20 %) and the amount absorbed by human under normal exposure conditions (0.5 %), considering the same base of maximal human exposure, a corrected safety factor may be calculated:

- The calculated daily human body load percutaneous absorption would be:

1.286 mg/kg x 0.5 % absorption = **0.00643 mg/kg b.w.**

taking a NOAEL of 24 mg/kg into account, the safety margin would be:

24 : 0.00643 = **3732**

- The calculated daily rat body load by percutaneous absorption would be:

1.286 mg/kg x 20 % absorption = 0.2572 mg/kg b.w.

taking the same NOAEL of 24 mg/kg into account, the safety margin would then be:

24 : 0.2572 = **93**

Table 1

Calculation of perfume retained on skin for average and high frequency users of products

	Product usage* (g/day)		Perfume Concn. %	Retention factor on skin	Perfume retained on skin (mg/day)	
	average	upper 90%ile			average	upper 90%ile
Bath Prepatations	1.81	9.50	2	0.01	0.36	1.90
Colognes, Toilet waters	0.55	0.93	5	0.9	24.75	41.85
Perfumes	0.10	0.40	18	0.9	16.20	64.80
Shampoos, rinses	12.02	29.40	0.5	0.2	12.02	29.40
Hair Sprays	0.93	2.13	0.15	0.3	0.42	0.96
Other Hair Preps	5.09	13.04	0.5	0.2	5.09	13.04
Soaps	2.47	3.72	1.2	0.2	5.93	8.93
Deodorants	0.42	0.67	0.75	1.0	3.15	5.03
Cleansing creams	1.04	2.60	0.5	1.0	5.20	13.00
Face/Body/Hand Preps	3.08	5.99	0.5	1.0	3.10	29.95
Moisturisers	0.45	0.87	0.5	1.0	2.25	4.35
Other Skin Preps	2.57	6.13	0.5	1.0	12.85	30.65
Suntan Preps	2.00	4.00	0.4	1.0	8.00	16.00
Air Fresheners	2.50	5.74	1.75	0.01	0.44	1.01
Household Detergents	61.12	140.31	0.25	0.001	0.15	0.35

* Calculated from CTFA figures based on a USA survey of product used per application and frequency of use for the average and upper 90th percentile of consumers.

Table 2

Calculation of Musk Xylene remaining on skin for average and high frequency users of products containing average and high levels of Musk Xylene in the perfume

	Musk Xylene in perfume (%)		Musk Xylene (MX) retained on skin (µg/day)			
	average 50%ile	upper 90%ile	av. use av. MX	upper use av. MX	av. use upper MX	upper use upper MX
Bath Prepatations	0.3	2.5	1.09	5.70	9.05	47.50
Colognes, Toilet Waters	0.1	1.5	24.75	41.85	383.63	648.67
Perfumes	0.2	0.5	32.40	129.60	81.40	325.62
Shampoos, rinses	1.5	2.0	180.30	441.00	239.40	585.55
Hair Sprays	1.5	1.7	6.27	14.39	7.27	16.69
Other Hair Preps	1.5	2.3	76.35	195.60	118.34	303.18
Soaps	1.5	3.8	88.92	133.92	224.79	338.55
Deodorants	0	1.5	0.00	0.01	46.23	73.74
Cleansing creams	0.4	1.5	20.80	52.00	77.09	192.72
Face/Body/Hand Preps	0.4	1.5	12.40	119.80	45.26	437.27
Moisturisers	0.4	1.4	9.00	17.40	30.71	59.38
Other Skin Preps	0.4	1.3	51.40	122.60	168.33	401.51
Suntan Preps	0.4	2.5	32.00	64.00	202.80	405.60
Air Fresheners	0	1.1	0.00	0.00	4.96	11.39
Household Detergents	0.8	4.1	1.25	2.87	6.29	14.43
	TOTAL (mg/day)		0.54	1.34	1.65	3.80

A 25: *HYDROXIBENZOMORPHOLINE*

1. General

Summary of SCC Evaluation of Submission I

The oral LD_{50} of Colipa A025 in mice was approximately 860 mg/kg body weight.

A 1 % solution on propylene glycol was considered to be „practically non-irritating“ to the rabbit eye and „slightly irritating“ to rabbit skin.

A sensitization test in guinea pigs induced and challenged with the pure material resulted in no reaction. There was no evidence of photosensitization when Colipa A025 was tested in guinea pigs at a concentration of 0.4 % in propylene glycol.

Slight signs of systemic toxicity were seen in rats administered Colipa A025 orally at 40 mg/kg body weight for 3 months. Administration to groups of rats at 0, 10, 100 and 1000 mg/kg body weight for one month resulted in dose-dependent nephrotoxicity at the 2 higher doses. No histopathological lesions were observed in the rats treated with 10 mg/kg body weight per day.

Topical application of a formulation containing Colipa A025 at 1.1 % (coded P25) in the presence of hydrogen peroxide twice a week for 13 weeks produced no evidence of systemic toxicity.

Topical application of the formulation containing Colipa A025 at 1.1 % (coded P25) in the presence of hydrogen peroxide to pregnant rats, at a dose level of 2 mg/kg body weight on days 1, 4, 7, 10, 13, 16 and 19 of gestation resulted in no embryotoxic or teratogenic effects.

The P25 formulation with hydrogen peroxide also gave no evidence of chronic toxicity or carcinogenicity following topical administration at 0.05 mg/cm^2 to mice once a week for 23 months.

Colipa A025 was negative in genotoxicity assays involving: gene mutation in *Salmonella* and the yeast, *S. Pombe* P1, unscheduled DNA synthesis in a human HeLa cell line (using both scintillation counting and autoradiographic methods) and an *in vivo* mice micronucleus test (400 mg/kg i.p.).

Dermal absorption was assessed using human epidermis *in vitro* in static diffusion cells. Mean values for penetration over 4 hours were 0.05, 0.048, 0.04 and 0.06 % in four separate assays.

Evaluation of the SCC:

Margin of safety: 580.
Classification: B.

Requirements:

- Chromosomal aberration assay in mammalian cells *in vitro*.
- Consideration of the possibility of nitrosamine formation.

This summary presents an evaluation of data presented in a Colipa Submission II document (dated September 1993), in the context of the SCC evaluation of November 1991 (revised December 1993), supported by examination of the individual Submission II study reports provided by Colipa on microfiche.

1.1 Primary name

Hydroxybenzomorpholine.

1.2 Chemical names

6-Hydroxybenzomorpholine.
INCI name: Hydroxybenzomorpholine.

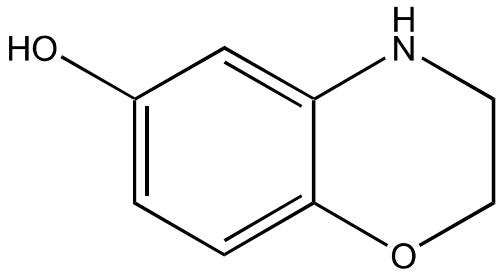
1.3 Trade names and abbreviations

Imexine OV (Chimex).

1.4. CAS no.

26021-57-8
EINECS no.: 247-415-5

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_9NO_2$
Mol weight: 151

1.7 Purity, composition and substance codes

Analytical data

The purity is stated to be not less than 98 % and not more than 100 % as determined by potentiometry. The Colipa Submission should state whether this is a specification. In all study reports where batch numbers and purity are cited, the purity is within this range. The analytical data presented either refer to batch nos op129 or op 59, or no batch number is given.

Possible impurities include:
- reagents and intermediate reaction products
  - 2,5-dimethoxyaniline (batch op 59: less than 0.1 %)
  - 2,5-(2,5-dimethoxyphenylamino)-ethanol (batch op 59: less than 0.1 %)
- solvent
  - isopropanol (batch op 129: less than 10 ppm)
- other
  - NaBr (batch op 129: 0.109 %)
  - Sulphated ash (batch no op 59: 1 %)
  - Heavy metals (batch no 129: less than 20 ppm).

Proportions indicated in parentheses refer to analysis of specific batches as indicated, and there is no comment as to whether this is representative.

1.8 Physical properties

Appearance: pink-mauve powder
Melting point: 115 °C
Odour: odourless

1.9. Solubility

Solubility at 25 °C

Soluble in 96 % ethanol and ethyl glycol.

Insoluble in water.

2. Function and uses

Colipa A025 is used in direct hair dye formulations at concentrations up to 2 %. Since the oxidative hair dyes are mixed with hydrogen peroxide before use, the concentration at application is 1 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

OECD guideline:	401
Species:	Ico rats OFA.SD (IOPS Caw)
Group size:	5 male + 5 female
Substance:	Colipa A025 in 0.5 % carboxymethylcellulose
Batch no:	op 90
Dose:	500, 1000, 2000 and 5000 mg/kg b.w. in a volume of 20 ml/kg
Observation period:	15 days
GLP:	Quality Assurance statement included

Groups of 5 male and 5 female rats received a single dose of 500, 1000, 2000 and 5000 mg/kg b.w. The animals were observed daily for 14 days for mortality and clinical abnormalities. Body weights and macroscopic observations were recorded, but histological examinations were not performed.

Results

At a dose of 5000 mg/kg b.w. all animals died within 4 hours. At 2000 mg/kg b.w. all animals died within 2 days of dosing. At 1000 mg/kg b.w. only 2 females animals died within 48 hours. No mortalities were reported at a dose of 500 mg/kg b.w.

Body weight gain was decreased in the male rats treated with 1000 mg/kg only. No analysis was carried out on the upper two groups because of the high number of mortalities.

At necropsy lung congestion was recorded in rats dying during the study. No abnormalities were seen in any other tissue.

The LD_{50} was reported to be between 1000 mg/kg and 2000 mg/kg.

Remark

This study was conducted in 1989 and should have been made available to the SCC for the evaluation of Submission I.

3.4 Repeated dose oral toxicity

OECD guideline:	407
Species:	Sprague Dawley OFA rat
Route:	oral
Group sizes:	10 male and 10 female
Substance:	Colipa A25 suspended in 2 % aqueous Polysorbate 80
Batch no:	op. 90
Dose:	0, 10, 100 and 1000 mg/kg b.w./day in a volume of 10 mg/kg b.w.
Exposure:	30 days - male, 31 days - female
GLP:	Quality Assurance statement included

The test substance suspended in hydrogel containing 2 % polysorbate 80 in sterile water (containing activated dimethicone as anti-foaming agent) was administered by gavage once daily to groups of Sprague Dawley rats (10/sex) for 30 days (males) and 31 days (females). The dose levels were 10, 100 and 1000 mg/kg b.w. The control group (10/sex) received the vehicle alone. All animals were sacrificed at the end of the study.

All animals were observed twice daily for mortality and clinical signs. Body weights and food consumption were recorded individually at weekly intervals. Ophthalmoscopic examination was performed on day 0 and at termination in the control and high dose group animals only. Blood samples were taken from all animals at day 0 and at termination for haematological and clinical chemistry investigations. Urine samples were collected on day 0 and during week 4. Organ weights were recorded for a number of tissues. Macroscopic examinations were carried out. A histopathological examination of tissues was undertaken.

Results

No treatment related mortalities were reported. Abnormalities reported in animals treated with 1000 mg/kg b.w. included; lethargy and excessive salivation, decreased body weight in males, decreased food consumption in the first few days of treatment in both sexes, a slight increase in the number of neutrophils in males, an increase in triglycerides (65 %) in females, dark discolouration and slight acidification of the urine and an increase in urinary proteins. An increase in both relative and absolute liver, kidney and testis weights were noted in the highest dose groups only. No ocular abnormalities or macroscopic abnormalities were noted.

There were no overt or biochemical signs of toxicity in animals treated with either 10 or 100 mg/kg b.w.

Histological examination revealed epithelial necrosis, basophilia and tubular dilation in the kidneys cortical tubules of male rats treated with 100 and 1000 mg/kg b.w. No effects were seen in animals treated with 10 mg/kg b.w. per day.

Remark:

Information on this study was available for the previous SCC evaluation of Colipa A025, from the Expert Panel of the Cosmetic Ingredient Review, and was used in calculation of the safety margin.

6. Teratogenicity

Embryotoxicity/teratogenicity study in the rat

OECD guideline:	414
Species:	Sprague-Dawley OFA rat
Route:	oral
Group size:	20
Substance:	Colipa A025 suspended in 2 % aqueous Polysorbate 80
Batch no:	op. 90
Dose levels:	0, 5, 50 and 500 mg/kg b.w. in a volume of 5 ml/kg b.w.
Administration:	days 6-15 of gestation
GLP:	Quality Assurance statement included

Doses (suspended in hydrogel) of 5, 50 and 500 mg/kg b.w. were administered to three groups of 20 pregnant Sprague Dawley rats. The control group was given the vehicle alone. On day 21 of gestation the dams were sacrificed.

The dams were observed for clinical signs of toxicity. Body weights and food consumption were measured. On day 21, complete autopsy and macroscopic examination of the organs and foetuses was carried out. Ovaries and uteri were examined. Foetal sex ratio, foetal body weights, number and position of implantations (live foetuses, early and late resorptions) and the number of corpora lutea were determined. Foetuses were examined for external, skeletal and visceral deviations.

Results

Dams: No mortalities were reported. There was a significant decrease in the body weight gain at 50 and 500 mg/kg b.w. No difference in either body weight gain or food consumption was seen in the lowest dose group when compared with controls. There were no differences in the fertility and pregnancy ratios and no abortion or any litter losses were reported.

Foetuses: The only reported observation was an increased number of foetuses with an extra rib (14th) in the highest dose group. The authors considered that this anomaly may be related to treatment but is usually considered as a minor abnormality and does not indicate that the test substance has teratogenic potential.

The highest dose level of 500 mg/kg b.w. was reported to be without foetotoxicity or teratogenicity.

Remark

We do not dispute the author´s conclusions on this study.

***In vitro/in vivo* hen´s egg screening test**

OECD guideline:	—-
Species:	Fertile egg from White Leghorn hen
Route:	injection into the albumen
Group size:	17-21
Substance:	Colipa A025 dissolved in egg albumen
Batch no:	op 90
Dose levels:	Day 1: 0.2 to 15 mg/egg Day 5: 0.1 to 5.0 mg/egg
Injection volume:	0.1 ml/egg
GLP:	—-

The test material was injected into the fertile egg on day 1 (before incubation) or on day 5. Non-viable eggs and hatched chicks (day 21) were examined for retardation and abnormalities.

Results

Dose-related mortalities occurred leading to LD_{50} estimates of 2.85 mg/egg at day 1 and 0.52 mg/egg at day 5. There was no evidence of teratogenic potential.

Remark

This is not accepted as a model for mammalian teratogenicity.

7. Toxicokinetics (incl. Percutaneous Absorption)

***In vitro* absorption studies**

The penetration of the compound through human breast epidermis on Franz-type diffusion cells was studied in four separate assays. Sections of the epidermis of human mammary skin was in contact with 0.625 % of the dye solution containing hydrogen peroxide for 30 minutes and then the skin was rinsed with an aqueous solution (2 % sodium lauryl sulphate in distilled

water) and dried. The concentration of dye penetrating the skin in the following 4 hours was measured by HPLC (detection limit 20 ng/ml). The penetration was determined in four studies - two with added hair and two with added p-phenylenediamine (described as "primary intermediate").

Each study involved 8 or 9 Franz cells. 4 µl of solution was applied to 2 cm^2 of skin which was equivalent to 0.125 mg dye/cm^2 of skin. The receiving fluid was physiological saline containing 0.01 % sodium ascorbate.

Results

	Presence of hair	Presence of p-phenylenediamine	Penetration as % of applied dose (with standard deviation)
Study 1	-	-	0.048 (0.030)
Study 2	+	-	0.050 (0.042)
Study 3	-	+	0.060 (0.023)
Study 4	+	+	0.040 (0.023)[(a)]

[a] In 6 of the 8 Franz cells in this study the concentration of dye in the receiving fluid was below the level of detection.

Remark

From the scatter of results about the mean it would appear that the results are near the limit of sensitivity of the method and no significant differences have been established between studies with and without added hair.

Information on these studies was available for the previous SCC evaluation of Colipa A025, from the Expert Panel of the Cosmetic Ingredient Review., and was used in calculation of the safety margin.

8. Mutagenicity

In vitro chromosomal aberration study in mammalian cells

The potential of Colipa A025 to induce chromosomal aberrations was investigated in Chinese hamster ovary (CHO) cells, in the presence and absence of S9 obtained from the livers of Aroclor 1254 pretreated male rats. Colipa A025 (batch no: op. 90; purity > 99.9 %) was dissolved in DMSO and applied to exponentially growing CHO cells at concentrations of 9.8, 19.5 and 39.1 µg/ml (without activation) and 39.1, 156 or 313 µg/ml (with activation). The negative control was DMSO and positive controls were mitomycin C (0.2 µg/ml, without activation) and cyclophosphamide C (20 µg/ml, with activation). The treatment period was 21 hours (without S9) or 4 hours (with S9). Cells were harvested after a total of 21 hours, with addition of colchicine for the final 2 hours. Chromosome breaks and exchanges were scored in 100 cells for each culture. The study was conducted according to OECD guideline no 473, in compliance with GLP.

Results

The test compound did not show any evidence of clastogenic activity. A decrease in mitotic index was seen at the highest concentrations tested.

Genotoxicity *in vivo*

Mouse bone marrow micronucleus test - i.p. administration

OECD guideline:	—
Species:	Swiss mouse
Group sizes:	40 treated and 20 controls - male only
Substance:	Colipa A025 dissolved in DMSO/distilled water (1/4 v/v)
Batch no:	—
Dose levels:	0 and 400 mg/kg b.w. in a volume of 10 ml/kg
Administration:	Single injection, with groups of mice sacrificed after 24, 48, 72 and 96 hours.
GLP:	—

Colipa A025 at 400 mg/kg b.w. (corresponding to approximately 50 % of the LD_{50}) was administered as a single i.p. injection. Mitomycin C (1 mg/kg b.w.) was used as positive control, the vehicle as negative control. The incidence of micronucleated polychromatic erythrocytes was evaluated 24, 48, 72 and 96 hours after administration. The slides from 6 test mice and 5 control mice (negative and positive) were evaluated for each time point.

Results

Colipa A025 did not increase the frequency of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse. The positive control functioned as expected. The test compound was reported to have no mutagenic activity. No mortalities were reported.

Remark

There is no indication as to whether there was a change in the ratio of polychromatic to normochromatic. It is not clear, therefore, whether the test substance reached the bone marrow (stem) cells. The study does not conform with the requirements of OECD guideline 474, which specified that mice of both sexes should be used.

Mouse bone marrow micronucleus test - oral administration

OECD guideline:	474
Species:	Albino CD mouse
Group sizes:	5 male and 5 female per time point
Substance:	Colipa A025 dissolved in arachis oil
Batch no:	EX70CXB
Dose levels:	0 and 400 mg/kg b.w. in a volume of 10 ml/kg
Administration:	Single gavage, with groups of mice sacrificed after 24, 48 and 72 hours.
GLP:	Quality Assurance statement included

Colipa A025 at 400 mg/kg b.w. was administered orally by gavage. Cyclophosphamide (50 mg/kg b.w.) was used as positive control, the vehicle as negative control. The incidence of micronucleated polychromatic erythrocytes and normochromatic erythrocytes was evaluated 24, 48 and 72 hours after administration for test animals and after 24 hours for negative and positive control animals. The slides from 5 mice were evaluated for each time point.

Results

Colipa A025 did not increase the frequency of micronuclei in polychromatic erythrocytes in the bone marrow cells of the mouse. The positive control functioned as expected. The test compound was reported to have no mutagenic activity. No mortalities were reported.

A significant change in the NCE/PCE ratio was observed 72 hours after treatment with Colipa A025, indicating toxicity to the bone marrow.

10. Special investigations

Contaminants

Ten industrially-synthesised samples of the Colipa A025 have been analysed for "total" nitrosamines using a proprietary method (Trew, 1992) developed from published procedures (Walters *et al.*,1978; Pignatelli *et al.*, 1987). Firstly "total" nitrite (if any in the sample) was converted to nitrogen by treating with sulphamic acid. "Total" nitrosamines were then converted to nitric oxide by a mixture of Hbr and acetic acid in boiling n-propyl acetate. After sparging by nitrogen gas from the boiling solvent and appropriate clean-up, the released nitric oxide was quantified by chemiluminescence detection against an authentic N-nitrosodiisopropylamine standard.

The "total" nitrosamine content of the sample (as ppb of nitric oxide) (C) was converted to the content of N-nitroso derivative (C') as follows:

$$C' \text{ (ppb of Nitroso derivative)} = \frac{C \times W}{30}$$

where

30 = molecular weight of nitric oxide

W = molecular weight of N-nitroso derivative being examined.

Result

Sample	Total nitrosamines as NO (ppb)	N-Nitroso derivative* (ppb)
op. 126	119	714
op. 127	123	738
op. 128	163	978
op. 129	179	1074
op. 130	295	1770
op. 131	436	2616
op. 132	149	894
op. 133	82	492
op. 134	163	978
op. 135	99	594

* N-Nitroso-6-hydroxybenzomorpholine

Remarks

These data were supplied in the Colipa Submission II, but were not supported by a detailed study report on microfiche. There is no information on GLP compliance.

Colipa seem to have only addressed the possibility that the test compound could form a nitroso derivative. From the information provided we cannot determine if this is a reasonable assumption or whether other nitrosamines could be formed during synthesis. No conclusions are made in the Submission on the acceptability of these levels.

11. Conclusions

1. Nitrosamine content of Colipa A25

Data has been submitted indicating that the N-nitroso derivative of Colipa A025 was present in 10 batches at level up to 2616 ppb. This is considerably in excess of the allowed maximum of 50 ppb nitrosamines in alkanolamines used in cosmetics. However, specific nitrosamines were not measured and it is therefore not possible to comment on the significance of this value. In this context, the apparent absence of genotoxicity Colipa A25 is reassuring.

2. Acute toxicity

The LD_{50} in rats was between 1 and 2 g/kg body weight.

3. Repeat dose toxicity study

The one-month oral toxicity study in rats given dose levels of 10, 100 and 1000 mg/kg b.w./day had been reviewed by the SCC previously. Examination of the study report confirmed that the low dose (10 mg/kg b.w./day) did not induce any adverse effect and corresponds to a NOAEL.

4. Teratogenicity

When administered orally to pregnant rats at dose levels of 5, 50 and 500 mg/kg b.w./day, Colipa A025 was found to be neither embryotoxic nor teratogenic. However, signs of delayed development of the foetuses (presence of a 14th rib) were seen in the high dose group. Decreased maternal body weight gain was reported at 50 and 500 mg/kg/day.

Colipa A025 was moderately toxic but had no teratogenic potential in the "*in vitro*/*in vivo* hen´s egg" screening test. This test is not an acceptable model for mammalian teratogenicity and it is not possible to comment on the implications of the results.

5. Genotoxicity

No evidence of genotoxicity was found *in vitro* in a chromosome aberration study in CHO cells, or *in vivo* in two micronucleus tests in mice - one with oral and the other with intraperitoneal dosing. Taking into account the negative results previously reported, Colipa A025 is considered not to be genotoxic.

6. Dermal absorption

In *in vivo* studies using Franz cells, formulations containing 0.625 % of A25 with or without another dyestuff (p-phenylenediamine), were applied in absence or in presence of hair. 0.04 % to 0.06 % of the applied compound was found in the receiving chamber according to experimental conditions. These studies had been reviewed by the SCC previously and used in calculation of the safety margin.

Conclusion

Colipa A025 (6-hydroxybenzomorpholine; INCI: hydroxybenzomorpholine) is used in direct hair dye formulations at concentrations up to 2 %. Since the oxidative hair dyes are mixed with hydrogen peroxide before use, the concentration at application is 1 %.

Information supplied in Submission II indicates that the nitrosamine content of Colipa A025 exceeds the maximum allowed for cosmetic ingredients. Colipa should be asked to explain this anomaly.

Overall evaluation of the data presented in Submission I and II does not show evidence of genotoxicity.

Other data provided in this submission were included in the previous SCC evaluation or do not modify the previous conclusions or calculation of the safety margin.

Classification: 2

Further classification of the contamination with nitrosamines is required.

Information should be provided on the representativeness of the batch used in relation to the commercial product.

OPINIONS ADOPTED DURING THE 68TH PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 20 December 1996

REFINED COAL TAR

1. Introduction

1. Submissions I and II contain information on:
   - the manufacturing process of refined coal tar and its aqueous extracts (25 %), ethanol extracts (25 %) and oil extracts (30 %);
   - analytical determination of benzo-alpha-pyrene;
   - carcinogenic PAH levels in the ingredients under consideration;
   - calculation of a safety factor for the use of refined coal tar in shampoos.

The risk evaluation report is based on the data available in the literature (toxicity in animals and epidemiological and clinical data) for coal tar and polyaromatic hydrocarbons.

No supplementary toxicological test has been carried out on refined coal tar or its extracts.

[The available data are generally interpreted by industry as showing that the risk is minimal, mainly as regards:
- the carcinogenic potential of benzo-alpha-pyrene (BαP) *demonstrated in certain highly susceptible mouse strains and with solvents that enhance cutaneous absorption;*
- cutaneous absorption, genotoxicity and tumour initiation potential of BαP, *reduced in the presence of other constituents present in the complex organic mixtures which constitute tars;*
- the toxic effects reported in man - increase in the risk of skin cancer, phototoxicity, etc. *observed in severe conditions of occupational or therapy-linked exposure;*
- the significance of the measurement of urinary metabolites and their mutagenic affect, notably *1-OH pyrene contested* as a quantitative biological marker of systemic exposure; human resistance to the potential carcinogenic effects of coal tars and PAHs *attributed to protection at cell level.*]

Submission III concerns an *in vitro* cutaneous absorption protocol for PAHs from a shampoo diluted to 10 %, submitted to the SCC for commentary.

Submission IV is an updated rationale to take account of recent draft OECD guideline on skin absorption *in vitro*.

2. The numerous data contained in the IARC monograph, as well as the very well documented report on the toxicological profile of PAHs, prepared in October 1993 for the US Department of Health and Human Services, give interesting elements for thought and discussion on:
   - PAH levels in crude and refined coal tars;
   - carcinogenicity and interactions with other substances;
   - metabolism of the PAHs and the carcinogenic action mechanisms;
   - biological markers;
   - epidemiological data;
   - calculation of the safety factor

and make it possible to prepare an opinion on the studies which could be required by the SCC.

3. It is interesting to examine the rules governing dangerous substances and dangerous preparations in the EU.

2. Discussion

1. Refined Coal Tar (RCT) and PAHs levels:

The manufacturing process mentioned in Submission II which consists of mixing the two fractions obtained by distillation of the crude coal tar, with the highest levels of PAHs of high molecular weight, does not lead to a reduction in the concentration of carcinogenic PAHs in the tar (RCT).

- The levels cited in Tables 1, 2, 3 and 4 (Submission II) for RCT are of the same order of magnitude, if not higher, than the levels cited in the IARC monograph for high temperature crude coal tars (CCT).

	RCT	**CCT**
Benzo(α)pyrene	6400 to 9500 ppm	5500 to 11000 ppm
Dibenzanthracene	1050 to 2010 ppm	~ 1000 ppm

— Nevertheless, the PAH levels are reduced in the aqueous, ethanol and oil extracts. The reduction is a direct function of the RCT concentration in these extracts.

NB: At least two coal tars of pharmaceutical quality correspond to the definition of the RCT ethanol *extracts*

— coal tar solution USP = coal tar + polysorbate 80 + ethanol (ethanol content 81 to 86 %)
— liquor picis carbonis = maceration of coal tar 20 % + quillaia 10 % in 90 % ethanol for 7 days + filtering.

This is, however, without relevance when discussing cosmetics.

2. Carcinogenicity

Animal studies have unquestionably demonstrated the skin tumour induction potential of
— benzo(a)anthracene
— benzo(b)fluoranthene
— benzo(j)fluoranthene
— benzo(a)pyrene
— chrysene
— dibenz(a,h)anthracene
— indeno(1,2 3-c,d)pyrene

acting as full carcinogens after skin exposure.

Benzo(a)pyrene (BαP) is a potent skin carcinogen and a skin tumours initiator in the mouse; it is often used as a positive control in bioassays of carcinogenicity.

By calculation the EPA has derived a relative potential based on mouse skin carcinogenicity. This potential is 1.11 for dibenz(a-b)anthracene, by comparison with 1.0 for the BαP.

PAH mixtures and cancer:

Since PAHs need metabolic activation by monoxygenases to induce carcinogenic effects, any alteration in the metabolic process can lead either to a reduction in toxicity (antagonistic interaction) by competition for the same metabolic activation enzymes, or to an increase in toxicity (synergic interaction) by competition for a metabolic disactivation phase.

Besides the antagonistic effects mentioned by COLIPA, co-carcinogenic effects have been observed in studies on interactions between carcinogenic PAHs, non-carcinogenic PAHs or weakly carcinogenic PAHs by skin application in the mouse.

Notably the studies have shown an increase in the cancer induction potential or tumour initiation potential of benzo-α-pyrene, by simultaneous application of pyrene or benzo(g, h, i)perylene.

Benzo(e)pyrene, fluoranthene and pyrene have also shown weak tumour promotion activity after initiation by benzo-α-pyrene and have led to the increased formation of DNA adducts (Toxicological profile for PAHs - USA).
Predicting the final resulting effect of multiple interactions in such a complex mixture is nearly impossible.

3. PAHs metabolism and carcinogenic action mechanisms

PAHs are classified as “alternants” (for example BαP) or “non-alternants” depending on the electronic density associated with their molecule, and are activated as final carcinogens through different biotransformation mechanisms.

In vitro tests have shown that intermediary diolepoxides of the “bay region” (between BαP carbons 11 and 12) are the final carcinogens for the “alternants” PAHs.
The biotransformation of PAHs in diolepoxides is induced by the enzymes associated with the cytochrome P450 system, notably Aryl Hydrocarbon Hydroxylase (AHH).

The diolepoxides form covalent bonds with the DNA and other cell macromolecules, provoking mutation and tumour initiation.

In vitro studies on human tissues indicate that the same activation mechanism may occur in man. AHH induction and the formation of the reactive intermediate - benzo-á-pyrene, 7.8-dihydrodiol - have been observed in the epithelial tissue of human capillary follicles. All the phases necessary for cell transformation and cancer induction via this mechanism have been demonstrated in human skin fibroblast cultures.

Several other factors seem to be involved in the ultimate expression of PAH toxicity and carcinogenicity, such as the cell immunity suppression through inhibition of prostaglandin synthesis.

4. Biomarkers

PAHs and their metabolites were measured in the urine of workers exposed to PAHs and in patients treated with coal tar.

The results of several studies indicate that 1-OH pyrene can be used as a biomarker of PAH exposure in man (Toxicological profile for PAHs - USA).

Several PAHs are genotoxic and indirect mutagens.

The results of three *in vivo* studies in the rat indicate that the mutagenicity of the excreta and the formation of DNA adducts in lymphocytes are useful biomarkers of BαP exposure in the rat.

In human clinical studies a significant correlation has been observed between the urinary excretion of PAHs and the mutagenic potential of the urines in the Ames test.

Measurement of the formation of DNA adducts in human lymphocytes has been proposed as a biomarker of the effects induced by BαP in man.

5. Epidemiological Data

The epidemiological data are controversial.

There is an apparent contradiction between the increase in cancer observed in the context of occupational exposure and the results of clinical studies of patients suffering from atopic dermatitis and psoriasis.

The authors try to explain why topical therapy with coal tar is not associated with a significant increase in skin and other cancers and put forward hypotheses on the mechanisms which determine the abnormal behaviour of the pathologic skins, notably:

- the abnormal immunity of patients suffering from atopic dermatitis and psoriasis;
- the abnormally low levels of the Aryl Hydrocarbon Hydroxylase enzyme (AHH) in patients suffering from atopic dermatitis, which might inhibit the conversion of PAH metabolites into active forms induced by AHH.

However it seems that the absence of an increase in the risk of non-melanoma skin cancer cannot be clearly established, since the results are biased by:

- better surveillance of the patient cohort by comparison with the general public in respect of skin tumours;
- the relatively recent inclusion of tumours of this type in the Cancer Register.

Moreover the absence of multivariant analyses in the studies on the treatment of psoriasis by Puvatherapy make it impossible to establish the relative role of ultraviolet radiation and tars in the induction of tumours.

The clinical data require an in-depth analysis. Experimental results on the mechanisms referred to above are necessary in order to determine whether it is appropriate to extrapolate to cosmetic use on healthy skin epidemiological data relating to pathological skin.

6. Safety Factor

In Submission II the calculation of the “safety factor” takes in account a partition coefficient of 10 and a rinsing coefficient of 10 in calculating the quantity of tar which reaches the scalp.

The BαP exposure level is calculated on the basis of a maximum concentration of 0.5 % BαP in the tar.
Cutaneous absorption is estimated at 1 %.

The concentration of 0.003 ng/cm^2/day benzo-α-pyrene is considered by industry to represent a negligible risk.

Two of these elements stand in need of correction.

1. The BαP concentrations in refined coal tars range from 0.64 % to 0.95 % (see Tables 1, 2, 3, 4. Submission II).
2. Percutaneous absorption of coal tars and BαP in humans seems to be higher than 1 %.
   - *In vivo* data in man indicate that after 6 hours of exposure, 20-56 % of a low dermal dose of PAH is absorbed.
   - An *in vitro* study evaluates permeation through the human viable skin after 24 hours at 3 % of the total radioactivity of the (^{14}C) BαP applied.

If we calculate systemic exposure to BαP after a single application of shampoo, taking these correctives into account, and on the basis of the calculation of the safety factor for hair dyes, we obtain:

- quantity of shampoo per application	=12 g
- quantity of RCT applied to the scalp	=12 g x 1 % x 1 %= 1.2 mg
- maximum quantity of BαP applied to the scalp	=1.2 mg x 1 % = 12 µg
- cutaneous absorption (in vitro)	=3 %
- systemic exposure per application	$=\frac{12\ \mu g \times 3}{100} = 0.36\ \mu g$
- average body weight	=60 kg
- systemic exposure to BαP per application per kg of body weight	$=\frac{0.36\ \mu g}{60\ kg} = 0.006\ \mu g$ BαP/kg
	= 6 ngBαP/kg per application

"Safety factor"

While some consider the dose of 0.003 ng/BαP/cm^2/day to be a tolerable limit corresponding to a negligible risk in the case of an unintentional and unavoidable exposure, the SCC cannot entertain laying down a negligible-risk dose limit for genotoxic carcinogens that form covalent bonds with the DNA, in the context of the voluntary use of cosmetic products on the skin.

7. Protocol on *in vitro* skin penetration in humans

The utility of this study depends on the sensitivity of the HPLC/FD method. If the quantitative detection limit were sufficiently low, the study would make it possible to confirm or refute the cutaneous absorption data available at present.

Studies on mammalian skins (including human skin) showed that, in all species, metabolic viability was a major factor involved in the *in vitro* skin permeation of surface applied BαP. Permeation was accompanied by extensive cutaneous "first pass" metabolism; both parent components and full spectrum of metabolites were founed in the receptor fluid from viable skin preparations.

A meaningful in vitro study should consider both diffusion and cutaneous biotransformation of the applied compound.

Otherwise, an adequate clinical cutaneous absorption study:

- by application during 28 days, of shampoos in normal conditions of use to a sufficient number of suitably selected human volunteers (non-smokers, etc.),
- drawing inspiration from van Schooten and Clonfero studies
- including measurement of the urinary PAH markers and their mutagenic and genotoxic effects

would provide information on the bioavailability of carcinogenic PAHs in normal conditions of use, resulting from the use of rectified coal tars and of their aqueous, ethanol and oil extracts.

Comments:

1. The van Schooten clinical study, which is contested by industry (see Submission IV) was conducted on the shampoo Resdan Forte, containing 56 ppm BαP.

2. Recently (July 1995) the KVW (Netherlands) published an analysis report on the determination of PAHs in 62 anti-dandruff shampoos. The maximum levels found in the shampoos containing tars currently on the market are:

BαP:	43.4 ppm (Resdan and Essex tar shampoos)
Dibenzanthracene:	3.2 ppm (Resdan)
	4.1 ppm (Essex tar)
Total carcinogenic PAHs:	> 100 ppm

8. Regulatory aspects

Directive 76/768/EEC

Directive 67/548/EEC

Directive 94/60/EEC

Directive 93/21/EEC

- The two constituents of the RCT mixture, coal tar pitch, high temperature (EINECS 266-028-2) and coal tar distillate (distillation range 130°-450°C) are entered in Annex I to Directive 67/548/EEC as category 2 carcinogenic substances under the numbers 648-055-00-5 and 648-047-00-1 respectively, with note M.

Note M says that a substance should not be classified as carcinogenic if it can be shown that it contains less than 0.005% (w/w) Benzo(α)pyrene (i.e. 50 ppm).

Category 2 carcinogenicity: Substances for which there is sufficient evidence to provide a strong presumption that human exposure may result in the development of cancer (Directive 93/21/EEC), Annex IV).

- Coal tar distillate (130°- 450°C) is entered in Annex II to Directive 76/768/EEC under No 38 under the name "anthracene oil".
- Directive 94/60/EEC, relating to restrictions (concentration and labelling warnings) on the marketing and use (by the final consumer) of certain dangerous substances and preparations does not apply to cosmetic products. However it is interesting to note that, for the other products to which the Directive applies, Category 1 and 2 carcinogenic substances listed in Annex I to Directive 67/548/EEC may not form part of preparations sold to the general public in an individual concentration greater than or equal to 0.1 % (Annex VI to Directive 93/21/EEC).

3. Opinion

1. Refined tars are complex ingredients which can be obtained by different methods.
2. According to the submissions received, Polyaromatic hydrocarbons (PAH) levels in refined coal tar in question (RCT) are in the same range as PAH levels in crude coal tar (CCT).
3. There is no doubt about the Carcinogenic potential of several PAHs entering the composition of RCT. Synergistic and antagonistic interactions between carcinogenic, weakly carcinogenic and non carcinogenic PAHs were reported in the literature.
   The final resulting effect of those multiple interactions is, however, not predictable.
4. Several studies have shown that PAHs enter the skin exposed to coal tar.
5. Aryl hydrocarbon hydroxylase induction is an important factor in the biotransformation of alternants PAHs into ultimate carcinogens.
   Induction increase of AHH activity was observed *in vitro* and *in vivo* in human skin exposed to coal tar.
6. The application of coal tar to skin may lead to dermal and systemic carcinogenesis.
7. Given the fact that numerous PAHs are genotoxic carcinogens, no safe level can be established.
8. Therefore it is the opinion of the SCC that crude or refined coal tars must not be present in cosmetic products.
9. The SCC is also of the opinion that there is an urgent need for evaluation of tars from sources other than coal.

Classification: 2a

OPINIONS ADOPTED DURING THE 71ST PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 24 June 1997

MUSK MOSKENE

1. General

1.1 Primary name

Musk moskene

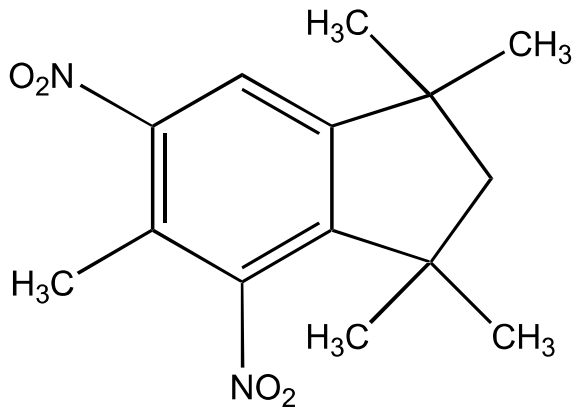
1.2 Chemical names

4,6-dinitro-1,1,3,3,5-pentamethylindane
1,1,3,3,5-pentamethyl-4,6-dinitroindane
1 H-Indene,2,3-dihydro-1,1,3,3,5-pentamethyl-4,6-dinitro-(CAS)

1.4 CAS no.

116-66-5
EINECS N°: 204-149-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{14}H_{18}N_2O_4$
Mol weight: 278.31

1.8 Physical properties

Physical form: pale yellowish or whitish-ivory coloured crystals or crystalline powder
Melting point: 131°C
Solubility in water: virtually insoluble
Vapor pressure: <0.001 mm Hg 20° C
Flash point: >200° F CC

2. Function and uses

Perfumery ingredient used at levels up to 0.25 % in alcoholic fragrances and up to 1 % in fine fragrances. The maximum concentration recommended in a fragranced cosmetic or toiletry product is about 0.025 % but Colipa indicates that most products contain much less.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

According to a 1972 study report, the LD_{50} in rat was greater than 5g/kg; the full report is not available.

3.2 Acute dermal toxicity

The dermal LD_{50} in rabbit was greater than 5 g/kg b.w.; the corresponding full report is not available.

3.8 Subchronic dermal toxicity

Results of a 90 day dermal toxicity in the rat -1990- are reported; Musk moskene application (unoccluded) was renewed daily at levels of 7.5 - 24.0 or 75.0 mg/kg b.w./day in phenyl ethyl alcohol; the treatment volume for the study was 2 ml/kg b.w./day; the test substance and/or the vehicle were applied on approximately 25 % of the body surface; the skin was clipped but not abraded; the animals were fitted in individual cages with collars to prevent ingestion.

Deaths were observed at the 3 levels of moskene as well as in the control group (vehicle). The clinical signs and symptoms observed were considered by the authors as non specifics and not attributable to the substance; no significant difference between treated and control animals nor dose related differences were observed in respect to body weight, haematology, clinical biochemistry, organ absolute weight and organ histopathology; statistical increase of the relative weight of the liver and kidneys occurred in males of the highest moskene dose; no neurotoxic alteration was objectivated.

According to the results, the NOAEL was at least 24 mg/kg b.w. for the males and 75 mg/kg b.w. for the females.

3.10 Chronic toxicity

No data available.

4. Irritation & corrosivity

4.1 Irritation (skin)

Skin primary irritation: A full strength solution of moskene applied for 24 hours under occlusion to intact or abraded rabbit skin was moderately irritating -1979-. The corresponding full report is not available.

A 10 % solution in petrolatum applied under occlusive patch for 48 hours produced a mild irritation in human skin -1985-.

Skin irritation by repeated application: In a 90 days study, Musk moskene was administered by dermal application (unoccluded) to groups of male and female rats on a daily basis at levels of 7.5, 24.0 or 75.0 mg/kg b.w./day in phenyl ethyl alcohol; the treatment volume for the study was 2 ml/kg b.w./day; the test and/or the control materials were applied weekly on approximately 25 % of the body surface, to the skin shaved; the treatment site was examined and weekly scored (according to the method of Draize) for erythema and edema.

No significant local effects were obeserved at the treatment area till to 75 mg/kg b.w./day.

4.2 Irritation (mucous membranes)

The potential irritant and/or corrosive effects of Musk moskene were evaluated on the eye of 6 rabbits by instillation of 0.1 ml (63 mg) of the test substance followed 30 seconds post instillation by a rinsing of the eye of 3 of the animals.

The test was performed according to OECD guideline n° 405. Light immediate conjunctivite occurred, resolved in all animals. Musk moskene was not considered an eye irritant – 1997 –.

5. Sensitization

Skin sensitization: A guinea pig sensitization test was performed according to the Maguire technique; on days 1 and 2 0.2 ml of 10 % moskene in petrolatum was applied to the skin; on day 4 0.1 ml of Freund´s complete adjuvant was injected intradermally; this was followed by the application of a third dose of the test substance.

Challenge application was made 21 days after the first dose and renewed 2 weeks later. No reactions were observed.

As part of a photosensitization study guinea pigs were treated under occlusion for 4 hours per day, 3 times weekly for 3 consecutive weeks with a 10 % moskene in acetone solution. The challenge application was made 2 weeks after the final induction treatment. No reactions were observed.

Photoallergy: In the same study 10 to 14 days after the final induction treatment and UVA irradiation, a challenge was done by application of the 10 % moskene in acetone solution, followed by the UVA exposure (320 - 400 nm) of the challenge site.

No effects were observed.

Photoallergic potential was investigated on Dunkin Hartley female guinea pigs on the basis of a maximization test using Freund´s complete adjuvant. Concentrations of 10 % in dimethyl acetamide/acetone/ethanol (4:3:3) were applied on the skin which was then irradiated with 100 Kjm^{-2} UV. The sequence was renewed twice. At challenge, the test substance was applied to fresh skin after its irradiation. A second confirmatory challenge was renewed a week later. There was a photoallergic response in all 12 animals treated with the positive control, Musk ambrette. There was a photoallergic reaction in only three animals to moskene

(at 10.1 % and on rechallenge only at 0.1 %). Cross reactivity was demonstrated on animals photoallergic to Musk ambrette.

Numerous studies were reported in the literature in order to assess the photoallergenic potential of Musk moskene in human. Some evidence of photocross reactivity to moskene in patients photoallergic to Musk ambrette are reported. Some individual cases of direct photoallergy to moskene are reported but such situations are limited, and results are negative in most cases.

There was a photoallergic response in all 12 animals treated with the positive control, Musk ambrette. There was a photoallergic reaction in only three animals to moskene (at 10, 1 % and on rechallenge only at 0.1 %). Cross reactivity was demonstrated on animals photoallergic to Musk ambrette.

6. Teratogenicity

No data available.

7. Toxicokinetics (incl. Percutaneous Absorption)

The results of various *in vitro* studies of the skin absorption are reported:

^{14}C moskene was applied (6 μlcm^{-2}) to 5 cm^2 of intact naked rat skin clamped to a penetration chamber. The horny layer was stripped from the skin and measured separately – a 3 % and 10 % solution in ethanol: acetone (1:1) were evaluated (180 μgcm^{-2} and 600 μgcm^{-2} respectively). The total penetration rate values are concentration and time dependant. After a contact time of 16 hours, they amount at 33.7 % and 23.3 % of the total applied dose.

With intact pig skin, after exposure times of 16 hours, the total absorption rates amount to 6.2 % and 4.1 % of the total applied dose; moskene shows then considerable lower total penetration rates in pig skin.

The total penetration during 16 hours of exposure is very low; a significantly higher portion of moskene was found in the stratum corneum than in the living skin layers.

In vitro absorption with stripped pig skin has also been investigated. ^{14}C moskene was applied (6 $\mu l/cm^2$) to 5 cm^2 for 6 hours; when the stratum corneum was removed by stripping, the total penetration rate of moskene increased while only traces of the labelled material were determined in the chamber liquid.

8. Mutagenicity

An Ames test -1980- was performed with and without metabolic activation (rat liver homogenate S9 fraction) in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100; at concentration of 33.3 - 100 - 333.3 - 1000 - 3333 and 10 000 µg/plate in dimethylsulfoxide, the test compound did not precipitate till to 100 µg/plate.

Chromosomic aberration was assessed with (6, 12, 24 and 36 µg/ml) and without (12, 24, 36 and 48 µg/ml) metabolic activation system (Araclor induced rat liver S9) in Chinese Hamster ovary cells (1995). No statistically significant increases in structural chromosome aberrations

or in numerical aberrations were observed at any of these dose levels in the non activated or activated studies.

9. Carcinogenicity

No data available.

10. Special investigations

Concentration in human tissues: When analysed in 32 human fat samples and 23 human breast milk samples from Germany, moskene was found at low levels in a few samples (no quantitative data are given).

Photoirritation: The photoirritating potential of moskene was assessed *in vitro* and *in vivo* methods:

- in the *in vitro* assay with *Saccharomyces cerevisiae*, according to the authors, no adverse effect was observed.
- in an *in vivo* method realized on guinea pig, 0.2 ml of a 25 % solution (vehicle not identified) was applied topically, then irradiated with black light tubes for 1 hour (1.2 - 1.8 m w/cm^2); no effects were observed.

According to the report of a study, guinea pigs were treated with 10 % moskene in acetone under occlusion for 4 hours per day, 3 times weekly for 3 consecutive weeks.

Subsequent to removal of patches at each treatment, the treated areas were irradiated for 2 hours with UVA. No reactions were observed after the irradiations; preparation was considered as not photoirritating.

In another study, moskene was applied as a 10 % solution in dimethylacetamide acetone/ ethanol (4:3:3); irradiation with fluorescent black lamps (300 - 400 nm) at the dose of 100 Kjm^{-2} followed the application. The results indicate that the substance was not an irritant or photoirritant under the conditions used.

11. Conclusions

Information concerning subchronic/chronic oral toxicity, teratogenicity/reproduction toxicity and carcinogenicity is not available.

Information concerning the short term studies comes from the 1979 RIFM monograph; no complete reports are available.

Most of the results given come from publications.

It can be assumed from the given information and from the results of the subchronic dermal toxicity that the Musk moskene is lightly irritating for the skin.

As this ingredient has no food use status, it makes sense, as suggested by industry, not to use it in lip products or in flavours for the oral hygiene products.

According to the given data, there was no evidence of significant photoirritating, sensitizing or photosensitizing potentials.

In a subchronic dermal toxicity study in rats, significant increase in liver and kidney relative weights were observed with males; the NOAEL was reported to be 24 mg/kg b.w./day for males and 75 mg/kg b.w./day for females.

As subacute, subchronic and chronic toxicity studies by oral route are not available, then a direct calculation of the safety margin cannot be made by means of the NOAEL expected from the 90 days oral study.

According to the results of an Ames test and the chromosomic aberration assay, Musk Moskene present no mutagenic potential.

According to these results, there is no evidence that Musk Moskene may be genotoxic or carcinogenic.

Absorption has been investigated in various *in vitro* studies with animals skin; no data are available on human skin:

In the rat according to concentration, 33.7 % and 23.3 % of the amount applied on the skin were absorbed during an application time of 16 hours.

With intact pig skin, the total absorption rate after 16 hours of exposure was 6.2 % and 4.1 % of the total amount applied.

Concerning the presence of traces of Musk moskene in human fat and breast milk, the results reported are questionable; according to the results, it can be at least suspected that Musk moskene was present. However, quantitative values, cannot be retained from such studies.

In all cases, the findings provide no evidence for toxicological evaluation. To solve the problem, new studies done with adequate methods and according to good laboratory and clinical practise should be undertaken.

In the absence of any useful new data, classification:

Classification: 2a

12. Safety evaluation

According to submission III, the quantity used and frequency of application for a range of cosmetic products were agreed by senior technical representatives of the cosmetics industry. It is considered that the range of products selected covers all those that are likely to be used in any one weekly period. Furthermore, the use quantities should be regarded as an exaggerated figure:

Calculation of Exposure to Moskene in Cosmetic Products

Type of cosmetic product	Application quantity in grams per application	Application frequency per day (c)	Retention factor (d) (%)	Fragrance mixture in product (e) (%)	Moskene in fragrance mixture (f) (%)	Moskene in product (%)	Exposure to moskene (mg/day)	Exposure to Moskene for 60 kg person (mg/kg/day)
Body lotion	8	0.71	100	0.4	5.7	0.0228	1.2950	0.0216
Face cream (a)	0.8	2	100	0.3	5.7	0.0171	0.2736	0.0046
Eau de toilette (b)	0.75	1	100	8.0	5.7	0.4560	3.4200	0.0570
Fragrance cream	5	0.29	100	4.0	5.7	0.2280	3.3060	0.0551
Anti-perspirant/ deodorant	0.5	1	100	1.0	5.7	0.0570	0.2850	0.0048
Shampoo	8	1	1	0.5	5.7	0.0285	0.0228	0.0004
Bath products	17	0.29	0.1	2.0	5.7	0.1140	0.0056	0.0001
Shower gel	5	1.07	1	1.2	5.7	0.0684	0.0366	0.0006
Toilet soap	0.8	6	1	1.5	5.7	0.0855	0.0410	0.0007
Hair spray	5	2	1	0.5	5.7	0.0285	0.0285	0.0005
							Total (g)	0.1452

Note (a): Including make up and foundation.

Note (b): The entry for eau de toilette includes all hydroalcoholic products (i.e., parfums, aftershaves, colognes, etc...). These products are not all used on one occasion, the quantity per application being inversely related to the fragrance concentration in the product. The figure for eau de toilette therefore covers all hydroalcoholic fragranced products.

Note (c): To allow comparison with the No Observed Adverse Effect Levels from animal studies, use is expressed as a daily exposure although in fact it is based on weekly figures in order to take into account of usage patterns which would not otherwise be evident. For example, a body lotion and a fragranced cream (i.e. a body lotion containing a higher level of fragrance) will not both be used on the same day. It has been estimated therefore that a body lotion may be used on five days per week (i.e., 0.71 times per day) and a fragranced cream on two days per week (i.e. 0.29 times per day). A similar calculation applies to bath products and shower gel.

Note (d): Retention factors for the skin are conservative estimates from known use of products, taking into account wash-off characteristics.

Note (e): The concentration of the fragrance mixture in a cosmetic product type has been determined by senior technical representatives of the cosmetic industry.

Note (f): The concentration of a fragrance ingredient in a fragrance mixture is based on data obtained by the fragrance industry from the examination of commercialized formulations containing the fragrance ingredient. The concentration used corresponds to the upper 97.5th percentile concentration of the fragrance ingredient in fragrance mixtures, a concentration which is in itself maximized because the products not containing the fragrance ingredient were not included as zero values in the distribution of the samples.

Note (g): Total consumer exposure to the fragrance ingredient is determined by adding figures f or the different product types expressed as mg/kg body weight/day. In view of all the above assumptions, this figure has to be regarded as conservative; it is most unlikely that a consumer will consistently use a number of different cosmetic products which are all perfumed with the upper 97.5th percentile level of the fragrance ingredient.

The more relevant data concerning the adverse effect was found in the 90 day dermal toxicity, in rat where the assumed NOAEL were 24 mg/kg b.w./day (males) and 75 mg/kg b.w./day (females).

The systemic NOAEL can be obtained from the results when corrected for dermal absorption of 24 % (worst figure) in the rat skin.

Considering 24 mg/kg b.w./day as the acceptable NOAEL by dermal route, the corresponding rat daily systemic exposure is:

24 mg/kg x 24 % = 5.8 mg/kg

The calculation of the margin of safety is then as follows:

Maximum amount of Musk moskene applied: 0.1452 mg/kg b.w./day

Maximum absorption through the human skin:

The pig absorption data may be used to estimate the absorption in human skin since pig skin is more relevant, physiologically to human skin. The assumed maximum absorption would be then 6.2 %.

Assumed human daily systemic exposure dose: 0.1452 x 6.2 % = 9.0 µg/kg b.w.

Margin of safety: $\frac{5800}{9.0} = 640$

MUSK TIBETENE

1. General

1.1 Primary name

Musk Tibetene

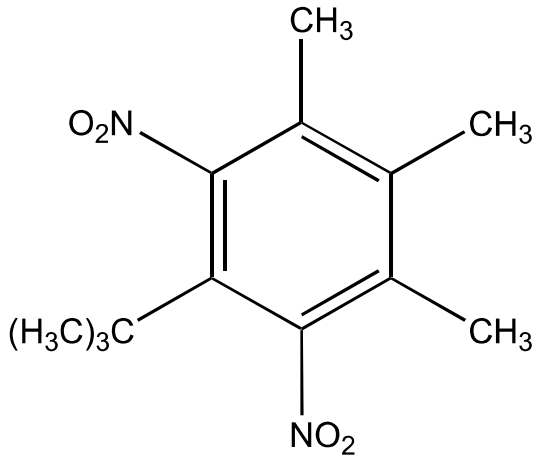
1.2 Chemical names

5-tert-Butyl-1,3,5-trimethyl-4,6-dinitrobenzene
1-tert-Butyl-2,6-dinitro-3,4,5-trimethyl benzene
Benzene,1-(1,1-dimethyl)-3,4,5-trimethyl-2,6-dinitro-(CAS)

1.4 CAS no.

145-39-1
EINECS N°: 205-651-6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{13}H_{18}N_2O_4$
Mol weight: 266.30

1.8 Physical properties

Appearance: pale yellowish crystals or yellowish-whitish coloured crystalline powder.
Melting point: 135 °C
Solubility in water: virtually insoluble
Vapor pressure: <0.001 mm Hg 20°C
Flash point: >200°F CC

1.9 Solubility

Virtually insoluble in water.

2. Function and uses

Fragrance ingredient used at levels up to 5 % in fragrance itself. Not all fragrances contain Musk Tibetene; according to the Colipa submission, most of them do not. When the fragrance is added to a cosmetic product, the concentration on Tibetene has to range to a maximum of 0.25 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Oral LD_{50}. According to a 1971 study, the LD_{50} in rat was above 6 g/kg b.w.; (6 groups of 5 Sprague Dawley rats); a first range finding study showed no death at level of 10 mg/kg.

3.2 Acute dermal toxicity

Dermal LD_{50}. The dermal LD_{50} in rabbit was above 10 g/kg b.w. (4 New Zealand White Rabbits).

Full report is not available.

3.4 Repeated dose oral toxicity

Acute toxicity by multiple dose was studied – 1985 – in wild trapper deer mice at a dose of 1.25 g/kg b.w./day for 3 days followed by an observation period of 4 days; it is recorded that the ingestion of this dose did not cause mortality in more than 50 % of the mice. It is however not known if any death occurred.

Full reports are not available.

3.8 Subchronic dermal toxicity

Results of a 90 day dermal toxicity in the rat are reported; Musk Tibetene application (unoccluded) was renewed daily at levels of 7.5-24.0 or 75.0 mg/kg b.w./day in phenyl ethanol alcohol; the treatment volume for the study was 2 ml/kg b.w./day; the test substance and/or the vehicle were applied on approximately 25 % of the body surface; the skin was clipped but not abraded; the animals were fitted in individual cages with collars to prevent ingestion.

Deaths of 1 female given 24 mg/kg b.w./day and five males given 75 mg/kg b.w./day as well as of the control group (vehicle) were reported. The clinical signs and symptoms observed were considered by the authors as non specifics and not attributable to the tested substance; no significant differences between treated and control animals nor dose related differences were observed in respect to body weight, haematology, clinical biochemistry, organ weight and organ histopathology. No neurotoxic alteration was objectivated.

According to the results, the NOAEL for Musk Tibetene was reported to be 75 mg/kg b.w./day as well for the males and females.

3.10 Chronic toxicity

No data are available.

4. Irritation & corrosivity

4.1 Irritation (skin)

In the same 1971 study Musk Tibetene powder was applied on normal and abraded moistened rabbit skin for 24 hours under occlusion; no irritation occurred (4 New Zealand White Rabbits).

Full report is not available.

Skin irritation by repeated application: In a 90 day study – 1988, 1990 – Musk Tibetene was administered by dermal application (unoccluded) to groups of male and female rats on a daily basis at levels of 7.5, 24.0 or 75.0 mg/kg b.w./day in phenyl ethyl alcohol; the treatment volume for the study was 2 ml/kg b.w./day; the test and/or the control materials were applied weekly on approximately 25 % of the body surface, to the shaved skin; the treatment site was examined and weekly scored (according to the method of Draize) for erythema and oedema.

No significant local effects were observed at the treatment area till to 75 mg/kg b.w./day.

4.2 Irritation (mucous membranes)

3 mg of Musk Tibetene were applied in the eye of 6 New Zealand White Rabbits and then ocular reactions were read according to the Draize method for 72 hours. Slight irritations appeared on the first hours following treatment but disappeared before the 24 hours reading.

5. Sensitization

Photoirritation: The photoirritating potential of Musk Tibetene was assessed - 1981, 1980 - in white female Hartley Guinea Pigs; concentrations of 5, 10 and 20 % of Tibetene when applied on the shaved skin which was then irradiated with fluorescent lamps (300 -430 nm) for 3 hours at dose range of 1.6 -7.6 10^7 ergs/cm^2. No photoirritations were observed on the 3 days thereafter.

According to the report of a study realized in 1986, guinea pigs were treated with 10 % Tibetene in acetone under occlusion for 4 hours per day, 3 times weekly for 3 consecutive weeks.

Subsequent to removal of patches at each treatment, the treated areas were irradiated for 2 hours with UVA. After no adverse effects were observed after the irradiations, preparation was considered as no photoirritating.

In another study -1988- Tibetene was applied as a 10 % solution in dimethylacetamide acetone/ethanol (4:3:3); irradiation with fluorescent black lamps (300-400 nm) at the dose of 100 KJm^{-2} followed the application. The results indicate that the substance was not an irritant or photoirritant under the conditions used.

Skin sensitization:
Several tests were reported on animals and humans:
The results of an open epicutaneous test performed in guinea pig were reported in 1979. Tibetene was applied topically on days 0-20 and a challenge was made after a 2 week rest period. No adverse effects were observed.

As part of a photosensitization study - 1986 - guinea pigs were treated under occlusion for 4 hours per day, 3 times weekly for 3 consecutive weeks with a 10 % Tibetene in acetone solution. The challenge application was made 2 weeks after the final induction treatment. No reactions were observed.

In 1970 a human maximization test has been performed on 25 volunteers with a 2 % Tibetene concentration in petrolatum. No reactions were observed after challenge. Each application following a local treatment with a 5 % Sodium Lauryl Sulfate under occlusion.

Two authors (1986, 1988) report that in patch test conducted on patients with eczema or dermatitis, 5 % Musk Tibetene produces no effects.

6. Teratogenicity

No data are available.

7. Toxicokinetics (incl. Percutaneous Absorption)

The results of various *in vitro* studies of the skin absorption investigations are reported 1984.

^{14}C Tibetene was applied (6 $\mu l/cm^{-2}$) to 5 cm^2 of intact naked rat skin clamped to a penetration chamber.

The horny layer was stripped from the skin and measured separately - a 3 % and 10 % solution in ethanol: acetone (1:1) were evaluated (180 $\mu g/cm^{-2}$ and 600 $\mu g/cm^{-2}$ respectively).

The total penetration rate values are concentration and time dependant. After a contact time of 16 hours, they amount at 28.1 % and 15 % of the total applied dose.

With intact pig skin, after exposure times of 16 hours, the maximum absorption rates amount to 4.5 % and 2.4 % of the total applied dose; Tibetene shows then considerable lower penetration rates in pig skin. The total penetration during 16 hours of exposure is very low; a significantly higher portion of Tibetene was found in the stratum corneum than in the living skin layers.

In vitro absorption with stripped pig skin has also been investigated. ^{14}C Tibetene was applied (6 $\mu l/cm^2$) to 5 cm^2 for 6 hours; when the stratum corneum was removed by stripping, the total penetration rate of Musk Tibetene increased while only a small portion of the labelled material was determined in the chamber liquid.

8. Mutagenicity

Ames test -1981- was performed with and without metabolic activation (rat liver homogenate S9 fraction) in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100; Musk Tibetene tested at concentrations of 50-100-150 and 200 µg/plate in dimethylsulfoxide, precipitated at 100-150 and 200 µg/plate.

Chromosomic aberration was assessed at the same dose levels of 4, 7, 13, 25 and 50 µg/ml with and without metabolic activations (Aroclor induced rat liver S9) in Chinese Hamster ovary cells -1995-. It was concluded that Musk Tibetene was weakly positive in the activated assay only for structural and numerical chromosomal aberrations at the 24 hour harvest time.

9. Carcinogenicity

No data are available.

10. Special investigations

Photoallergy: In the same study from 1986 10 to14 days after the final induction treatment and UVA irradiation, a challenge was done by application of the 10 % Tibetene in acetone solution, followed by the UVA exposure (320-400 nm) of the challenge site. No effects were observed.

Photoallergic potential was investigated in 1988 on Dunkin Hartley female guinea pig on the basis of a maximization test using Freund´s complete adjuvant. Concentrations of 10 % in dimethyl acetamide/acetone/ethanol (4:3:3) were applied on the skin which was then irradiated with 100 KJm^{-2} UV. The sequence was renewed twice. At challenge, the test substance was applied to fresh skin after its irradiation. A second confirmatory challenge was renewed a week later. Only 1 of the 12 animals reacted to both challenge at concentrations of 10 % and 1 % but not 0.1 %. Musk Tibetene was reported to have only a very weak photoallergic potential in the guinea pig.

The potential for cross photosensitivity was assessed in 1979 on 2 patients photosensitive to Musk Ambrette by means of photo patch tests and exposition to a Wood´s lamp delivering 6 mW/cm^2 at a peak output 365 nm. No evidence of cross sensitivity was observed.

In a clinical test performed on 18 patients -1984- previously diagnosed as photosensitive to Musk Ambrette, Tibetene was applied at a concentration of 5 % in petrolatum under occlusive patches, for two days and then, removed. The patch sites were then irradiated on the second day with UVA lamps delivering a dose of 1 J cm 6^{-2} over a period of 2.5 minutes. No evidence of cross reactivity to Musk Tibetene was found.

A positive effect was reported in 1/8 patients suspected of suffering from photoallergic contact dermatitis, using the photopatch technique recommended by the SPDRG – 1985 –.

Different other workers report also that no or mild reactions were observed with Musk Tibetene in various vehicles at concentrations of 1.0 % up to 10.0 %.

Concentration in human tissues: Musk Tibetene has been analysed in 32 human fat samples. The test compound was not found in normal human fat at post mortem.

Musk Tibetene has also been analysed in 23 human milk samples from West Germany. The test compound was detected in none of the samples.

11. Conclusions

Information concerning subchronic/chronic oral toxicity, teratogenicity/reproduction and carcinogenicity studies are not available.

Information concerning the short term studies comes from a 1971 RIFM monograph from which detailed data are not available.

However, it can be assumed from the given information and from the results of the subchronic dermal toxicity that Musk Tibetene is not irritating for the skin but lightly irritating for the mucous membrane.

According to the given data, there was no evidence of significant photoirritating sensitizing potentials; Musk Tibetene had also shown no photosensitizing potential according to a maximizating study made on guinea pigs; clinical workers have however reported some cases of weak photoallergenicity on human.

In a subchronic dermal toxicity study, no significant differences between treated and control animals as well as for the systemic effects as for the local tolerance, at the highest dose applied to the skin. The NOAEL was then considered to be at least 75 mg/kg b.w./day.

No subacute, subchronic or chronic toxicity by oral route are available; then a direct calculation of the safety margin cannot be made by means of the NOAEL expected from a 90 day oral study.

As this ingredient has no food use status, it makes sense, as suggested by industry, not to use Musk Tibetene in lip products or in flavours for the oral hygiene products.

Musk Tibetene showed no evidence of mutagenic potential in the absence or presence of metabolic activation, according to the results of the Ames: however Musk Tibetene was weakly positive for structural and numerical chromosome aberration assay using CHO cells.

Absorption has been investigated in various *in vitro* studies with animal skin; no data are available on human skin:
- in the rat, according to concentration, 28.1 % and 15 % of the amount applied on the skin were absorbed during a contact time of 16 hours;
- with intact pig skin, the total absorption rates after 16 hours of exposure were 4.5 % and 2.4 % of the total amount applied.

No Musk Tibetene was detected in human fat or breast milk but the results reported are questionable.

In the absence of any useful new data:

Classification: 2 a

12. Safety evaluation

According to submission III, the quantity used and frequency of application for a range of cosmetic products were agreed by technical representatives of the cosmetic industry. It is considered that the range of products selected covers all those that are likely to be used in any one weekly period. Furthermore, the use quantities should be regarded as an exaggerated figure:

Type of cosmetic product	Application quantity in grams per application	Application frequency per day (c)	Retention factor (d) (%)	Fragrance mixture in product (e) (%)	Tibetene in fragrance mixture (f) (%)	Tibetene in product (%)	Exposure to Tibetene (mg/day)	Exposure to Tibetene for 60 kg person (mg/kg/day)
Body lotion	8	0.71	100	0.4	6	0.024	1.3632	0.0227
Face cream (a)	0.8	2	100	0.3	6	0.018	0.2880	0.0048
Eau de toilette (b)	0.75	1	100	8.0	6	0.480	3.6000	0.0600
Fragrance cream	5	0.29	100	4.0	6	0.240	3.4800	0.0580
Anti-perspirant/ deodorant	0.5	1	100	1.0	6	0.060	0.3000	0.0050
Shampoo	8	1	1	0.5	6	0.030	0.0240	0.0004
Bath products	17	0.29	0.1	2.0	6	0.120	0.0059	0.0001
Shower gel	5	1.07	1	1.2	6	0.072	0.0385	0.0006
Toilet soap	0.8	6	1	1.5	6	0.090	0.0432	0.0007
Hair spray	5	2	1	0.5	6	0.030	0.0300	0.0005
							Total (g)	0.1529

Note (a): Including make up and foundation.

Note (b): The entry for eau de toilette includes all hydroalcoholic products (i.e., parfums, aftershaves, colognes, etc...). These products are not all used on one occasion, the quantity per application being inversely related to the fragrance concentration in the product. The figure for eau de toilette therefore covers all hydroalcoholic fragranced products.

Note (c): To allow comparison with the No Observed Adverse Effect Levels from animal studies, use is expressed as a daily exposure although in fact it is based on weekly figures in order to take into account of usage patterns which would not otherwise be evident. For example, a body lotion and a fragranced cream (i.e. a body lotion containing a higher level of fragrance) will not both be used on the same day. It has been estimated therefore that a body lotion may be used on five days per week (i.e., 0.71 times per day) and a fragranced cream on two days per week (i.e. 0.29 times per day). A similar calculation applies to bath products and shower gel.

Note (d): Retention factors for the skin are conservative estimates from known use of products, taking into account wash-off characteristics.

Note (e): The concentration of the fragrance mixture in a cosmetic product type has been determined by senior technical representatives of the cosmetic industry.

Note (f): The concentration of a fragrance ingredient in a fragrance mixture is based on data obtained by the fragrance industry from the examination of commercialized formulations containing the fragrance ingredient. The concentration used corresponds to the upper 97.5th percentile concentration of the fragrance ingredient in fragrance mixtures, a concentration which is in itself maximized because the products not containing the fragrance ingredient were not included as zero values in the distribution of the samples.

Note (g): Total consumer exposure to the fragrance ingredient is determined by adding figures for the different product types expressed as mg/kg body weight/day. In view of all the above assumptions, this figure has to be regarded as conservative; it is most unlikely that a consumer will consistently use a number of different cosmetic products which are all perfumed with the upper 97.5th percentile level of the fragrance ingredient.

The more relevant data concerning the adverse effect was found in the 90 day dermal toxicity, in rat where the assumed NOAEL were 75 mg/kg b.w./day (males) and 75 mg/kg b.w./day.

The systemic NOAEL can be obtained from these results when corrected for dermal absorption of 15 % (worst figure) in the rat skin.

Considering 75 mg/kg b.w./day as the acceptable NOAEL by dermal route, the corresponding rat daily systemic exposure is:

75 mg/kg x 15 % = 11.25 mg/kg

The calculation of the margin of safety is then as follows:

Maximum amount of Musk Tibetene applied: 0.1529 mg/kg b.w./day

Maximum absorption through the human skin: The pig absorption data may be used to estimate the absorption in human skin since pig skin is more relevant, physiologically to human skin. The assumed maximum absorption would be then 4,5 % (worst figure).

Assumed human daily systemic exposure dose: 0.1529 x 4.5 % = 6.9 µg/kg

Margin of safety: $\frac{11250}{6.9} = 1630$

STRONTIUM CHLORIDE

1. General

1.1 Primary name

Strontium chloride hexahydrate

1.2 Chemical names

INCI : Strontium chloride

1.3 Trade names and abbreviations

Strontium chloride hexahydrate

1.4 CAS no.

Strontium chloride hexahydrate = 10025-70-4
Strontium chloride = 10476-85-4

1.5 Structural formula

Cl-Sr-Cl $6H_2O$

1.6 Empirical formula

Emp. Formula : $Cl_2Sr.6H_2O$
Mol weight : 266.62

1.7 Purity, composition and substance codes

Purity : 97.4 %

1.8 Physical properties

Substance code :
Appearance : white and deliquescent coarse crystals
Melting point :
Boiling point :
Density : 0.9 g/cm^3
Rel. vap. dens. :
Vapour Press. :
Log P_{ow} :

1.9 Solubility

Soluble in water.

2. Function and uses

Strontium chloride in a maximum concentration of 6.6 % in salt, correspondig to 2.1 % elementary strontium, is intended to be used in cosmetic products (hair, face care products) because of its inherent properties and technical advantages to improve tolerance of cosmetic products.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

One study showed that the general behaviour and body weight gain of animals were not affected and that no deaths occurred when the Strontium chloride was administered in rats at the 2000 mg/kg dose level by the oral route in purified water.

3.2 Acute dermal toxicity

One study showed that the general behaviour and body weight gain of animals were not affected and that no deaths occurred when the Strontium chloride was administered in rats at the 2000 mg/kg dose level by cutaneous application under a semi-occlusive dressing for 24 hours.

3.7 Subchronic oral toxicity

In a 90-day study in rats performed at the dose levels of 75, 300, 1200 or 4800 ppm, the non-toxic level was assessed to be 300 ppm. At higher dose levels thyroid weights were increased and at 4800 ppm, it was also noted a depletion of hepatic glycogen.

4. Irritation and Corrosivity

4.1 Irritation (skin)

A study performed in rabbits showed that no skin irritation was noted after one application at a concentration of Strontium chloride of 12.7 %.

4.2 Irritation (mucous membranes)

A study performed in rabbits showed that no ocular irritation occurred after one application of Strontium chloride at a concentration of 12.7 %.

In vitro studies i.e. assessment of irritancy by the hen's egg chorioallantoic membrane test using fertile chicken eggs (HET CAM) and assessment of ocular irritancy by the bovine corneal opacity and permeability test (BCOP) showed both concentrations of 6.5 or 13 % of Strontium chloride were not irritant.

5. Sensitization

A sensitization study (1996) performed in guinea-pigs according to the method established by Buehler under the following conditions:

- induction by topical application on day 1 (first induction), day 8 and day 15 at a concentration of 50 %;
- challenge by topical application on day 29 at a concentration of 50 % did not induce any sensitization in guinea-pigs.

7. Toxicokinetics (incl. Percutaneous Absorption)

- *In vitro* percutaneous absorption of Strontium chloride

An aqueous gel containing 250mM of Strontium chloride (^{89} Sr Cl_2) was tested *in vitro* on human skin (split-thickness skin obtained by dermatomisation and cut between 250 and 400 µm) using Franz cells by application of 10 mg/cm^2 (mean dose of Strontium applied was 196 ± 8.8 eq.µg/cm^2) during 24 hours. Twelve cells were tested. Two results were excluded (one due to a bad total recovery and the other one due to the bad quality of the skin). On the 10 Franz cells retained for interpretation, it was observed a weak diffusion of the radioactivity through the skin (receptor fluid) 2.7 ± 1.9 % of the applied dose, i.e. 5.3 ± 4.0 eq.µg/cm^2.

The penetrated amount after the study time which is the sum of the amount in the receptor fluid + that found in the dermis (2.3 ± 1.4 %, i.e. 4.5 ± 2.8 eq.µg/cm^2) was : 5.0 ± 3.1 % of the applied dose, i.e. 9.8 ± 6.4 eq.µg/cm^2 of Strontium.

- Percutaneous absorption, excretion balance and tissue distribution of ^{89} Sr Cl_2 after topical administration to female hairless rats

An aqueous gel containing 250mM of Strontium chloride (^{89} Sr Cl_2) and approximately 920 µCi.g^{-1} was applied during 4 hours at the dose of 10 mg of formulation/cm^2. After this contact time, the treated area was washed and the rats were placed in metabolism cages for 96 hours.

The mean (± S.D.) dose of Strontium applied was 223 ± 41 eq.µg/cm^2.

The amount recovered on the skin surface at the end of administration represented 94 ± 5.3 % of the applied dose.

Only a low amount of radioactivity was found in urine and faeces for each time of collection and no radioactivity was found in organs and tissues 96 hours after the end of the administration.

The total amount which has crossed the stratum corneum during 4 days represented 1.49 ± 1.57 % of the dose applied. Only traces of Strontium were still present in the stratum corneum 96 hours after the application (0.02 ± 0.02 % of the dose applied).

These results demonstrated that the absorption of Strontium was very low after a topical administration.

The good recovery : 95 ± 5.1 % validates these results.

8. Mutagenicity

An Ames test (1996) performed on five strains *of Salmonella typhimurium* (TA 1535, TA 100, TA 1537, TA 1538 and TA 98) in absence as well as in presence of metabolic activation showed that Strontium chloride was not mutagenic.

An *in vivo* micronucleus test (1996) showed that the Strontium chloride did not induce damage to the chromosomes or the mitotic apparatus in bone marrow cells when administered twice at 24-hour interval by oral route in mice up to 2000 mg/kg/day.

10. Special investigations

- *In vitro* studies

Cytotoxicity was determined in an *in vitro* assay performed on pulmonary fibroblasts of Chinese hamsters (V79 cells). The test substance was put into contact with the cells during 24 hours. Thereafter, the proteins and the quantity of neutral red incorporation were measured. According to the CI 50 obtained, the test substance was considered not to be cytotoxic.

11. Conclusions

Classification: 1

(maximum concentration of 6.6 % in salt, corresponding to 2.1 % elementary strontium, in "rinse off" hair products, e.g. shampoo, and face care products)

12. Safety evaluation

CALCULATION OF SAFETY MARGIN *

STRONTIUM CHLORIDE

Based on a daily usage volume of	**a) 800 mg (face cream)** **b) 120 mg ("rinse off" hair products, e.g. shampoo),**	
containing at maximum:	**a) + b) 6.6 % $SrCl_2,6H_2O$**	
Maximum amount of ingredient applied:	**I (mg) =**	**a) 52.8 mg $SrCl_2,6H_2O$** **b) 7.92 mg $SrCl_2,6H_2O$**
Typical body weight of human:	**60 kg**	
Maximum absorption:	**A (%) =**	**a) + b) 5 % (human skin)** **1.5 % (rat skin)**
Absorption:	**I (mg) x A (%) =**	**a) 0.792 - 2.64 mg** **b) 0.119 - 0.396 mg**
Systemic exposure dose (SED):	**I (mg) x A (%) / 60**	**a) 0.0132 - 0.044 mg/kg** **b) 0.00198 - 0.0066 mg/kg**
No Observed Adverse Effect Level (mg/kg/day): (90 days, rat, oral route)	**NOAEL**	**= 30**
Safety margin:	**NOAEL / SED**	**= a) 682 - 2273** **b) 455 - 1515**

*** based on a topical application**

P 91: *3-IODO-2-PROPYNYL BUTYLCARBAMATE*

1. General

1.1 Primary name

3-iodo-2-propynyl butylcarbamate

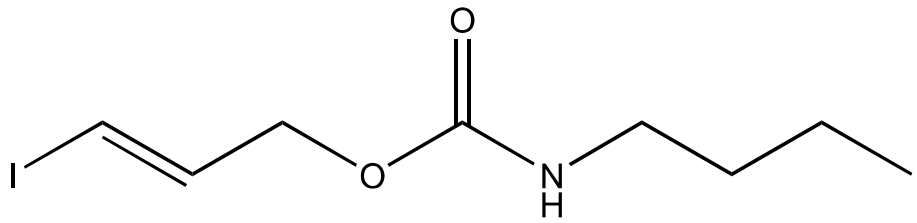
1.2 Chemical names

3-iodo-2-propynyl butylcarbamate
Iodopropynyl butylcarbamate

1.4 CAS no.

55406 53 6

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_8H_{12}NO_2I$

1.9 Solubility

It has a low solubility in water (156 ppm at 20°C) and is soluble in organic solvents.

2. Function and uses

lt is proposed for use as a preservative in all types of cosmetic products at levels up to 0.05 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

The substance has moderate acute toxicity by the oral route with LD_{50} values of 1056 mg/kg in female rats and 1798 mg/kg in male rats when given in corn oil. No deaths and only minimal signs of toxicity were seen at 500 mg/kg or below.

3.7 Subchronic oral toxicity

In a subchronic study, rats were given 20, 50 and 125 mg/kg by gavage in corn oil 5 days a week for 13 weeks. In addition a satellite group was given the top dose and allowed a 28 day recovery period prior to autopsy. No compound related mortality was observed. The only signs of toxicity seen were a reduction in weight gain of the males at 125 mg/kg. No effects were seen on haematology, clinical chemistry nor on ophthalmological examination. At autopsy a significant increase in liver weight was seen at 125 mg/kg. Histological examination showed hepatocyte enlargement at 125 mg/kg which was believed to be due to enzyme induction. Effects on weight gain and liver weight were reversible, with recovery being noted in a satellite group. The no-effect level in this study was 50 mg/kg.

4. Irritation & corrosivity

4.1 Irritation (skin)

In a skin irritation study in rabbits (4 hours exposure, occluded dressing) slight erythema and severe edema were reported at 4 hours but the effects were transient with animals returning to normal by 48 hours.

The potential of P 91 to produce primary skin irritation in humans following a single topical application was examined. Amounts of 0.2 ml corn oil containing l % and 3 % P 91 were applied onto the upper back, nonabraded skin of six subjects using occlusive patches. The patches were removed 24 hours after application and skin readings were performed 30 minutes, 24 hours and 48 hours after patch removal.

The l % solution of P 91 was slightly irritating to the majority of the study pannel (one subject showed no irritation, four subjects showed faint erythema and one subject showed moderate erythema 30 minutes after patch removal). The 3 % solution was moderately irritating to the majority of the pannel (two subjects showed faint erythema and four subjects showed moderate erythema 30 minutes after patch removal). There was no evidence of edema in any of the subjects, and erythema was generally less intense or no longer apparent 24 and 48 hours after patch removal.

The potential of concentrations of 0.0, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 % P 91 in corn oil to produce primary skin irritation in humans following a repeated topical application (three times over a five day period) was examined. Amounts of 0.2 ml P 91 in corn oil were applied onto the upper back, nonabraded skin of 7 subjects using semi-occlusive patches (1 x 1 inch). The patches were removed 24 hours after application. Skin readings were performed 24 hours (first two applications) or 48 hours (final application) after patch removal. In all subjects, observation of all treated areas remained negative throughout the test.

The potential of P 91 to produce primary skin irritation or sensitization in humans following repeated topical application was examined. Amounts of 0.2 ml corn oil containing 1 % P 91 were applied onto the upper back, nonabraded skin of 170 subjects using semi-occlusive patches (1 x 1 inch). Treatment was conducted three times a week for a total of ten applications (24 hours contact per application); skin readings were made 24 or 48 hours after patch removal. Challenge (1 % P 91 in corn oil) was conducted on both the application site and a virgin site 14 days after the tenth application; each site was evaluated 24 and 48 hours after application.

No significant irritation was observed during induction. Upon challenge, all test areas remained negative in all subjects.

The potential of a formulation containing P 91 to produce primary skin irritation or sensitization in humans following repeated topical application was examined. The study was conducted with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission). Amounts of 0.2 ml corn oil containing 1:50 and 1:100 aqueous dilutions were applied 10 times (3 times/week) onto the upper back, nonabraded skin (1 inch by 3/4 inch) of 51 subjects using semi-occlusive patches. The patches were removed 24 hours after application. Challenge application (1:50 and 1:100 aqueous dilutions) were made to the induction site and to a virgin site, 14 days after the last application and skin readings were performed 24 hours and 48 hours after patch removal. The dilutions of a 2.6 % formulation of P 91 (viz. 0.026 % and 0.05 % P 91) did not induce any skin reactions throughout the study. The significance of this study for assessment of the sensitization potential of P 91 is doubtful.

4.2 Irritation (mucous membranes)

Severe effects were noted in an eye irritation study in rabbits. The substance (0.1 g) produced moderate to severe hyperaemia, chemosis, discharge and corneal opacity for 7-13 days in most animals; in one instance the opacity remained until termination of the experiment at day 21. lf the compound was washed out of the eye 20-30 seconds post instillation, only transient irritant effects were seen.

The eye irritancy of a 0.5 % solution of P 91 in corn oil as well as the effects of 0.5 % P 91 in a baby shampoo have been tested in rabbits. Groups of 6 animals were used in each case. No signs of any irritant effects were seen with the corn oil formulation. In the case of the baby shampoo, slight irritant effects were seen for about 24-48 hours, but similar effects were seen in ‘control’ baby shampoo that did not contain P 91. Thus 0.5 % P 91 in corn oil or in a baby shampoo formulation did not produce eye irritation.

5. Sensitization

Skin sensitization potential has been investigated in a guinea pig maximisation test. Induction concentrations were 10 % by the intradermal route and 50 % by the topical route. Challenge was with 0.01 % in petrolatum (a concentration of 0.05 % was reported to produce a slight irritant effect). There was no evidence of sensitization in any test animal. Two further Magnusson & Cligman maximisation tests have been carried out on formulations containing 0.5 % test compound. In the first study induction concentrations of 0.05 % (i.d.) and 0.5 % (topical) were used. In the second study the concentrations were 0.1 % and 0.5 %, respectively. The intradermal doses were reported to produce some irritation. In both studies challenge was with a 0.5 % formulation. There was no evidence of sensitization in either test. These studies suggest that P 91 does not have potential for sensitization.

The potential of P 91 to produce photosensitization was examined in guinea pigs (adapted Buehler method). Induction was conducted with a 5 % (w/v) formulation in PEG400 (0.3 ml over a 25 mm area, occluded during four hours, three times a week for three consecutive

weeks). Thirty minutes after removal of the occlusive dressing, animals were irradiated with UVA and UVB for two hours. Appropriate control groups were included. Challenge and re-challenge of treated and naive animals was conducted with 5 % (w/v) in PEG400 (+UV), 12 and 19 days after induction, respectively.

Equivocal evidence of photosensitization was obtained. After primary challenge positive results were obtained (3/10 animals showed grade 1 (slight but definate) erythema whereas all other animals in the naive and induction group showed a $\pm$ (barely perceptible) reaction). However, the three grade 1 responses were not re-elicited after rechallenge (maximum grades of $\pm$ were observed in both the induction and the naive group). Clear conclusions can not be drawn, the more so as the dose used for induction may have been relatively low.

A guinea pig photosensitization study was conducted with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission).

Induction was conducted with a 25 % solution (0.1 ml over the nuchal area, non-occluded, five times a week for two consecutive weeks). The animals were irradiated with UVB (4.5 J/cm^2; a unusually high dose) and UVA (10 J/cm^2). Control animals were not irradiated during induction. Challenge of treated and naive animals was conducted with 2.5 %, 5 % and 25 % aqueous solutions (+UVA), 17 days after induction.

Irritation was not observed at the start of the induction phase but reached maximum severity (erythema and edema) after four treatments. Following challenge with the 25 % solution, clear skin effects were observed in all animals of the induction group but not in the naive animals. No skin effects were observed at the 2.5 % or 5 % test sites following challenge. It was concluded that the test article was a photoallergen at 25 %. The significance of this study for assessment of the photosensitization potential of P 91 is not clear.

The potential for P 91 to absorb light in the ultraviolet and visible spectrum (in the range of 190 to 800 nm) was determined. Maximum absorbance occurred at 191 nm (extinction coefficients were 6570 L/mol-cm at pH 5 and approx. 6000 L/mol-cm at pHs 7 and 9). A smaller absorbance peak was detected at 227 nm (extinction coefficents were approx. 500 L/mol-cm at pHs 5, 7 and 9). No other wavelength maxima were detected.

A quantitative structure activity relation (QSAR) analysis was conducted to evaluate the potential of P 91 or structurally related chemicals to produce sensitization in humans. The Contact Allergens Database Evaluation System (CADES), the national Library of Medicine (NLM) and the STN International online databases were searched.

No references indicative of skin sensitization were found for P 91 and structurally related chemicals (e.g. those containing carbamic acid H_2NCOOH, butyl carbamate, propynyl-iodide moiety R-CH_2CCI, or propynyl-halogen moiety). The closest structurally related compounds with potential skin sensitization are the dithiocarbamates ($H_2NC(S)_2$) such as the fungicides Maneb, Zineb and Ziram.

A QSAR analysis, focussed on the aliphatic carbamate and iodoacetylene moieties of P 91, was conducted to assess the potential to produce sensitization in humans.

No evidence was found indicating that P 91 is sufficiently protein reactive to initiate a sensitization reaction. Also no reference to either allergy or irritation by P 91 was found in a MEDLINE search.

In a literature search no evidence was found for cross-reactions of 3-Iodo-2-Propynyl butyl Carbamate with dithiocarbamates used in the rubber industry. This information is, however, considered unsatisfactory proof for the absence of cross sensitivity.

A human cross sensitization study was conducted to determine the potential for P 91 to elicit skin contact cross sensitization reaction in humans with an existing allergy to dithiocarbamate compounds. Ten volunteers with a history of sensitivity to Thiuram Mix (European Standard Patch Test battery No. 3, consisting of 0.25 % w/w of each of tetramethylthiuram monosulfide, tetramethylthiuram disulfide and dipentamethylene thiuram disulfide), received a single application of a 0.1 % concentration of P 91 in soft yellow petrolatum (0.2 ml; 3 cm^2 area; occluded; 24-hr period of contact), as well as a patch containing petrolatum alone (vehicle control). The sites were examined 48 and 96 hours after application. No noticeable skin reactions were observed in any of the 10 volunteers. It was concluded by the authors that P 91 does not cause cross sensitization reactions in humans with a known sensitivity to dithiocarbamate compounds. It should be noted, however, that the sensitivity of the subjects to dithiocarbamates was not confirmed, the challenge concentration of P 91 (0.1 % in petrolatum) was rather low, and that exposure lasted for only 24 instead of 48 hours.

A second human cross sensitization study was conducted in humans with an existing allergy to dithiocarbamate compounds. A preliminary irritation screen conducted with 10 volunteers showed that concentrations (0.3 % of P 91 in soft petrolatum) (0.2 ml, 3 cm^2 area, occluded, 48-hr period of contact) were irritating.

Twelve volunteers with a history of sensitivity to Thiuram Mix, received single applications of a 0.1 % concentration of P 91 in soft yellow petrolatum (0.02 ml, FINN chambers (8 mm), occluded contact period: 48 hr). Appropriate control sites were included. To confirm the existence of allergy to dithiocarbamate compounds, to volunteers were co-challenged with 1 % Thiuram Mix, 1 % zinc dibutyldithiocarbamate and 1 % zinc diethyldithiocarbamate on separate sites. The sites were examined 48 and 96 hours after application.

Nine volunteers were clearly confirmed as being Thiuram sensitive with 5 of these also reacting to one or both of the zinc carbamate formulations.

None reacted positively to P 91, confirming that P 91 is not a cross sensitizer in human with Thiuram sensitivity.

6. Teratogenicity

Teratogenicity studies have been carried out in both the rat and the mouse. In the study in rats compound was given on day 6-15 of gestation at dose levels of 20, 50 and 125 mg/kg by gavage in corn oil. The only effect seen in maternal animals was a transient reduction in weight gain at the top dose. The only effect seen on the developing offspring was delayed ossification of cranial bones at the top dose, with no significant increase in malformations at any dose level. The no-effect level was 50 mg/kg. A similar dosing regime was used in the study in mice. No

compound related signs of toxicity were seen in the maternal animals nor in the developing offspring at any dose level. The no-effect level was 125 mg/kg.

6.2 Two-generation reproduction toxicity

A two generation reproductive toxicity study has also been carried out in the rat. Groups of 25 animals of each sex were given test compound in the diet at 120, 300 and 750 ppm, together with a similarly sized control group. After a 14-week pre-mating period the parental animals in each generation were mated and the females allowed to rear their offspring until weaning. No compound-related effects were seen at any dose level on clinical chemistry or at necropsy. Slightly reduced weight gain was seen in the males at 750 ppm during the pre-mating period in both the initial generation and the F1 generation. No effects on mating performance or fertility were seen at any dose level apart from a reduction in live birth index (= no. of pups alive at day 1/ total number of pups) at 750 ppm in either generation, while a marginal effect was also noted at 300 ppm in the F1 generation. Postnatal growth of the offspring however was not affected and no effects were seen on the development of the offspring. The no-effect-level in this study was 120 ppm test compound in the diet (roughly equivalent to a dose of 10 mg/kg b.w./day).

7. Toxicokinetics (incl. Percutaneous Absorption)

In a percutaneous toxicity study in rabbits, a single dose of 2 g/kg applied as an aqueous paste and using a 24-hour occlusive dressing did not result in deaths. The only signs of toxicity seen were slight irritant effects at the application site.

The potential skin absorption of P 91 was examined in an *in vitro* skin penetration study with previously frozen, excised human cadaver skin (thickness 400-800 µm; epidermis + papillary dermis). 5 µl of a 0.1 % solution of ^{14}C-labelled P 91 in acetone (approx. 6 µg P 91/cm^2 skin) were applied to six samples from each of four donors (total 24 skin samples). Since a constant air flow was maintained through the evaporation cell (10 cell volumes/min) it may be assumed that the acetone (5 µl) instantly evaporated. The amount of radiolabel in the receptor fluid bathing the visceral side of the dermis was determined periodically during the 24-hour experimental period. Excess radiolabel on the epidermal surface of the skin was removed (by two successive tape strips) 24 hours after application. Radiolabel evaporating from the epidermal surface was trapped in vapour traps and quantified periodically during the 24-hour experimental period.

The mean ($\pm$ SD; n = 24) skin penetration (the sum of radioactivity recovered in the dermis and receptor fluid) was 54 $\pm$ 12 % (38 $\pm$ 5 %, 54 $\pm$ 10 %, 55 $\pm$ 3 % and 68 $\pm$ 6 % for the respective individual donors). Peak penetration into the receptor fluid occurred within 28 hours of application for all donors. 14 $\pm$ 3 % of the applied radioactivity evaporated from the skin surface during the 24-hour experimental period. Overall recovery of radioactivity was 87 $\pm$ 10 % of the applied radioactivity.

Under the conditions of this study 54 % of a 6 µg/cm^2 dose of P 91 penetrated excised human skin during a contact period of 24 hours. It may be noted that P 91 was delivered to the skin as a thin film during a full 24 hour contact period. Vehicles used to formulate P 91 as well as the time they remain on the skin will likely influence skin absorption of P 91.

8. Mutagenicity

The mutagenic potential of the compound has been investigated in a number of studies. Negative results were obtained in the Salmonella assay versus strains TA1535, 1537, 1538, 98 and 100 but this study was limited by investigating only 3 concentrations (6.2 - 55.6 µg/plate) since the two higher concentrations used were toxic. However an additional plate incorporation assay has been carried out using 5 concentrations in the range 1-333 µg/plate against TA 1537, 98 and 100 and concentrations of 1-1000 µg/plate against TA 1535. In all cases the top concentration resulted in some evidence of toxicity to the bacteria. Studies were carried out in the presence and absence of rat S-9. Negative results were obtained with all strains. In addition the ability of the compound to produce unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro* has been investigated. UDS was determined by autoradiography, with 8 concentrations in the range 3-13.5 µg/ml (resulting in 84 %-25 % viability) being used and the results were confirmed in an independent experiment. There was no evidence for any induction of UDS. The potential for the compound to produce chromosome damage has been investigated in an *in vivo* micronucleus test using a comprehensive protocol. Single oral dose levels of 200, 660 and 2000 mg/kg were given to mice by gavage in corn oil and bone marrow cells harvested at 30, 48 and 72 hours post dose, and the frequency of micronuclei in polychromatic erythrocyte cells analysed. Toxic effects (lethality) were noted at both 660 and 2000 mg/kg (2 deaths after 72 hours at 660 mg/kg and 9 deaths at 30-72 hours at 2000 mg/kg). There was no evidence of any increase in micronuclei at any dose level or harvest time. These four studies provide no evidence to suggest that the compound has any significant mutagenic potential.

10. Special investigations

A phototoxicity study was conducted in guinea pigs with an experimental formulation stated to contain only 2.6 % P 91 besides other active ingredient(s?) and 24 % ethanol (the composition of the formulation tested was not presented in the submission). Aliquots (0.1 ml) of 2.5 %, 12.5 %, 18.75 % and 25 % aqueous formulations were applied topically on the depilated dorsal skin of 10 guinea pigs. Thirty minutes after application the animals were irradiated with UVA. No irritation was observed with 2.5 % and 12.5 % concentrations, with 18.75 % questionable erythema was noted in a number of animals while questionable to minimal erythema was noted with 25 % in a number of animals. Based on these results the formulation was not considered to be phototoxic.

The significance of this study for assessment of the phototoxicity of P 91 is not clear.

The comodogenic potential of P 91 was examined in 12 human subjects with a history of acne. The test substance (0.1 % P 91 in white cream) and the controls (blank patch, vehicle control and positive control Acetulan) were applied to 4 x 4 cm areas on the upper back (non-abraded skin) using occlusive tape for three times a week for four weeks, resulting in a 28-day continuous period of exposure. Folicular biopsy samples were taken at the end of the exposure period and comodeone density was determined stereomicroscopically. Several subjects showed moderate erythema at the 0.1 % P 91 site during treatment.

Acetulan increased the production of comodones in this study. It was stated that 0.1 % P 91 in white cream was not comedogenic, but the scoring scale was not clearly presented.

Pharmacokinetic studies have been carried out in the rat following oral and intravenous administration using ^{14}C radio-labelled material. Following iv administration the principal route of elimination was by exhalation as carbon dioxide (57 %) and in the urine (32 %). The compound was essentially completely absorbed following oral administration, with 51 % of the dosed radioactivity being excreted in the urine and 38 % exhaled as carbon dioxide within 96 hours. Peak plasma levels occurred within 2 hours. Following absorption levels of activity were highest in the level and kidneys, but declined relatively rapidly with no evidence to indicate that the compound would present any potential for accumulation. Metabolic studies indicate that it is rapidly metabolised to carbon dioxide and compounds other than 3-iodo-2-propenyl butyl carbamate.

The compound is a carbamate and studies have been carried out to investigate whether significant cholinesterase (ChE) activity inhibition occurs in rats following intravenous administration. P 91 was given in PEG400: water vehicle at 2-16 mg/kg and blood samples were taken and analysed for erythrocyte ChE activity at 15, 30 and 60 minutes and 5 hours post dosing. No effects on blood cholinesterase activity were observed.

Data on minimum inhibitory concentrations of 3-iodo-2-propynyl butyl carbamate demonstrated the efficacy of this compound at levels $\leq$ 0.1 %.

11. Conclusions

The test substance has relatively low acute oral toxicity ('harmful if swallowed' according to EEC criteria) and is not harmful following acute dermal exposure.

It is a mild to moderate skin irritant in rabbits. In humans, exaggerated exposure conditions (1 % - 3 % P 91, 24 hour occlusion) resulted in transient, slight to moderate irritation. Repeated semi-occluded application of formulations containing levels up to 1 % P 91 did not induce any skin reactions in humans.

The pure substance is a severe (corrosive) eye irritant; however, formulations containing 0.5 % did not produce any eye irritation.

No evidence of sensitization was obtained in a Magnusson & Kligman maximization test. A human repeated insult patch test with 1.0 % P 91 in corn oil did not reveal any sensitizing potential. In a literature search, no evidence was found for cross-reaction of P 91 with dithiocarbamates used in the rubber industry. P 91 did not cause cross sensitization reactions in two separate studies in humans with Thiuram sensitivity.

Equivocal evidence of photosensitization was obtained with pure substance and with a formulation containing 2.6 % P 91 besides other active ingredient(s). QSAR analysis did not reveal evidence of sensitizing potential for P 91 or closely related compounds.

P 91 (0.1 % in white cream) was not found to be comedogenic in humans.

The ultraviolet-visible absorption spectrum of P 91 showed two absorbency peaks (at 191 and 227 nM).

Mutagenic potential has been investigated in Salmonella assays for gene mutation, in a study to investigate Unscheduled DNA Synthesis (UDS) in rat hepatocytes *in vitro,* and in an *in vivo*

micronucleus test. Negative results were consistently obtained. There was no evidence of teratogenic potential in studies in two species (rats and mice). In a 2 generation reproductive toxicity study in rats a reduction in life birth index was observed. The no-effect-level in this study was 120 ppm in the diet (c. 10 mg/kg b.w./day).

An *in vitro* skin penetration study indicated considerable (ca. 54 %) dermal absorption. The compound is well absorbed orally but is rapidly metabolised and excreted.

Conclusion:
- With the below restrictions (max. concentration 0.05 %; not to be used in oral hygiene and lip products), the safety margin of P 91 would be acceptable.

Classification: 1; max. concentration 0.05 %; not to be used in oral hygiene and lip products.

12. Safety evaluation

CALCULATION MARGIN OF SAFETY

In Submission VII (April 1996) industry proposed to restrict the fields of application as well as the concentration of P 91 in the following way:
- the concentration will be reduced to 0.05 % (instead of the 0.5 % originally requested),
- P 91 would not be allowed for use in oral hygiene products and lip products.

With these restrictions the calculation of the Margin of Safety is as follows:

- Exposure to oral hygiene products:	not applicable
- Exposure to eye products:	0.06 g
- Exposure to non-rinse products:	20.3 g
- Exposure to rinse-off products:	1.7 g
- Total systemic exposure (SED):	22.06 g x 0.05 %[(1)] x 54 %[(2)] = 5.956 mg P91 /human/day = 0.0993 mg/kg b.w.[(3)]/day

(1) Maximum level of use (0.05 %) is assumed.

(2) 54 % skin absorption is assumed.

(3) 60 kg/human is assumed.

Margin of Safety = NOAEL/SED = 10/0.0993 = 101

S 27: *ISOPENTYL-P-METHOXYCINNAMATE*

1. General

1.1 Primary name

Isopentyl-p-methoxycinnamate

1.2 Chemical names/IUPACname

Isoamyl-p-Methoxycinnamate
Isopentyl-p-Methoxycinnamate
2-Propenoic Acid, 3-(4-Methoxyphenyl)-, 3-Methylbutyl Ester

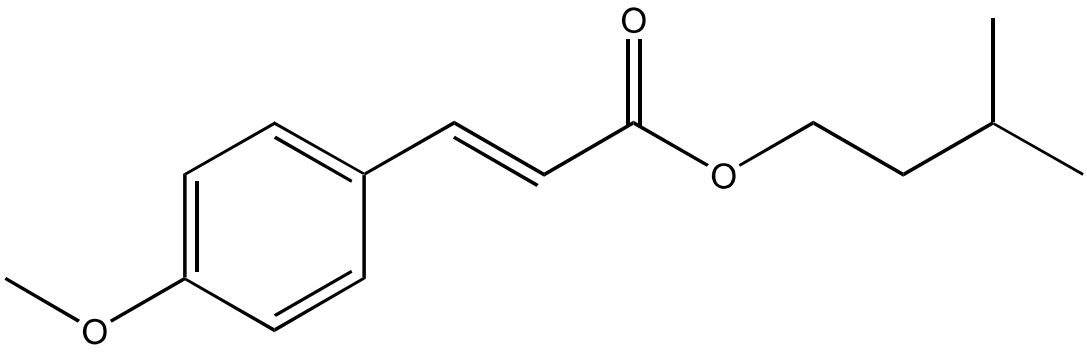
1.3 Trade names and abbreviations

Trade Name: NEO HELIOPAN E 1000
Abbreviations: NHE1000, HR90/656083, HR91/656083, HR94/656083

1.4 CAS no./Einecs/Elincs no./INCI name

CAS 71617-10-2/275-702-5/-Einecs
Isoamyl-p-Methoxycinnamate (INCI)

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{15}H_{20}O_3$
Mol weight: 248

1.7 Purity, composition and substance codes

98 % min.

1.8 Physical properties

Subst. code: $C_{15}H_{20}0_3$
Appearance: Clear, colourless to yellowish liquid

Melting point: <-30 °C
Boiling point: 170 °C / 2 mbar
Density: (25/25) 1.039 g/ml
Absorption max: 308 nm
Rel. vap. dens.: n.a.
Vapour Press.: < 1 Pa (< 0.01 mbar)
Log P_{ow}: 4.33 (calc.)

1.9 Solubility

Soluble (all proportions): Paraffin oil, olive oil, EtOH, Isopropanol, Cetiol V, Miglyol 812
Insoluble: H_2O, H_2O/EtOH 10-50 %

2. Function and uses

UV Absorber in sun protection products, max. 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. Oral. Values of 9.6 to 9.9 g/kg b.w. were found. No details are given.

3.2 Acute dermal toxicity

Acute dermal toxicity testing was carried out according to OECD guidelines. No abnormalities were found up to 20 g/kg b.w.

3.4 Repeated dose oral toxicity

Subacute toxicity. Rat: A 3 week oral toxicity study was carried out as a range finding study. Four groups, each of 5 m and 5 f animals, were used. The doses were 0.3, 0.9 and 2.7 ml/kg b.w./day suspended in 0.8 % hydroxypropylmethylcellulose and given by gavage. (In mass units, 312, 935 and 2805 mg/kg b.w./day). There were no deaths. There was decreased weight gain in both sexes at the high dose. All animals were subjected to necropsy. At the top dose, the absolute and relative weights of the spleen and thymus were significantly decreased in both sexes. In males, the weights of the gonads were significantly reduced at the top dose. At 2.7 ml/kg b.w./day in males, and at 2.7 and 0.9 ml/kg b.w./day in females, the weights of the liver were increased significantly, and those of the spleen and thymus reduced.

(b) Relative organ weights: In the text of the report the relative weights of spleen, thymus and gonads in males are stated to be decreased significantly at the top dose, and of the spleen and thymus in females. In the tables of the report, increased relative liver weights are seen at all dose levels, most pronounced in males at 2.7 ml/kg b.w. and in females at 2.7 and 0.9 ml/kg b.w. The NOAEL may be 0.3 ml/kg b.w./day.

3.5 Repeated dose dermal toxicity

Guinea pig: Twelve animals were used. The material applied is not specified: it may have been undiluted a.i. It was rubbed into the clipped skin of the flank for 30 seconds daily for 5 days. The test is stated to have been negative; no details are given.

Man: Thirty subjects were tested by applying undiluted a.i. to the skin of the back or of the inside of the forearm, followed (probably) by occlusion for 24 hours. No irritation is said to have been produced. No details are given.

Man: Tests were carried out on 65 m and 45 f patients hospitalised for various skin diseases. Three concentrations of a.i. in soft paraffin were tested: 1%, 5 % and 10 %. They were applied to disease free areas of skin of the back by means of a Finn chamber. Contact time was 24 hours; reading was at 24 and 48 hours. In 15 subjects, the test was repeated one or more times. No adverse reaction was found in any test.

3.7 Subchronic oral toxicity

Rat: Thirteen week oral study. Following a preliminary study, the doses chosen were 0, 20, 200 and 2000 mg/kg b.w./day, administered daily by gavage 7 days a week for 13 weeks. Four groups of animals were used, each containing 15 m and 15 f. All animals were subjected to necropsy after sacrifice, and animals dying during the trial were subjected to necropsy as soon as possible after death. A wide range of tissues was fixed, and all from the control and top dose groups were subjected to histological examination. There were four deaths during the experiment: 1 control, 2 at 20 mg/kg b.w./day, and 1 at 200 mg/kg b.w./day. Weight gain was reduced in all animals at the top dose. Haematological changes were found, which were rather variable; in summary, it may be stated that the haemoglobin and MCHC values were increased at the top dose in both male and female animals at the end of the first and third months. There were many changes in the values obtained by clinical chemical analysis. The main ones, which may be significant, were: at 1 month, AP and GOT were increased at the top dose in both sexes, and cholesterol was reduced. The same finding was made at 3 months, and in addition the GPT was raised in female animals at that time. There were no urinary abnormalities.

Organ weights: (a) Absolute weights. At the top dose, both sexes showed increase in the weight of the liver; in females, the weight of the spleen was reduced, and in males the weight of the testis was reduced. (b) Relative weights. At the top dose, the weights of the liver and kidneys were increased. In males, the weight of the heart was increased and that of the spleen and adrenals slightly reduced. In females, the weight of the spleen was reduced.

The histological findings at the top dose showed patchy areas of increase in size of hepatocytes with clear cytoplasm and large nuclei. There was also increased iron-containing pigment in the spleen of both sexes and in the Kupfer cells of the liver in females. These changes were not seen at the lower dose levels. In sum the findings indicate that at the top dose there are effects on the liver, and possibly increased breakdown of red cells. The NOAEL is 200 mg/kg b.w./day. This appears to have been a well conducted study carried out according to OECD guidelines.

4. Irritation & corrosivity

4.2 Irritation (mucous membranes)

Chick: Applications of 0.2 ml of dilutions of a.i. in olive oil were made to the chorioallantoic membrane. The text gives data for tests in 1 egg only for each of the concentrations 1%, 10 % and control. The results were negative. This test is not yet officially recognized for this purpose.

Rabbit: Eight animals were subjected to a Draize test. A 50 % solution in olive oil was instilled into the conjunctival sac. In 4 animals rinsing was carried out. The result was reported as negative. No details are given.

5. Sensitization

Guinea pig: Twelve animals were used. The concentration used is not stated: it may have been undiluted a.i. It was rubbed into the flank skin for 30 seconds daily, 5 days a week, for 3 weeks. After a 5 day rest, the a.i. was applied to the skin of the opposite flank daily for 3 days. The test is reported as negative. No details are given.

A Magnusson-Kligman maximisation test was carried out according to GLP in 30 male guinea pigs, of which 10 were used as controls. The active ingredient was of a purity of 99.1 % as determined by GLC.

In groups of 2 animals preliminary tests were carried out to determine suitable doses for the main experiment.

(a) A concentration of active ingredient was sought which when injected intradermally would cause mild to moderate erythema and no evidence of systemic toxicity when assessed at 24 and 48 hours after injection. The concentration chosen was 5 % in arachis oil.

(b) A concentration of active ingredient for topical induction was sought which, in animals treated with injections of Freund´s complete adjuvant 10 days previously, would procedure mild to moderate erythema following occlusive exposure for 48 hours (Animals had been treated identically with the control animals of the main study for ten days prior to the test.). The most suitable concentration was found to be undiluted active ingredient.

(c) A concentration of active ingredient for the challenge application was sought which was the highest non-irritant concentration when applied to the skin of the flanks for 24 hours with occlusion (Animals had been treated identically with the control animals of the main study before the application.). The concentrations chosen were undiluted active ingredient; a lower concentration of 75 % active ingredient in ethanol/diethylphthalate 1:1 was also selected to ensure that a non-irritant concentration had indeed been determined.

The main study was conducted in 20 animals. The intradermal induction injections, 3 on either side of the midline in the shoulder area, and each of 0.1 ml volume, were: Freund´s complete adjuvant diluted 1:1 with distilled water; 5 % of active ingredient in arachis oil; and 5 % of active ingredient in diluted Freund´s complete adjuvant. Reading was at 24 and 48 hours. After 7 days, the topical induction was carried out in the same area as the intradermal injection, and consisted of undiluted active ingredient with occlusion for 48 hours. Reading was at 1 and 24 hours. On day 21, the challenge application was made on the flanks; on one side undiluted

active ingredient was used, and on the other a 75 % solution in ethanol /diethylphthalate 1:1. The application sites were occluded for 24 hours. Reading was at 24 and 48 hours after removal of the occlusive dressing. There was no evidence of sensitization in any animal, test or control, at any time after the challenge. The author gives a table of results with the test in the same laboratory over the preceding 2 years using known sensitizers: alpha-hexylcinnamaldehyde, 2-mercaptobenzothiazole, ethyl-4-aminobenzoate, and 2,4-dinitro-chlorobenzene. These gave strongly positive reactions.

Man: Ten subjects had undiluted a.i. applied twice weekly to the same site for 7 applications. After 12 days a challenge application with undiluted a.i. was made. No abnormality was found. No details are given.

6. Teratogenicity

Fertile hen's eggs: Groups of 20 eggs were tested. The dose applied was contained in 0.1 ml of olive oil. The amounts applied were 0, 0.25, 0.625, 2.5 and 6.25 µl a.i. per egg. Injections were given into the white of the egg on day 1 of incubation in one series and on day 5 in another. The LD_{50} of injections on day 1 was 5.8 µl, and on day 5, 1.15 µl (approximately 120 and 25 ppm respectively). Deaths of embryos during the incubation were dose related. Following hatching, the chicks were anaesthetised and bled. The only abnormality found was a statistically significant reduction of blood glucose at 0.25 and 6.25 µl, but its biological significance is doubtful [This test is not regarded as adequate for an evaluation of teratogenic or embryotoxic effects. In addition, injections are usually made into the yolk sac, or sometimes into the air space, and not into the white of the egg, as here.]

Rat: A study of the teratogenic and embryotoxic properties of the a.i. was carried out according to GLP. The a.i. was dissolved in 3 ml of olive oil and given daily by gavage in doses of 0, 0.25, 0.75 and 2.25 ml/kg b.w./day, from days 6 to 15 (inclusive) after mating. A positive control was used: tretinoin, similarly administered, at a dose of 15 mg/kg b.w./day. At day 20 the animals were killed by ether anaesthesia and subjected to post mortem examination. The foetuses were weighed, and about half of them were subjected to visceral examination and the remainder to skeletal examination.

The chief findings in the dams during the experiment were: a loss of weight in the high dose animals; an increase in water consumption in the high dose animals throughout the experiment, and in the low and intermediate dose animals in the second half of the experiment; a decrease in food intake in the intermediate dose animals in the first half of the experiment, and in the high dose animals throughout the experiment; and a dose related increase in hair loss in all dosed groups and in the positive control animals.

At necropsy, the weight of the adrenal glands was increased in the high dose animals; the weight of the liver was increased in the low dose animals, but this was not thought to be of biological significance.

The effect on foetuses were as follows. There was a dose related increase in intra-uterine mortality. There was a fall in foetal weights in the high dose animals and in the positive control animals. This was a well conducted study, and the a.i. does not show any teratogenic activity; the NOAEL was 780 mg/kg b.w./day. The positive control animals showed numerous foetal abnormalities.

7. Toxicokinetics (incl. Percutaneous Absorption)

Rat: Five experiments in all are reported; they are designated by the author by the letters A, B, C, D and E.

Experiment A: A 10 % formulation of ^{14}C a.i. in a w/o emulsion was applied (weight of formulation applied 210 mg) to the clipped skin of 3 m and 3 f rats for 24 hours, covering an area of 2.5 x 3.5 cm (this area was the same for all the subsequent experiments). A non-occlusive dome was applied over the area. A large number of organs was examined after sacrifice, but the account is confusing. The authors seem to suggest that absorption may be determined by summing the radioactivity in carcass + urine + faeces; this amounts to 11.24 %. Although there seems to have been some radioactivity in the various organs examined, the data given do not permit of any calculation of the amounts.

Experiment B: The same formulation was used in 1 female animal (weight of formulation applied 230 mg). The area was covered with an occlusive polyethylene sheet for 3 days. The total amount of radioactivity over the period in urine + faeces was 15.8 %. The carcass value was 0.7 %, so that the total absorbed over the period is taken to be 16.5 %.

Experiment C: A 10 % o/w formulation was used (weight of formulation applied 220 mg). One female animal was tested. A non-occlusive dome was sutured to the skin under anaesthe-sia, and the preparation allowed to remain in contact for 7 days. The total of the percentages of radioactivity for urine + faeces over the period was 64.8 %.

Experiment D. This was the same as C except that a 10 % w/o formulation was used (weight of formulation applied 180 mg). The total of radioactivity for urine + faeces over the period was 70.5 %.

Experiment E: One animal was used. A 10 % o/w emulsion was used (weight of formulation applied 200 mg) and the area of application covered with a non-occlusive dressing. After 6 hours, the area of application was washed and the dressing reapplied, and allowed to remain in place for 7 days. The amount found in the urine + faeces over the period was 3.18 % of the amount applied.

The report is difficult to interpret. It may be concluded that over a period of 6 hours, about 3 % of a.i. is absorbed from an application area of 8.75 cm^2, using a 10 % formulation; over 7 days about 70 % is absorbed.

Man: After 30 minutes exposure to formulations containing 10 % a.i., the skin was repeatedly stripped at the site of application. It is stated that OECD guidelines were followed. The formulations were w/o emulsions, one of which contained 13.5 % of liquid paraffin; the other contained 10.5 % of liquid paraffin + 3 % “Eusolex 8020” (the sunscreen 4-isopropyldibenzoylmethane). The a.i. was labelled with ^{14}C. About 3 mg of each formulation was applied without occlusion to two different areas of the forearm, each measuring 2 cm^2. The period of exposure was 30 minutes. The subjects were 2 males and 4 females. Using the first formulation, the amount in the first two strips were 42.27 % and 13.28 % respectively. (The area of application is not stated to have been washed before stripping). The authors suggest this may be ignored as being present only in the most superficial layers of the skin. The remaining strips yielded 42.21 % of the applied radioactivity. The amounts found in the strippings with the second formulation were not significantly different. There was a significantly higher amount of radioactivity in the strippings from the females than from the males. There was slight or definite erythema for up to 24 hours in

4 of the subjects treated with the first formulation. The results are difficult to interpret; if the results in all the strippings are taken into account, the formulae developed by Rougier and his coworkers suggest an absorption of 60 to 70 % over 4 days, or 60 to 70 mg/kg b.w.

Pig skin *ex vivo*. Two formulations were tested, o/w and w/o lotions each containing 10 % active ingredient. A glass cell was used to clamp areas of pig skin of 5 cm^2; the rate of application of lotion was 4 mg/cm^2 of skin, that is, 400 µg of active ingredient per cm^2. Temperature was controlled at 32 °C. The skin sections were 3 to 4 mm thick; they were not treated before use. The receptor fluid was physiological saline to which was added 1 % bovine serum albumen and 0.02 % gentamicin. The active ingredient was estimated by HPLC: the limit of detection was 0.06 µg/ml, and the limit of determination was 0.2 µg/ml. The recoveries were 90 % for the o/w preparation and 85 % for the w/o preparation. The results are given in "standardised" form: that is, presumably corrected for recoveries, since the figures given in the results for any particular layer of skin always add up to 100 %.

Samples were taken at 3, 6, 16 and 24 hours after application of the formulation. From the context it is clear that each experiment was carried out for a specific time interval, since the sampling involved removal of the skin from the cell to have various procedures carried out on it which would have made it impossible to continue the experiment. The samples were obtained as follows:

(a) Surface: gentle scraping with a spatula, and threefold wiping with cotton wipes;
(b) Stratum corneum: 15- to 20-fold stripping with adhesive tape;
(c) Epidermis and dermis: heating of the skin disc for 45 seconds on a hot plate at 80 °C, followed by separation of dermis and epidermis with forceps;
(d) Dermis: 3 punch samples of dermis (presumably following step (c) above).
(e) Two aliquots of receptor fluid.

The experiments were carried out in triplicate.

The results were as follows: (percentages found in various compartments, normalised, means of 3 experiments)

(a) o/w formulation:

	3 h	**6 h**	**16 h**	**24 h**
Surface	84.4	67	63.5	63
Strat. corn.	14.3	30.6	33.3	34.6
Epidermis	1.2	2.2	2.9	2.2
Dermis	0.1	0.2	0.3	0.2
Fluid	0	0	0	0

(b) w/o formulation:

	3 h	**6 h**	**16 h**	**24 h**
Surface	76.1	61.2	45.5	41.9
Strat. corn	21.7	34.4	47.1	48.1
Epidermis	2	4	6.8	9
Dermis	0.2	0.4	0.6	1
Fluid	0	0	0	0

A graph gives the coefficients of variation of the means for each of the compartments (except on the skin surface). For the w/o preparation this was up to 70 % at 6 hours, but otherwise about 10 % to 25 %; for the w/o preparation the coefficient at 3 hours was between 5 % and 15 %, but it increased gradually from 6 to 24 hours, being maximally between 15 and 50 % at the last mentioned time. On the whole, these are not very high, considering only 3 samples were involved at each time.

If the penetration of the active ingredient into the receptor fluid is taken as the amount of percutaneous absorption, this may be regarded as effectively zero for both formulations. If the amounts in the dermis and epidermis at 6 hours are taken as indicating the amount of absorption (regarded as representative of use patterns) a reasonable figure for the percentage absorption would be 4.4 %.

8. Mutagenicity

Ames test: A standard Ames test was carried out, using a.i. dissolved in DMSO, up to 10 mg/plate. No evidence of mutagenic activity was found. With strains TA 1538 and TA 98, the level of revertants was some 3 to 5 times higher after activation, both with the vehicle control and the a.i. This may be related to the fact that the investigator used phenobarbitone + 5.6-benzoflavone as an inducting agent, instead of the customary Aroclor.

A second test using strains TA 98, TA 100, TA 1535 and TA 1537 was carried out. In this case precipitation was noted at levels greater than 5 mg. There was no evidence of mutagenic activity.

Mouse: Micronucleus test. The dose levels were 750, 1500 and 3000 mg/kg b.w., dissolved in olive oil and given as a single intraperitoneal injection. All animals showed toxic effects, most marked at the top dose. There was no evidence of abnormal micronucleus formation.

Human lymphocytes *in vitro*. The test was carried out according to GLP standards. Human lymphocytes were cultured and exposed to concentrations of a.i. in DMSO determined by preliminary toxicity testing, as follows: without activation 0, 10, 30, 100 µg/ml; with activation 0, 30, 100 and 300 µg/ml. The top doses gave 55 % to 70 % toxicity. Positive controls were cyclophosphamide and mitomycin C. Tests were carried out in duplicate. The cells were exposed to a.i. for 24 hours; they were then washed and cultured for a further 24 hours. At least 100 metaphases from each culture were counted.

There was a slight tendency to an increase in the number of gaps with increasing dose of a.i., but the authors report the test as negative, by comparing the values with those of the historic controls.

Test for photomutagenic activity. A test was carried out, according to GLP, using two strains of S. typhimurium: TA 1537 and TA 102.

The tests were carried out in the same manner as the conventional Ames test. The positive control for TA 1537 was chloropromazine, and for TA 102, 8-methoxypsoralen. The active ingredient and 8-methoxypsoralen were dissolved in DMSO; there was a tendency for the development of precipitates of the active ingredient at 5000 µg/plate. Chloropromazine was dissolved in water. Each experiment was carried out twice. Metabolic activation was not

used. The sensitivity of the strains to mutagenic effects was confirmed, before each set of experiments, by using plates containing 9-aminoacridine for TA 1537 and cumenehydroperoxide for TA 102, in each case without ultraviolet radiation. A xenon arc was employed to produce the ultraviolet radiation, and the intensity of the radiation was measured at the level of the plates. The values were (experiment 1): UVA/UVB 6.9/0.48, 13.8/0.96, 20.7/1.44, and 41.4/2.88 mJ/cm^2. There were trifling differences between these values and those measured in the second experiment. The doses of active ingredient used were 8, 40, 200, 1000 and 5000 µg/ plate.

With TA 1537, some toxicity was seen at 40 µg/plate and above; precipitation was noticed at 5000 µg/plate. In the absence of ultraviolet radiation, there was no increase in the number of revertants in any plate. In the presence of ultraviolet radiation, there was an increase in the number of revertants with chloropromazine, but none with the active ingredient.

With TA 102, toxicity was not found, but precipitation occurred at 5000 µg/plate. In the absence of ultraviolet radiation there was no increase of revertants; when ultraviolet radiation was used, there was an increase in revertants with 8-methoxypsoralen, but not with the active ingredient. There was no evidence of photomutagenesis.

10. Special investigations

Tests for capacity to produce phototoxicity and photoallergy

Guinea pig. Fifty animals were used in a maximisation procedure, according to the method of Guillot et al. GLP guidelines were followed. From preliminary experiments, it was decided to use a 50 % solution of a.i. in ethanol/DEP 1:4 as a nonirritant concentration for the tests. Irradiation was delivered from two lamps, which produced wavelengths from 285 to 400 nm. The two control groups (Ia and Ib) consisted each of 3 m and 2 f animals, and were treated identically with the respective test groups except that they were not irradiated. The two test groups (IIa and IIb) each contained 10 m and 10 f animals. Animals of group IIa had applications of the solution containing a.i.; those of group IIb had vehicle only. Both of these groups were irradiated.

(a) Phototoxicity. A single application of 0.5 ml of the solution of a.i. (test animals) or of vehicle (control animals) on a piece of gauze 2 cm x 2 cm was made to the depilated skin of the back. After 90 minutes, this was removed, and, in animals of groups IIa and IIb, immediately followed by irradiation. This consisted of exposure to both lamps for 5 minutes, followed by a 90 minute exposure to the lamp producing the longer wavelengths. The total irradiation was 12.5 J/cm^2, and amounted to a minimal erythema dose. The site was inspected after 24 hours. Any reaction was compared with that produced in the area surrounding the patch, which had also been exposed to a m.e.d.

(b) Photoallergy: Four days after the first test, using the same animals, intradermal injections of Freund's complete adjuvant (diluted with saline 50/50) were made at each corner of the site previously tested. The patches and irradiation were repeated. Further applications of patches and irradiation were made on days 7 and 9. A rest period of 14 days ensued. On day 23, a new site on the back was depilated and patches applied as before. The irradiation on this occasion, however, was from the lamp producing the longer wavelengths only, for 90 minutes. Tests on

other Guinea pigs had shown that this irradiation did not of itself produce any skin reaction. Readings were made at 6, 24 and 48 hours.

Result: There was no evidence of any phototoxic or photoallergic reaction in any animal. There were no formal positive controls, but in an appendix the findings of a series of experiments using the same protocol are given. In these a wide range of chemicals capable of producing phototoxic and photoallergic reactions was tested (e.g. 8-mop, 5-mop, angelica extract; and promethazine, 3, 5, 4-tribromosalicyclamide, etc.). These gave the expected positive results.

Man: Ten subjects had undiluted a.i. applied by means of an occluded patch for 24 hours. The area was then exposed to UV irradiation of an intensity slightly below the m.e.d. No abnormality was seen. No details are given.

Test for capacity to induce chromosomal aberrations in mammalian cells *in vitro* in the presence of ultraviolet radiation

An investigation was carried out according to GLP in Chinese hamster ovary cells in tissue culture at various concentrations of active ingredient and in the presence and absence of ultra-violet radiation. Ultraviolet radiation was produced by a "Heraeus" (xenon) arc producing SSR, and the doses of UVA and UVB were determined by an Osram "Centra" meter. Allowances were made for the absorption of ultraviolet radiation by the culture flasks and by the culture medium. The active ingredient was dissolved in DMSO, and then diluted in culture medium.

(a) Preliminary test for phototoxic effects. It was found that a concentration of 160 µg/ml in culture medium, the active ingredient precipitated, although the precipitate redissolved at 37 °C. This concentration of active ingredient was therefore the highest used in a preliminary study to determine possible phototoxicity. The concentrations used were the maximum at 160 µg/ml, and then a series dilutions such that each concentration was X 0.7 of the next highest concentration; 12 concentrations were used, the lowest being 3.164 µg/ml. A previous study (not reported in detail) had suggested that doses of ultraviolet radiation of 1500 and 750 mJ/cm^2 would be suitable. Although the details are somewhat obscurely expressed, it is probable that at 1500 mJ/cm^2 the ration of UVA to UVB was about 15.

Flasks were incubated in the dark with the active ingredient or with the control chemicals: solvent only, 4-nitroquinoline-1-oxide or 8-methoxypsoralen. Irradiation was for 3 hours, at 750 and 1500 mJ/cm^2, after which the cultures were washed and re-fed; harvesting (following addition of colchicine) was at 17 hours. Slides were examined for evidence of mitotic inhibition compared with the control solutions (defined as a reduction of the number of mitoses, preferably dose related, compared with the controls; 1000 cells should be examined). The mitotic index showed a fall at concentrations of active ingredient of 38.42 µg/ml and above. Since the effects of the ultraviolet radiation were similar at both 750 and 1500 mJ/cm^2, in the main study (following the protocol) ultraviolet radiation at 1500 J/cm^2 alone was used.

(b) In the main study, the highest concentration of active ingredient used was 80 µg/ml. Lower concentrations were such that each concentration was less than the next highest concentration by a factor of 0.75; 8 concentrations in all were used, the lowest being 10.68 µg/ml. Experiments were carried out in duplicate at each level, in the absence and presence of ultraviolet radiation. At each concentration, 200 cells were scored, except in

the positive controls (50) and in the range finder experiment (100); at the higher concentrations in the range finder experiment, the preparations did not provide a sufficient number of metaphases to score the full number.

The results showed no increase in aberrations compared with the negative controls (solvent, and 8-methoxypsoralen in the absence of ultraviolet radiation); the positive controls (8-methoxypsoralen in the presence of ultraviolet radiation, and 4-nitroquinoline-1-oxide) gave substantial increases in aberrations. The results with the negative controls were consonant with the historical control levels in the laboratory. The test was negative.

11. Conclusions

Acute and subchronic toxicity are low. Tests for irritation of mucous membranes and skin were negative. A maximisation test for sensitization in the guinea pig was negative. The results of the tests for photoallergenicity permit the deduction that sensitization is unlikely; the substance is a very rare allergen and photoallergen in clinical practice. Tests for teratogenicity were negative. There was no evidence of mutagenic or photomutagenic activity in tests with S. typhimurium, nor in human lymphocytes in culture or in a micronucleus test in the mouse. A test for the production of photomutagenesis in CHO cells *in vitro* was negative. While earlier tests for percutaneous absorption might be interpreted as indicating marked percutaneous absorption, a recent well conducted study on pig skin *ex vivo* suggests limited absorption.

Classification: 1

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

ISOPENTYL-*P*-METHOXYCINNAMATE
S 27

Based on a usage volume of 18 g, containing at maximum 10 %

Maximum amount of ingredient applied:	**I (mg)= 1800 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%)= 4.4 %**
Dermal absorption per treatment:	**I (mg) x A (%)= 1800 mg x 4.4 % = 79.2 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg** **1800 mg x 4.4 % / 60 kg = 1.32 mg/kg b.w.**
No observed adverse effect level (mg/kg): **(13 week rat study, oral)**	**NOAEL = 200 mg/kg b.w./day**

Margin of Safety: NOAEL / SED = 200 mg/kg b.w. / 1.32 mg/kg b.w. = 150

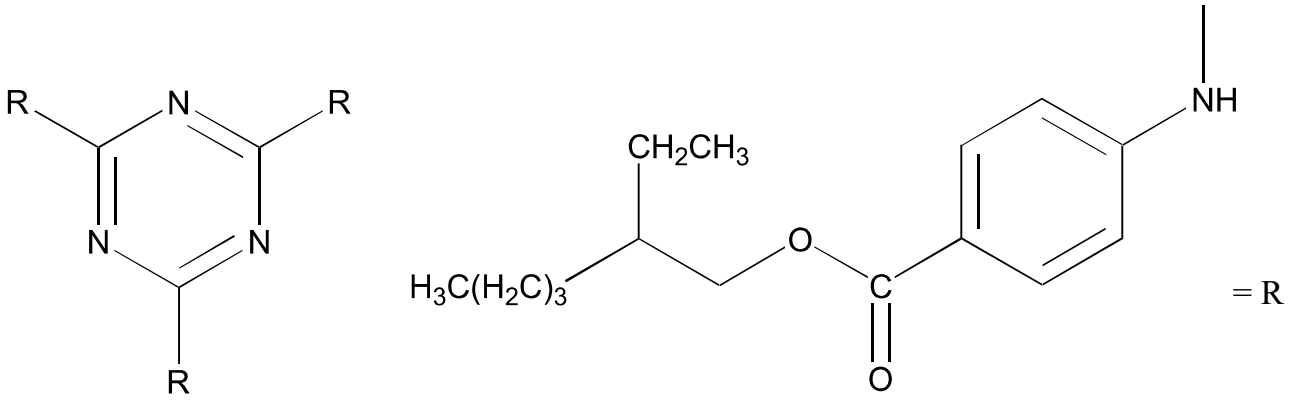
S 69: *ISOCTYLTRIAZONE*

1. General

1.1 Primary name

2,4,6-trianilino-(p-carbo-2'-ethylhexyl-1'-oxi)1,3,5-triazine.

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{48}H_{66}N_6O_6$.
Mol weight: 823.1.

1.7 Purity, composition and substance codes

Stated by manufacturer to be more than 98 % pure.

1.9 Solubility

Insoluble in water; soluble in isopropyl myristate, olive oil, ethanol.

2. Function and uses

Used as a stabiliser in light sensitive plastics, dyes, etc. Proposed use level in sunscreen preparations: up to 5 %. Absorption maximum 312 nm.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat and mouse. The acute oral toxicity was in general greater than 10 g/kg b.w. Dermal application up to 2 g/kg b.w. did not cause any abnormality.

3.7 Subchronic oral toxicity

Rat. Oral. In a 13 week study, groups of 10 male and 10 female animals were given 0, 1000, 4000 and 16000 ppm in the diet. There was a dose related increase in the weights of the liver in female animals only. However, there was no evidence of liver damage on histological examination, and clinical chemistry tests were normal; because of these findings, and the fact that they occurred in female animals only, the liver changes are considered not to be significant, and the no effect level is put at 16000 ppm, or about 1150 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Two groups of 6 animals were used, one group with scarified skin and one group without. A 10 % dilution of a.i. in olive oil was applied for 24 hours with occlusion. There was definite erythema in 4/6 animals with scarification, and slight erythema in 2/6 animals with intact skin. No abnormality was found after 7 days.

In another experiment, groups of 3 male and 3 female NZW animals were used. A 50 % suspension of a.i. in physiological saline was applied to intact and scarified skin with occlusion for 24 hours. Vehicle controls were used. No abnormality was found.

In another experiment, groups of 6 males and 6 females were used; 3 of each sex had scarification of the area of application. Concentrations of up to 2 % of the a.i. were applied for 24 hours with occlusion; the material was formulated in various o/w creams, in emulsions, and in a formulation used commercially. The last had no adverse effects, but the concentrations of a.i. were only 0.9 % and 1.8 %. The emulsions and o/w preparations showed slight erythema and oedema in the first few days, but the maximum Draize score at any time was 2.

In another experiment, a 50 % suspension in water was applied under semi-occlusive conditions for 24 hours to 3 animals. There was no evidence of irritation.

Guinea pig. A commercial preparation containing 2 % of a.i. was applied daily for 5 days. No abnormality was found.

Man. Fifty subjects were tested, 18 males and 32 females. Concentrations of 5 % and 10 %, formulated as emulsions and as oily solutions, were applied for 24 hours with occlusion. There was one reaction to the 5 % solution in oil. Otherwise no abnormality was found.

4.2 Irritation (mucous membranes)

Rabbit. Four standard Draize tests are reported. Evaluation is uncertain in two of the experiments, because of doubts about the concentrations used. Slight changes were found with a 10 % solution in olive oil, with and without rinsing. Findings were normal after 48 hours. A suspension of (probably) 50 % in saline caused no abnormality. A suspension of (probably) 41 % caused slight changes only. In another study, up to 50 % in olive oil was used; no abnormalities were found. Overall, the substance appears to be only slightly irritating to mucous membranes, if at all.

Chick chorio-allantoic membrane. The probable concentrations of a.i. used were 0.64 % and 6.4 %. No abnormalities were found at either concentration.

5. Sensitization

Guinea pig. A commercial preparation containing 2 % of a.i. was used. It was applied daily to the skin, 5 days a week for 3 weeks. After a 2 weeks rest, the same preparation was applied 3 times to a fresh site. No reaction was seen.

A Magnusson-Kligman maximisation test was carried out in 40 animals, 20 test and 20 controls. The induction concentration of a.i. was 5 % in olive oil intradermally, and 60 % dermally, with occlusion for 48 hours. The challenge was made with 40 % solution in olive oil. There were no significant differences between control and test groups.

Man. Sixty subjects were tested by applications of a commercial preparation containing 2 % a.i., applied for 24 hours with occlusion. No reaction was seen. Of the 60 original subjects, 10 had the test material applied to the same sites 5 times at intervals of 48 hours with occlusion. The application was repeated after 10 days rest and again after a further 10 days. No reaction was seen.

Test for production of allergy.
Man. A 1 % solution of a.i. in olive oil was applied to the skin of a panel of 8 subjects known to be allergic to para-aminobenzoic acid derivatives. No reaction was produced. No details given.

6. Teratogenicity

Tests for developmental and teratogenic effects.
Chick embryo. Two series of experiments were carried out, injections being made on day 1 and day 5 respectively, the doses being lower for the latter. The LD_{50} was 45 mg (day 1) and 25 mg (day 5). Mortality was dose related. There was a significant increase in the length of the metatarsus, and some biochemical changes, in chicks of the group given 10 mg on the fifth day.

Rat: The compound was tested for teratogenic effects in groups of 25 female Wistar rats, in accordance with GLP (OECD 1981; Chemicals Act Bundesgesetzblatt 1990; 87/302/EEC). The doses chosen were justified as follows: (a) The acute oral toxicity was greater than 10 g/kg b.w.; (b) the oral rat 90 day study revealed a NOAEL greater than 1150 mg/kg b.w./day. The top dose (4) was therefore taken as 1000 mg/kg/day; the intermediate dose (3) 400 mg/kg/day; and the lowest dose (2) 100 mg/kg b.w./day, together with a vehicle control group (1). The authors accept that the top dose might not cause maternal toxicity, but justify the selection of this dose in that the procedure may be considered as a limit test in accordance with the OECD guidelines.

A number of rats failed to become pregnant, so that the groups consisted of the following numbers of pregnant females: 25 (1); 21 (2); 21 (3); and 24 (4). Dosing was by gavage in a constant volume of olive oil.

Animals were examined clinically every day, and weighed weekly; food consumption was recorded. Ophthalmological examination was carried out in groups 1 and 4 at the beginning of the experiment and before sacrifice; clinical chemistry and haematological investigations were carried out before sacrifice. (However, the results of the ophthalmological, chemical and haematological tests are not given).

Sacrifice was at day 20 post coitus. All dams were examined macroscopically. The livers and uteri were weighed. The uteri were examined for implantations and numbers of foetuses; the corpora lutea were counted. The foetuses were classified as: live; dead implantations; early resorptions; late resorptions; dead foetuses (i.e., fully developed but not showing signs of life). The foetuses were weighed and sexed, and subjected to macroscopic examination. The placentae were weighed and examined; the membranes and amniotic fluids were examined. Approximately half the foetuses were prepared for soft tissue examination, and the rest for skeletal examination. There is no mention of histological examinations.

The results of the above examinations showed no evidence of any changes which could be attributed to the test substance; indeed, the autopsies on the dams showed no significant abnormalities of any sort. The foetal examinations showed no evidence of any teratological effect. The findings of the study are consonant with the historical controls of the laboratory.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man. A 0.5 % solution of a.i. in ethanol/hexane was applied to the forearms of 8 subjects. (Note, however, that the maximum permitted use level is 5 %.) After 30 minutes the area was repeatedly stripped, 20 times in all. The concentration of a.i. in each stripping was estimated by HPLC. The authors state that in 20 strippings, 87 % of the applied material was recovered. The area treated was between 1 and 2 cm^2. If one calculates from these figures the percutaneous absorption using the method proposed by Rougier et al, and extrapolating to the full body surface area, this gives an absorption of between 18 and 37 mg/kg b.w.

A photoacoustic method was used to measure penetration into the skin. As this method is not as yet a validated one for this purpose, it was not possible to evaluate it.

Man *ex vivo*. A study was carried out according to GLP, using human skin from various body sites, and both male and female subjects. The epidermis was separated from the rest of the skin following brief immersion in water at 60° and mounted in Franz cells, maintained at 32° to 35°; the stratum corneum faced outwards. The area for application was 3.1 cm^2. For the main experiments the receptor fluid was ethanol/water 50/50. In each experiment, the integrity of the membrane was determined by looking for diffusion of $3H_2O$ at the beginning and the end of the experiment; in these cases the receptor fluid was physiological saline. The active ingredient was applied as a 5 % formulation, of which the composition is given; it is probably an o/w emulsion. Two volumes of application were used: 1 mg formulation/cm^2 (= 0.05 mg active ingredient/cm^2) and 10 mg formulation/cm^2 (= 0.5 mg/cm^2). Each concentration was tested on 5 different skin samples. The formulation was applied on a cotton wool swap in the appropriate volume and the outer chamber sealed with "Parafilm". Samples of the receptor fluid, which was continuously stirred, were taken at (hours) 0, 1, 2, 4, 8, 24, 48 and 72; the volumes of sample removed were replaced by the same volumes of receptor fluid. The active ingredient was estimated by the extinction at 309 nm. After the experiment, the remaining formulation was removed by gentle blotting; the skin was washed twice with ethanol; the receptor chamber was washed twice with ethanol; and the "Parafilm" and the skin were extracted with dioxan or with dioxan/ethanol/water. The concentrations of active ingredient in all these washings were also estimated.

Results: Balance studies showed rather a large variation, but the means gave an acceptable balance. In all the skins, there was a rapid initial flux over about 3 hours; thereafter a very low continuous flux was noted until 72 hours in all had elapsed. There were substantial differences between the skins in the amounts absorbed over 72 hours, but the total amounts absorbed were small, and were similar for the high and the low volumes of application. Of the skins used, inspection of the graphs suggested that female breast skin had the highest absorption rate, and female abdominal skin the lowest, but the number of experiments was inadequate to establish these observations as scientifically correct. After 72 hours, the mean cumulative absorption was approximately 1.4 μg/cm^2 (low dose) and 1.1 μg/cm^2 (high dose).

If it was assumed that the average absorption in use would be 1.25 μg/cm^2, the total body exposure would be about 0.35 mg/kg b.w. The experiments seem to have been carefully carried out to an acceptable protocol.

8. Mutagenicity

A standard Ames test was carried out. The maximum concentration used was 5000 μg/plate (limit of solubility, 500 μg/plate). There was no evidence of mutagenicity.

A test for the production of chromosomal aberrations *in vitro* was carried out in a culture of V79 cells. The investigation was carried out according to GLP. Activation was achieved by using an "Aroclor" induced rat liver preparation. The active ingredient was dissolved in DMSO, and dilutions of this were made in water. Precipitation occurred between 50 and 100 μg of active ingredient per ml of water, and the latter was used as the top dose. Exposure to the active ingredient was for 18 hours, except in the case of cultures where activation was used; there, the exposure was for 4 hours. The usual cell cycle for this cell line is 14 hrs, so that the harvest time of 18 hrs was within the recommendations; in case exposure to the active ingredient might interfere with the cell cycle, or that it might act at an unexpected stage of the cycle, harvest times of 28 hrs were also tested, at the top dose of active ingredient. Positive controls were ethylmethanesulphonate and cyclophosphamide.

All tests were in duplicate; cytotoxicity was looked for by setting up parallel tests in which the cell survival at 100 μg/ml was compared with the survival using the vehicle only.

Following preliminary tests, the active ingredient was tested at levels of 10, 33 and 100 μg/ml, with a vehicle control. Results with the 18 hour or 28 hour harvest showed no significant increase in aberrations at any concentration tested, with or without activation, nor any significant change in mitotic index, although there was a good deal of variation in the index. There was no evidence of cytotoxicity. The positive controls showed marked increase in aberrations. The test was negative.

A test for photomutagenicity was carried out using the tryptophan-dependent organism *E. coli* WP2. The experiments were carried out according to GLP. The organism was tested for absence of the pKm101 gene (ampicillin resistance). Ultraviolet radiation was obtained from an Osram "Vitalux" lamp, with the use of a glass filter for irradiation with UVA only. The ultraviolet irradiation was measured by use of an Osram "Centra" meter. The active ingredient was dissolved in DMSO and then added to water in the required amounts. Concentrations of 1000 and 5000 μg/plate regularly produced precipitation, and 1000 μg/plate was therefore used as the top dose. Positive controls were 8-methoxypsoralen and 4-nitroquino-line-1-oxide 20 and 500 μg/plate,

respectively. Activation was not used. All experiments were carried out in triplicate (negative controls in quintuplicate). No evidence of toxicity was found at any concentration.

Following a range finding experiment, the following concentrations of active ingredient were tested (µg/plate): (a) 1.6, 8, 40, 200, 1000; (b) 62.5, 125, 250, 500, 1000 (to study closer spacing in the higher values). The levels of ultraviolet radiation were: (UVA/UVB, mJ/cm^2): control; 5.3/1.8; 11.1/3.8; 230/0; 460/0.

There was no significant increase in revertants at any dose of the active ingredient, with or without ultraviolet radiation; the positive controls were strongly positive. The test was negative.

A test for chromosomal aberration using CHO cells in culture, with exposure to ultraviolet radiation, was carried out according to GLP (1989, UK). The active ingredient was dissolved in DMSO to form a stock solution; this was further diluted in DMSO and filtered, after which the dilute DMSO solution was added to the media in appropriate amounts. Preliminary experiments indicated that 80 µg/ml was about the solubility limit of the active ingredient, and this was used as the top dose. The range finding study used 0.1 log ratios from 80 µg/ml downwards, testing 13 dose levels. For the main study, the doses selected were said to be 32.77, 40.96, 51.2, 64 and 80 (µg/ml), but in fact results are presented for concentrations of 51.2, 64 and 80 µg/ml only. The concentrations of the positive control compounds were: 8-methoxypsoralen 3.125 µg/ml and 4-nitroquinoline-1-oxide 0.25 µg/ml. Ultraviolet radiation was obtained from an Osram "Vitalux" lamp, and the intensity of the radiation measured with an Osram "Centra" meter. The doses of ultraviolet radiation were: UVA 200 and UVB 33 mJ/cm^2; and UVA (filtered through glass) 700 mJ/cm^2. The absorption spectra provided show that the plastic of the flask absorbed strongly below about 280 nm, and the glass similarly below about 325 nm. All tests were carried out in duplicate, and at each dose level 200 metaphases were examined, except in the case of the positive controls, where 50 metaphases were examined.

Cultures were first incubated for a day; then the active ingredient (or positive control compounds) were added; after an interval of not less than 15 minutes and not more than 2 hours, exposure to ultraviolet radiation was carried out (where appropriate). Two hours later the cultures were washed and re-fed, and cultured for further 18 hours. Colchicine was added 90 minutes before harvest.

No mitotic inhibition was found at any level of active ingredient. There was no evidence of toxicity. No increase in the number of aberrations was found at any dose of active ingredient, and the positive controls were strongly positive. Apart from the positive controls, the numbers of aberrations were within the historical values previously found in the laboratory. The test was negative.

Mouse. A micronucleus test was carried out at a dose of 2100 mg/kg b.w. There was no evidence of micronucleus production.

10. Special investigations

Test for tolerance on repeated use.
Man. In 45 subjects, of whom 14 had sensitive skin and allergic conditions, a commercial formulation containing 2 % a.i. was applied daily. During 3 weeks of exposure, no adverse reaction was seen.

Test for capacity to produce phototoxic and photoallergic effects.
Guinea pig. Dunkin Hartley albino animals were used. Tests for phototoxicity and photoallergenicity were carried out in the same animals, according to the method of Guillot et al. (1985) J. Toxicol. 4, 117.

Two groups of animals were used. Group 1 consisted of 3 male and 2 female animals which were treated with the a.i. but not irradiated. Group 2 consisted of 10 male and 10 female animals which were both treated and irradiated.

(a) Phototoxicity. The a. i. was applied to the clipped skin of the interscapular area over about 4 cm^2, with occlusion for 90 minutes. The animals of group 2 were then irradiated with 2 lamps: one with a spectrum of 400 to 310 nm ("UVA lamp") and one with a spectrum of 350 to 285 nm ("UVB-lamp"). The dose of UVB radiation was chosen to be a minimal erythema dose. Both lamps were used for 5 minutes (energy produced 0.43 J/cm^2) at a distance of 10 cm from the skin. This was followed by irradiation with the UVA lamp for 90 minutes at a distance of 5 cm from the skin; energy released 12 J/cm^2; total energy thus 12.5 J/cm^2, of which 1 % was UVB. Readings were carried out 6 and 24 hours after irradiation.

(b) Photoallergy. On day 4, following wax depilation, the applications were repeated in the same way to both experimental groups, but as well 4 intradermal injections of 0.1 ml of FCA, diluted 50/50 with saline, were made at the sides of the application site. The applications were then repeated on days 7 and 9. Animals of group 2 were irradiated with the UVA and UVB lamps as before, for 15 minutes at 5 cm, and with the UVA lamp for 40 minutes at 5 cm, after the removal of each patch. Total energy 7.1 J/cm^2 and UVB 6 %.

After a 14 day rest period, a new area of skin was treated with a.i. as before. Animals of group 2 were then irradiated with the UVA lamps for 90 minutes at 5 cm (energy released 12 J/cm^2). Reading was at 6, 24 and 48 hours after the irradiation. No abnormality was found in any of the experiments.

No contemporaneous positive controls were used, but the investigators present tables of experiments carried out under identical conditions in their laboratory, in which the effects of various phototoxic and photoallergenic compounds are recorded and shown to be positive.

Guinea pig. Two groups of 9 animals were used. The concentration of a.i. was probably 0.5 %, and 0.5 ml was applied to the skin of the neck for 15 minutes. The positive control was 2 % 3,3',-4,5-tetrachlorosalicylanilide (TCSA). Applications were followed by 15 minutes from UV irradiation from a quartz lamp 75 cm from the site. The procedure was repeated 5 times. After a 10 day rest, 2 challenge applications were made, the test being the same as the induction application, but the positive control being 0.1 % TCSA, followed by irradiation as before. Later, the test and positive control solutions were applied as follows: "1 % of emulsion Ka99 and 0.1 % TCSA in 8 % soap solution... Soap solution without the test agent was applied as a control to the opposite flank of the test animals..." (This seems to indicate that the concentration of a.i. on this occasion was 0.02 %). Again, irradiation was applied. There was no evidence of photosensitization. The positive control, however, gave either very weak reactions or none.

Miscellaneous tests.
Rat. Doses up to 500 mg/kg b.w. by mouth had no effect on blood pressure, or on carrageenan-induced oedema of the paw.

11. Conclusions

The concentrations of a.i. used in some of the tests for irritation of mucous membranes and for sensitization were low in relation to the proposed use level. On the whole, however, these tests were acceptable, and negative. Acute oral and dermal toxicity were low. A subchronic oral study in the rat gave a NOAEL of about 1150 mg/kg b.w./day (the highest dose tested). An Ames test and a micronucleus test in the mouse were negative. Tests for chromosomal aberration *in vitro*, and for photomutagenicity and photoclastogenicity, were negative. Tests for phototoxicity were negative. One test for photoallergenicity could not be interpreted since the wavelengths of the light used were not given, and the positive controls gave anomalous results. In a second test for photoallergenicity, positive controls were not used, but historical evidence from the same laboratory suggests that the results may be taken to be negative. A test for teratogenic activity was negative. Tests for percutaneous absorption, using the stripping method, suggested a relatively high absorption, but this is not a validated method. Similarly an experiment using a photoacoustic method could not be evaluated. A careful set of experiments according to GLP, using human skin *in vitro*, suggested a low absorption, of about 1.5 % of the amount of active ingredient applied.

Classification: 1.

CALCULATION OF MARGIN OF SAFETY

Amount of formulation applied = F = 18000 mg.

Concentration of active ingredient = C % = 5 %.

Total amount of active ingredient applied = F x C/100 = I = 900 mg.

Percentage absorption = A % = 2.8 or 0.55.

Total absorption (mg) = I x A/100 = 25.2 or 4.95.

SED = 0.42 or 0.08 mg/kg b.w.

NOAEL = 1150 mg/kg b.w.

Margin of safety = 2700 or 14000.

S 73: *PHENOL,2-(2H-BENZOTRIAZOL-2-YL)-4-METHYL-6-(2-METHYL-3-(1,3,3,3,-TETRAMETHYL-1-(TRIMETHYLSILYL)OXY)DISILOXANYL)-PROPYL)*

1. General

1.1 Primary name

Phenol,2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-methyl-3-(1,3,3,3,-tetramethyl-1-(trimethylsilyl)oxy)disiloxanyl)-propyl)

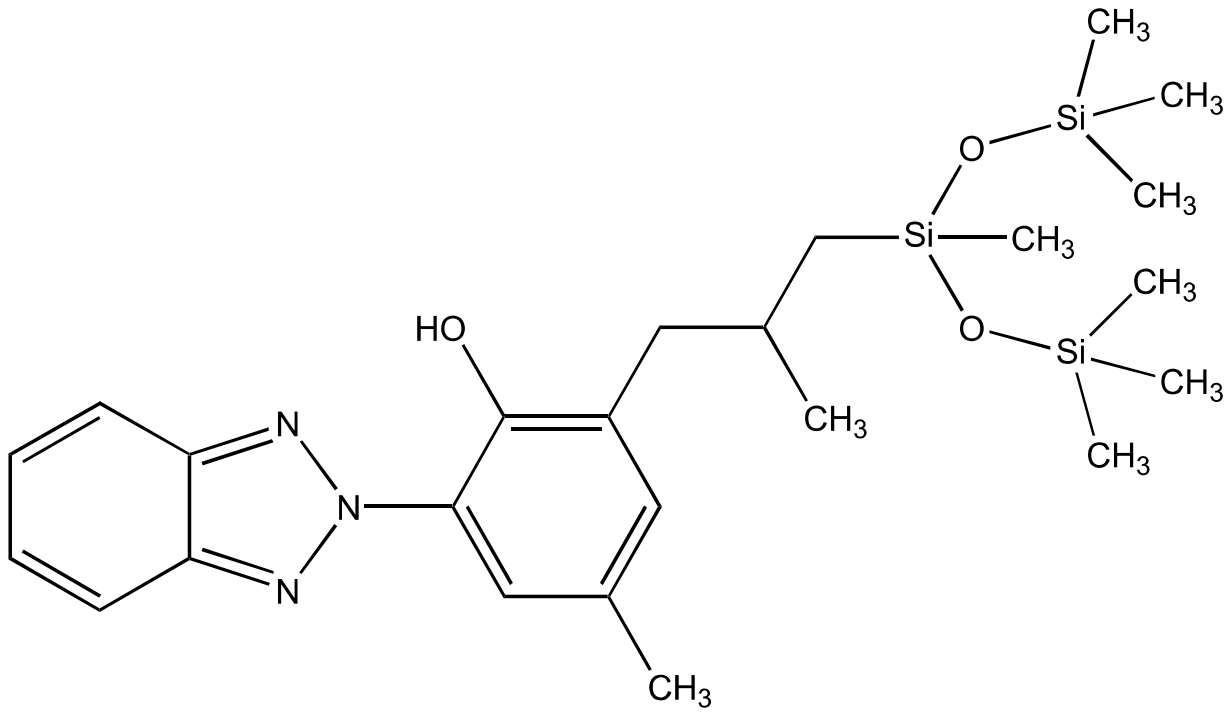
1.2 Chemical names

Phenol,2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-methyl-3-(1,3,3,3,-tetramethyl-1-(trimethylsilyl)oxy)disiloxanyl)-propyl)

1.4 CAS no.

155633-54-8

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{24}H_{39}N_3O_3Si_3$
Mol weight: 501.855

1.8 Physical properties

Appearance: white crystalline powder
Melting point: 46.3 °

1.9 Solubility

Soluble in ethanol, 95 % ethanol, acetone, DMSO; insoluble in water.

2. Function and uses

Proposed for use at a maximal concentration of 15 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Rat. The experiments were carried out according to GLP, using active ingredient of a purity of 99 %. 5 male and 5 female animals were used. The active ingredient was suspended in 4 % methylcellulose + 1 % "Tween 80" and administered as one dose of 2000 mg/kg b.w. by gavage. Clinical examination was carried out four times on day 1 and daily thereafter for 14 days in all. There were no abnormal clinical findings and no deaths. At necropsy, no abnormalities were found. Body weight and body weight gain corresponded to the normal ranges for rats of this age in the laboratory. It was concluded that the LD_{50} was greater than 2 g/kg b.w.

3.7 Subchronic oral toxicity

Rat. Oral. Four groups of 10 male and 10 female Sprague Dawley rats (IcoIbm:OFA (SPF)) were used (group A). The doses of active ingredient, administered daily by gavage, were (mg/kg b.w./day) 0, 100, 300 and 1000. The active ingredient was suspended in water/methylcellulose/Tween 80. The homogeneity and the stability of the active ingredient in the vehicle were established. In addition to the animals of the main study, groups of 5 male and 5 female rats were similarly treated with 0 and 1000 mg/kg b.w./day for 13 weeks, after which a 4 week drug free follow up was carried out (group B) and two groups of 5 male and 5 female rats were similarly treated and used to obtain plasma samples at 4 and 13 weeks of treatment (group C). The doses chosen were based on a preliminary 14 day study, not reported in the documents provided.

Clinical observations were carried out daily. Food consumption and body weights were measured weekly. Ophthalmological examinations were carried out on animals of group A before testing began, and in the control and top dose animals of this group at week 13.

Blood for haematological and biochemical investigations was taken from animals of group A at 6 and 13 weeks, and from animals of group B at 13 and 17 weeks. Specimens of urine were taken from these groups at the same intervals. Full haematological examinations were carried out, as well as tests for coagulation. A large range of biochemical variables was estimated.

Numerous tests were also carried out on urine. Samples of blood were taken from animals of group C at 4 and 13 weeks for the determination of plasma levels of active ingredient. These animals were sacrificed at week 13 and not subjected to further examination. (According to the protocol, the samples were to be transmitted to the sponsor for estimation of plasma levels of the active ingredient, but if these estimates were carried out, they do not seem to be reported in the documentation submitted.)

All animals of groups A and B were subject to necropsy, at 13 and 17 weeks respectively. Organ weights were recorded. A large number of tissues was fixed, and histological examination was carried out on approximately 30 of these, together with any gross lesions noticed at necropsy.

Results

There was one death: a male animal in the control group. The cause of death could not be determined at autopsy.

There was a slight significant increase in food consumption in males at the low and high doses of active ingredient in the first week of treatment. There had been some increase in food consumption in the high dose animals before the experiment had begun, and in view of this, and the absence of such increase at the intermediate dose in the first week, and subsequently during the experiment, the finding was thought not to be of biological significance. Relative food consumption was not affected. There was no effect on body weight, or body weight gain. Some abnormal clinical signs were noted, but these were distributed evenly through the groups, and were those to be expected in rats of this strain under the experimental conditions. Ophthalmoscopic examination revealed no abnormalities.

Organ weights are given as absolute and relative to body weight and brain weight. In the main experiment, there were no significant changes in any of the values. In the recovery animals, sacrificed at 17 weeks, there was a significant (0.05) increase in pituitary weight in males, and a decrease in females. These changes were regarded by the authors as probably not of biological significance.

Necropsy and histological examination showed no important differences between control and test animals, with one possible exception. In the main experiment, “myofiber necrosis” in heart muscle was found in 4 male control animals but not in any females of this group; at 1000 mg/kg b.w./day, it was found in 5 males and 3 females. In the recovery animals, the same finding was found in 1 male and 1 female control animal, and 4 male animals receiving 1000 mg/kg b.w./day. However, the changes do not seem to have been severe. The only other difference between the control and dosed groups was the finding of a schwannoma in the ear of a recovery male animal treated with 1000 mg/kg b.w./day. The author of the report did not consider the finding of this rare tumour of any biological significance.

Haematological investigations revealed significant changes in some groups, but the absolute changes were small, and there was no dose relationship. A possible exception to this is found in the reticulocyte fluorescence ratios in female animals of group B: after 4 weeks recovery (i.e. without administration of the active ingredient), the high and middle fluorescence ratios were significantly reduced, and the low fluorescence ratio was significantly increased. In these cases the differences were marked in absolute terms, but the level of significance was less than 0.05.

The implications of this finding are unclear, and the reticulocyte and nucleated red cell counts were unaffected. Probably it is of no biological significance.

The changes in clinical chemistry findings are stated to be not of biological significance. The following statement is found in the text (probabilities in square brackets; [0.05] means "less than 0.05", etc.).

"The following statistically significant effects were recorded for clinical biochemistry data in the animals of group 4 (1000 mg/kg) at 6 and/or at least 13 weeks of treatment when compared with the controls:
- The glucose level was slightly lower by 12 % in females [0.01] after 13 weeks.
- The total cholesterol level was slightly higher in males [0.05] at 6 weeks (+21 %) and after 13 weeks (+23 %).
- Slightly higher creatine kinase activity in males [0.01] at 6 weeks.
- Slightly higher sodium concentration in females [0.01] at 6 weeks.
- Slight changes in some plasma protein fractions of the protein electrophoretic pattern (relative and/or absolute). This was characterised primarily by an increased alpha 1-globulin fraction in females [0.05] at 6 weeks, decreased alpha 2-globulin fraction in both males [0.05 - 0.01] and females [0.05] at 6 weeks and in males [0.05] after 13 weeks, increased beta globulin fraction in both males and females [0.05 - 0.01] at 6 and after at least 13 weeks, and decreased gamma globulin fraction in females [0.05] at 6 weeks.

At termination of this treatment-free recovery period these findings were found to be reversed.

The above differences recorded in the animals of group 4 (1000 mg/kg) were of minor degree and suggest metabolic adaptations.

No toxicological relevance is therefore associated with any of these findings.

All other statistical differences in the results of the haematology, clinical biochemistry and urinalysis data were considered to be incidental and unrelated to the treatment, and of normal biological variation for rats of this strain and age (and see also historical control data for untreated Sprague Dawley (SPF) rats, Attachment 4, pp. 323)."

There was no evidence of changes in the urine following treatment.

The authors suggest that the no observed effect level (NOEL) was 300 mg/kg b.w./day, and that the no observed adverse effect level (NOAEL) was 1000 mg/kg b.w./day.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit. Three rabbits of strain CRL:KBL(NZW)BR were used: 1 male and 2 female. The experiments were carried out according to GLP, and the active ingredient was of at least 98 % purity.

An area of about 100 cm^2 of dorsal skin was clipped. If there were no visible skin lesions on inspection, the animal was accepted for the test. A dose of 0.5 mg active ingredient was applied over about 6 cm^2 of the prepared skin, and covered with a surgical gauze dressing 3 cm x 3 cm

in area. This was in turn covered by a semi-occlusive dressing. After 4 hours, the dressing was removed, and the area of application was rinsed with warm tap water. Reading was at 1, 24, 48 and 72 hours after removal of the dressing. Scoring was based on the intensity of erythema or eschar formation produced, and on the degree of oedema. The maximum score, according to the protocol, was 8. Staining and corrosion of the skin were also looked for. The mean score for each animal over 24-72 hours was zero, and there was no evidence of staining or corrosion. The active ingredient was therefore adjudged to be non-irritant.

4.2 Irritation (mucous membranes)

Rabbit. A standard Draize test was carried out in 3 rabbits, 1 male and 2 female, of strain CRL:KBL(NZW)BR, according to GLP. The purity of the active ingredient was greater than 98 %. A dose of 0.1 mg of the active ingredient was placed in the left conjunctival sac of each animal, after which the lids were held closed for about 1 second. Rinsing was not carried out. Reading was at (hr) 1, 24, 48 and 72. A conventional scoring system was used, and the means of the readings at 24, 48 & 72 hours calculated. The maximum score attainable, according to the protocol, was 13. The mean values in this experiment were: 1, 1 & 0.67. There was no staining of the sclera, conjunctiva or cornea. The active ingredient was therefore adjudged to be non-irritant.

5. Sensitization

Guinea pig. This investigation followed the Magnusson-Kligman maximisation method, under GLP conditions. Guinea pigs of the strain G0H1:SPF (Himalayan spotted) were used. The investigation falls into 4 parts as follows. A: A pre-test investigation. B: The main study. C: A contemporaneous positive control study using 2-mercaptobenzothiazole as the positive control substance. D: A contemporaneous positive control study using alpha-hexylcinnamaldehyde as the positive control substance. The test material was of a purity of greater than 98 %.

A. The pre-test:
   (a) Intradermal exposure. Two male guinea pigs were used (In the table of results, these animals are specified by number, viz., 848 and 849. But in the tables, animal 848 is stated to be male on two occasions and female on one, and animal 849 is stated to be female on two occasions and male on one. Probably these are typing errors, and both animals were male). Both flanks were clipped, and intradermal injections (volume 0.1 ml) were made at concentrations of active ingredient of 5 %, 3 % and 1 %, suspended in corn oil/acetone 20/80. Reading at 24 hours showed slight but equal changes at all concentrations, and a concentration of 5 % was selected for intradermal injection in the mean study.
   (b) Epidermal exposure. Four male guinea pigs were used. The flanks on either side were clipped and shaved. The active ingredient was suspended in corn oil/acetone 20/80. Four patches of filter paper were soaked with the vehicle containing concentrations of active ingredient of 85 %, 80 %, 70 % and 60 % respectively, and these were applied to both flanks. The patches were covered with aluminium foil and occluded for 24 hours. Reading was at 24 and 48 hours after removal of the occlusion. No reaction of any kind was found, and a concentration of active ingredient of 85 % was chosen for epidermal application in the main test.

B. The main test. Thirty male animals were used, 20 as test and 10 as control. Skin in the interscapular region was clipped free of hair, and 3 injections, each of 0.1 ml, were made at each lateral edge of an area of skin about 6 x 8 cm in area. For the test group the injections were: 1:1 mixture of Freund´s complete adjuvant and physiological saline; active ingredient, 5 % in corn oil/acetone 20/80; active ingredient diluted to 5 % in a 1:1 (v/v) mixture of Freund´s complete adjuvant and physiological saline. For the control group, the injections were: 1:1 (v/v) mixture of Freund´s complete adjuvant and physiological saline; corn oil/acetone 20/80; 1:1 (w/w) mixture of corn oil/acetone (20/80) in a 1:1 (v/v) mixture of Freund´s complete adjuvant and physiological saline (The report notices that this last mixture conforms with OECD 406, but that 92/69 EEC recommends the use of Freund´s complete adjuvant without dilution, in both test and control systems.). One week later, the same area was prepared and pretreated with 10 % sodium lauryl sulphate in liquid paraffin. On the following day, a patch of filter paper, saturated with 85 % active ingredient in corn oil/acetone 20/80, was applied to the skin and allowed to remain for 48 hours under conditions of semi-occlusion. The animals of the control group were treated similarly except that no active ingredient was used. Readings were made 24 and 48 hours after removal of the patches.

The challenges were applied to clipped 5 x 5 cm areas on each flank of both test and control animals. The challenges were 85 % active ingredient in corn oil/acetone 20/80 to the left flank, and vehicle only to the right flank. Semi-occlusive dressings were applied and left in place for 24 hours. Readings were at 24 and 48 hours after removal of the dressings.

The results were as follows: after the injections, there were no differences between the test and the control animals. No reaction was found to the epidermal induction applications. No reaction was found to the challenge applications in any animal. No deaths occurred, and there was no effect on body weights.

C. Positive control test using 2-mercaptobenzothiazole. The procedure was similar to that of the test. Ten female animals were used as negative controls, and 20 for the test. For intradermal induction, the active ingredient was used as a 5 % solution in mineral oil; for the epidermal induction 25 % in mineral oil, and for the challenge 15 % in mineral oil. No reaction was found after challenge in the control animals: 95 % of the test animals had grade 1 to 3 erythema following challenge.

D. Positive control test using alpha-hexylcinnamaldehyde. Conditions and experimental procedures were as for the preceding control *(supra)*. The concentration for intradermal induction was 5 % active ingredient in PEG 400. For epidermal induction, 10 % in PEG 400 was used. For the challenge, 3 % in PEG 400 was used. No abnormality was found in the control animals. After 24 hours, 75 % of test animals showed grade 1 erythema, and after 48 hours, 45 %.

The conclusion of the author was that there was no evidence that the active ingredient had sensitizing activity in this test.

6. Teratogenicity

Test for capacity to produce embryotoxicity and teratogenic effects.

Rat. A study to determine whether the active ingredient displayed any teratogenic or embryotoxic effect was carried out according to GLP in Sprague Dawley rats (IcoIbm; SPF). Groups

of 25 female animals were used. The active ingredient, of a purity not less than 98 %, was administered by gavage, in a constant volume, daily from days 6 to 15 post coitum. The suspending medium was 4 % methylcellulose + 1 % Tween 80 in water. Sacrifice was at 21 days. Following preliminary studies, the doses administered (mg/kg b.w./day) were: group 1, 0; group 2, 100; group 3, 300; group 4, 1000. The homogeneity and content of active ingredient in the suspension were controlled by chemical analysis: the concentrations of active ingredient were very close to the required values at every dose level.

Clinical observations were made twice daily. Two animals were sacrificed prematurely for ethical reasons: one animal in the control group at day 20 (necropsy showed enlarged spleen, and liver changes), and one at day 9 in the top dose group (injury during dosage). The numbers of animals per group pregnant, and available for evaluation, were: group 1, 24; group 2, 23; group 3, 25, group 4, 24.

Food consumption was measured during post coital days 0-6, 6-11, 11-16 and 16-21. Body weights were recorded daily.

All animals were subject to gross necropsy, and the weights of the uteri and contents were recorded: the body weight gains are given for the entire animal and also for the weights of the animals minus the weights of the uteri ("relative body weight gain"). The foetuses were removed and weighed, and their viability observed. One half of the foetuses were fixed for tissue examination, and one half were treated with alizarin for skeletal examination. The uteri were inspected for signs of implantations and resorptions.

Results:

Dams:

(1) Clinical examinations. No spontaneous deaths occurred. No dose related clinical abnormalities were noted. There were various abnormal signs, distributed through all groups: none was serious (except for animals sacrificed prematurely, *vide supra*).
(2) Food consumption. There was a slight significant decrease in group 4 animals during days 6-11 post coitum, but since this was not found at other times, and was in any case slight in absolute terms, it was not attributed to the active ingredient.
(3) Body weights, body weight gains, and relative body weight gains, showed no differences between the groups.
(4) Post mortem findings showed only anomalies commonly seen in these animals, and no differences between the groups.

Foetuses:

(1) The numbers of foetuses (Group, n) were: 1, 374; 2, 366; 3, 369; 4, 397.
(2) All foetuses were alive. There were no significant differences between foetal numbers per litter. Foetal weights showed a slight significant increase in weight in male and female foetuses of dams of group 3 only. There was no evidence of any differences in the numbers of deaths in the embryonic or foetal stages in any group.
(3) External examinations gave the following results. Groups 1 & 2: no abnormality seen. Group 3: 1 animal had rudimentary tail, 1 animal had caudally flexed left forepaw. Group 4: 1 animal had caudally malpositioned left hindlimb.

(4) Sex ratios: there were no differences between groups.
(5) No important differences were found between groups in external or visceral examinations. Skeletal developmental changes (group, %) were: 1, 17; 2, 22; 3, 36; 4, 28.
(6) The distributions of the conceptuses in the uteri, in respect of sex, resorptions and implantation sites, showed no differences between the groups.

Figures for four earlier experiments in the same laboratory, involving 1090 foetuses, are given. The results of the experiment under consideration were consonant with these. It was concluded that there was no evidence of teratogenic or embryotoxic activity following administration of the active ingredient.

7. Toxicokinetics (incl. Percutaneous Absorption)

Percutaneous absorption.

Human skin **ex vivo**. Human female abdominal skin, obtained at plastic surgery, was preserved at -20 °C. In all, samples of skin were obtained from 6 donors. Each sample was used in a diffusion cell, and the 6 experiments were conducted twice. Before each skin sample was used, it was allowed to thaw, and by means of a dermatome converted into a membrane about 0.45 µm thick. The thickness of each sample was measured 7 times, and the means of these measurements recorded. The means of these 6 values were 0.464 µm in the first experiment and 0.456 µm in the second.

The skins were mounted in a diffusion chamber with a surface area of 2 cm^2, and with a receptor compartment of volume 3 ml. The chambers were maintained at 32 °C, and the receptor fluid was constantly stirred. The composition of the receptor fluid is given as: PBS buffer free of calcium and magnesium; Volpo N20; Instamed; water. The maximum solubility of the active ingredient in the fluid was 4.695 µg/ml, and the limit of detection was 100 ng/ml.

The active ingredient was incorporated in a formulation of which the composition is given; it appears to be an o/w emulsion, and the concentration of the active ingredient was 10 %. The weight of formulation applied to the skin was measured by difference. The mean amount of formulation applied to each skin in the first set of 6 experiments was 9.85 mg, and in the second 10.25 mg. This figure should be corrected on the assumption that maximally 2 mg/cm^2 should be applied. Thus the amount of formulation effectively applied was about 2 mg/cm^2, and the amount of active ingredient about 0.2 mg/cm^2. Diffusion was allowed to proceed for 16 hours. The amount of active ingredient on either side of the skin membrane was carefully maesured: extensive rinsing, and stripping of the skin on the donor side, was carried out. The balance calculations show that the recovery of the applied doses of active ingredient was about 100 %, with a range of 94.85 % to 104.09 %; the mean and standard deviation of the values given may be calculated to be 99.54 % ± 2.66. The receptor fluid showed no active ingredient to be present, and the permeation is thus set at the limit of detection, 100 ng/ml, so that approximately 0.8 % of the applied dose may be assumed to be absorbed.

8. Mutagenicity

A test for mutagenic activity was carried out according to GLP, using the micro-organisms *S. typhimurium* (strains TA 1535, 1537, 98 and 100), as well as *E. coli* WP2 and WP2uvrA.

Each experiment was carried out in triplicate, and 2 separate experiments were carried out. The purity of the active ingredient was greater than 99 %. An initial experiment with and without activation showed that concentrations of active ingredient of up to 5000 µg/plate did not cause toxicity (in strains TA98 and 100) and this was taken as the top concentration. The active ingredient was dissolved in acetone and no precipitation was seen. The concentrations tested (µg/plate) were: 33.3; 100; 333.3; 1000; 2500; 5000. Activation was by the use of rat liver S9 fraction following prior treatment with "Aroclor 1254". Positive control chemicals (with the solvent used and the concentration per plate in brackets) were: without activation: for TA 1535 & 100, sodium azide (water, 10 µg); for TA 1537 & 98, 4-nitro-*o*-phenylene-diamine (DMSO, 10 µg); for WP2 and WP2uvrA, methylmethanesulphonate (water, 5 µg).With activation: 2-aminoanthracene (DMSO, 2.5 µg for *S. typhimurium* and 10 µg for *E. coli*). Negative controls and solvent controls were also used.

Two independent experiments were carried out, each in triplicate. There was no evidence of reversions, and the positive controls gave strongly positive results. The experiment was negative.

A test for production of chromosomal aberrations *in vitro* was carried out according to GLP, using Chinese hamster V79 cells. The purity of the active ingredient was greater than 99 %. Preliminary tests for toxicity were carried out according to two methods.

(a) A XTT assay. The cells were incubated for 20 hours with doses of active ingredient ranging from 0.3 to 50 µg/ml, with and without activation. The yellow tetrazolium salt XTT (not further identified in the text) was added to the cultures after 20 hours, and further incubation was for 4 hours. The effect of the active ingredient on the cells was indicated by the absorbance of the culture medium. The rationale was that mitochondrial activity catalysed the formation of a yellow dye from the tetrazolium salt. In the absence of activation, no effect was found; with activation a fall in absorbance occurred, most marked at 30 and 50 µg/ml.

(b) In a second test for toxicity, the number of cells surviving 24 hours of incubation with concentrations of active ingredient from 100 to 5000 µg/ml was measured, and expressed as a percentage of the solvent control values (The use of a concentration of 5000 µg/ml is to satisfy the Japanese guidelines. In practise, precipitation of the active ingredient occurs at 30 to 50 µg/ml in the culture medium). In this test, without activation, there was a fall in the percentage of viable cells between concentrations of the active ingredient of 100 to 1000 µg/ml; at concentrations of 3000 and 5000 µg/ml, the number of normal cells was higher than in the solvent control. With activation, there was a fall in the number of viable cells from 500 to 5000 µg/ml of active ingredient, except for 3000 µg/ml, where the number of viable cells was much the same as in the solvent control.

In the main part of the study, two similar experiments were carried out. The only difference between them was that the top concentration of active ingredient in the first experiment was 1000 µg/ml, and in the second 5000 µg/ml. Each experiment was carried out in duplicate. Harvesting was carried out at 18 hours (concentrations of active ingredient from 3 to 1000 and 3 to 5000 µg/ml) and 28 hours (concentrations of active ingredient of 30 and 1000 µg/ml, and concentrations of 30, 1000 and 5000 µg/ml). In the case of experiments with activation, exposure to the S9 mix was for 4 hours only. Positive controls were used in each experiment (ethylmethanesulphonate for cultures without activation, and cyclophosphamide for cultures with activation).

The results showed that with 18 hour harvest, there was a fall in the mitotic index of about 20 % at all concentrations of active ingredient, although this was not entirely regular: at 28 hours the tendency was for the mitotic index to be increased. When activation was used, the effect on the mitotic index was about the same but there was no definite tendency to an increase at higher concentrations of active ingredient. Chromosomal aberrations (excluding gaps) were subjected to statistical test. This shows that the only significant increase (p less than 0.05) was found with 30 µg/ml active ingredient with activation in the first experiment. The authors regarded this as not of biological significance; the test was considered to be negative.

Test for photomutagenic activity.

An investigation was carried out according to GLP, using active ingredient of greater than 99 % purity. The organism used was *E. coli* WP2 Trp^-. In the light of a previous study (*vide* test for mutagenic activity, *supra*), no preliminary test for toxicity was carried out; the doses of active ingredient chosen (µg/plate) were: 33.3, 100, 333.3, 1000, 2500, 5000. The positive control was 8-methoxypsoralen, 125 µg/plate.

In a preliminary experiment, bacteria were exposed to SSR for varying periods of time, followed by plating on selective medium, in 2 replicates. It was found that an exposure of 10 seconds gave an increase of 2.6 times in the number of revertants. This dose of radiation was approximately equivalent to 9 mJ/cm^2 UVA and 1 mJ/cm^2 UVB, and this was then used throughout the main experiments.

In the first experiment, 2 replicate plate incorporation tests were carried out, each using 3 plates. In the second experiment, a pre-incubation test was carried out, in which, after irradiation, the bacteria were incubated with the appropriate concentrations of active ingredient in test tubes at 37° for 60 minutes; after this the organisms were plated in triplicate and incubated for 48 hours in the dark.

There was no evidence of an increase in revertants in any of the test plates, compared with the negative controls; 8-methoxypsoralen gave strongly positive results. The test was negative.

A test for photomutagenicity was carried out using Chinese hamster ovary cells *in vitro*. The ultraviolet radiation used was SSR derived from a Honle GmbH instrument; the intensity of the radiation was determined by meters supplied by the same manufacturer. The active ingredient was dissolved in acetone to make a stock solution; this stock solution was then added to the culture medium in appropriate amounts. Precipitation was noted at concentrations of active ingredient in the culture medium greater than 10 µg/ml, and this concentration was therefore chosen as the top dose. The lower doses were 1, 3 and 5 µg/ml, but it appears that the results of exposure to 5 µg/ml were not evaluated.

The following preliminary tests were carried out:

(a) Six doses of ultraviolet radiation, between (UVA/UVB, mJ/cm^2) 100/6 and 400/24 were tested. There were no chromosomal aberrations at 200/12, and numerous aberrations at 300/18; these doses of ultraviolet radiation were used for further preliminary testing and in the main study.

(b) A preliminary test for toxicity induced by the active ingredient showed no reduction of cell numbers, and no decrease of mitotic indices, at 10µg/ml of active ingredient in the culture medium.

The main experiments were carried out in duplicate. After 2 days of incubation of the cells in culture medium, the latter was replaced by phosphate buffered saline containing the appropriate concentrations of the active ingredient. After a further 30 minutes of incubation, exposure to ultraviolet radiation was carried out. In the first experiment, the intensity of the irradiation was 200 mJ/cm^2 UVA and 12 mJ/cm^2 UVB.

In the second experiment, two intensities of ultraviolet radiation were used: 200 mJ/cm^2 UVA and 12 mJ/cm^2 UVB and also 300 mJ/cm^2 UVA and 18 mJ/cm^2 UVB. Ten minutes after the irradiation, the phosphate buffered saline was removed and culture medium reintroduced. The cultures were treated with colcemid 19 and 27 hours after the commencement of the treatment, and harvest was after a further 3 hours, i.e. harvesting at 22 and 30 hours. For a positive control, 8-methoxypsoralen was used.

The results show:

(a) A strongly positive response to 8-methoxypsoralen.

(b) Two statistically significant increases in aberrations: at 3 µg/ml without radiation and also at 1 µg/ml with 200/12 mJ/cm^2 (both in the second experiment following 22 hours of incubation).

(c) A substantial increase in aberrations at the higher dose of ultraviolet irradiation in the second experiment. In view of the lack of a dose correlation, and the fact that one of the increases occurred without prior irradiation, the findings (under (b), *supra*), were not regarded by the author as biologically significant, and the test was regarded as negative.

10. Special investigations

Test for production of micronuclei.

Mouse. Groups of 5 male and 5 female mice were used. Since a dose of 2000 mg/kg b.w. had shown no effect in an acute toxicity experiment (*antea*), preliminary toxicity testing was not carried out, and this dose was used as a maximum. Two lower doses at 0.5 log intervals were used: 670 and 200 mg/kg b.w.; a control group received vehicle only. The positive control was cyclophosphamide.

The active ingredient, of a purity of greater than 99 %, was suspended in Methocell/Tween 80 and given orally in a constant volume of 10 ml/kg b.w. (probably by gavage, although this is not stated). All animals were sacrificed at 24 hours, except for the top dose animals: two groups were treated at this dose, and sacrifice was at 24 and 48 hours. The results show that there was no increase in micronuclei in the femoral marrow in any animal, except in the positive control animals, which showed marked increases in micronucleated cells. There was no effect on the ratio between polychromatic and normochromatic cells. The test was negative.

Test for capacity to induce photoallergenicity.

Guinea pig. A study was conducted according to GLP, using active ingredient of a purity greater than 98 %. The investigation used 34 male animals: 4 for a pretest, 10 control animals and 20 test animals. Contemporaneous positive controls were not carried out, but animals of this strain are tested yearly in the laboratory to determine whether the strain had retained its

photoallergenicity. The positive control agent used was 3,3',4'5'-tetrachlorosalicylanilide, and a report of the latest such test (February 1995) was presented in the submission.

The light sources were: UVA, 10 and 20 J/cm^2: Philips "Actinic" TLD lamp; UVB, 1.8 J/cm^2: Philips "UV-B-Sunlamp TL". Radiation spectra are not given.

Pretest. To determine the highest non-irritant concentration of the active ingredient, a phototoxicity test was performed in 4 animals. Both flanks were shaved. Test areas of 2 cm^2 were delineated and the sites were pretreated with a 2 % solution of DMSO in ethanol. After 30 minutes, the sites of the left flank were treated with acetone solutions of the active ingredient at concentrations of 85 %, 80 %, 70 % and 60 %. The flank was then exposed to 20 J/cm^2 of UVA radiation. The right flank was than treated with active ingredient in the same concentrations, but the skin in this area was not irradiated. Reading was at 24, 48 and 72 hours after exposure. No effect was seen at any level of exposure to active ingredient, and so 85 % in acetone was chosen as the dose for the main experiment.

Main test. An area of skin in the nuchal area was shaved and a test site of 6 to 8 cm^2 was delineated. At each corner an injection of 0.1 ml Freund´s complete adjuvant and physiological saline 1:1 was given intradermally. The active ingredient (85 % in acetone) was then applied to the delineated area. The site was then exposed to radiation: 1.8 J/cm^2 UVA and 10 J/cm^2 UVB. The epidermal induction (active ingredient + irradiation) was repeated 4 times more: on days 3, 5, 8 and 10. The control animals received the intradermal injection described above, but no epidermal applications, or irradiations were carried out.

The animals of both control and test groups were shaved on the flanks, and on the following day, 3 weeks after the commencement of the induction, anaesthetised. The active ingredient, 85 % in acetone, was applied to the left flank, and the area was then exposed to 10 J/cm^2 of UVA radiation. The right flank was treated with the active ingredient, but was not irradiated. Reading was at 24, 48 and 72 hours after exposure. The reactions to the intradermal injections were the same in control and test animals; following repeated applications of active ingredient in the test group, some scaling and desquamation was found in the areas of application, which was attributed to the acetone. No reaction suggestive of photoallergy was found, either in the control or test animals, in exposed areas. One animal of the control group died on day 22. Body weight showed some differences: the mean body weights at the end of the experiment were (pretest) 670 gms; (control) 620 gms; (test) 580 gms. The author did not consider these findings biologically significant.

The positive control test used 3,3'4',5-tetrachlorosalicylanilide as a photoallergen. No details are given of the method; it is stated to be that described. The active ingredient was dissolved in ethanol. Four concentrations were tested in each animal: 0.01, 0.03, 0.1 and 0.3 %. The account is somewhat obscure, but it is clear that the test animals showed marked reactions, and the control animals very few.

11. Conclusions

The tests were carried out in accordance with GLP, and the purity of the ingredient was shown to be greater than 98 % in the tests. The compound seems to have low acute and subchronic toxicity. There is no evidence from animal experiments that the compound is irritant to skin or

mucous membranes, nor is there any evidence of allergenic or photoallergenic activity. Tests for mutagenicity and photomutagenicity are negative. A test for teratogenic activity in the rat is negative. In a test for percutaneous absorption carried out in human skin **ex vivo**, the amount absorbed was at most 0.8 % of the amount applied.

Classification: 1.

12. Safety evaluation

CALCULATION OF MARGIN OF SAFETY

Amount of formulation applied to skin = 18000 mg = F.

Concentration of active ingredient = 15 % = C.

Total amount of active ingredient applied = F x C/100 = I = 2700 mg.

Percentage of active ingredient absorbed = 0.8 % = A %.

Total amount absorbed = I x A/100 = 21.6 mg.

Typical body weight = 60 kg.

Systemic exposure dose = SED = (I x A/100)/60 = 0.36 mg/kg b.w.

Margin of safety: if 1000 mg/kg b.w. is taken as the NOAEL, 2700; if 300 mg/kg b.w. is taken (NEL), 800.

OPINIONS ADOPTED DURING THE 72ND PLENARY MEETING OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY, 14 October 1997

S 3: *ETHOXYLATED ETHYL-4-AMINOBENZOATE*

1. General

1.1 Primary name

Ethoxylated Ethyl-4-Aminobenzoate

1.2 Chemical names

Ethoxylated Ethyl-4-Aminobenzoate

1.5 Structural formula

1.6 Empirical formula

Emp. formula: $C_{59}H_{111}NO_{27}$
Mol weight: 1266.6

1.7 Purity, composition and substance codes

The compound is manufactured by reacting the ethyl ester of para-aminobenzoic acid with ethylene oxide. Free ethylene oxide is then blown away by a stream of nitrogen. The content of ethylene oxide in the end product is less than 1 ppm. Purity greater than 99 %.

1.8 Physical properties

Appearance: A clear slightly viscous yellow liquid at room temperature.

1.9 Solubility

Soluble in water; poorly so in ethanol or anhydrous isopropanol.

2. Function and uses

Proposed use level: up to 10 %.

TOXICOLOGICAL CHARACTERISATION

3. Toxicity

3.1 Acute oral toxicity

Acute toxicity is low: in the mouse (i.p.) and the rat (oral) the LD_{50} is greater than 1.9 g/kg b.w. Exposure of rats to air saturated with a.i. for up to 8 hours produced no abnormality.

3.7 Subchronic oral toxicity

Rat: A 3 month test using a.i. in the diet was carried out according to GLP in groups of 10 male and 10 female Wistar rats. The dose levels were 0, 1000, 4000 and 16000 ppm, approximately 70, 290, 1130 mg/kg b.w./day (males) and 80, 360 and 1350 mg/kg b.w./day (females).

The main abnormal findings were as follows. The total bilirubin in dosed males fell progressively with dose. There was no obvious reason for this. Histological examination of the liver showed cellular infiltration and fatty changes in all groups, including controls; and tubular mineralisation of the kidneys was found in all female animals, both test and control. It was concluded that no drug related abnormality had been produced.

4. Irritation & corrosivity

4.1 Irritation (skin)

Rabbit: A patch with about 0.5 ml of test solution was applied to the shaved skin of the back. With undiluted a.i., exposure was for 1, 5 and 15 minutes and 20 hours; using 10 % and 50 % aqueous solutions, exposure was for 20 hours. The undiluted material caused slight erythema which faded over 8 days. The diluted solutions caused no irritation. Undiluted a.i. or 10 % or 20 % aqueous solutions were applied to the inner skin of the ear in groups of 2 animals for 20 hours. The results were similar to those of the preceding experiment.

A patch soaked in 50 % aqueous solution of a.i. was applied for 8 hours a day for 5 days, always to the same area. No abnormality was produced.

Groups of 6 male albino rabbits were used; sites on either flank were prepared, and those on the left side scarified. A 20 % aqueous solution was applied on a patch for 24 hours without occlusion. Reading was at 24 and 48 hours. There was very slight erythema in 4/6 animals.

Two animals had 6 applications in a week of a 50 % aqueous solution to an area of 36 cm^2 of depilated dorsal skin. Each application was for 8 hours. There was no evidence of irritation.

Man. Twenty subjects, some suffering from skin disease, were tested. Undiluted a.i. and aqueous solution of 1 %, 5%, 10% and 50% were applied on patches for 20 hours over an area of 1 cm^2. No irritation was produced.

4.2 Irritation (mucous membranes)

Rabbit: The undiluted a.i. was applied to the conjunctiva in a dose of 50 mm^3. There was a slight redness and opacity at 1 hour and 24 hours, but appearances were normal at 8 days. The use of 10 % and 50 % aqueous solution was followed by no abnormality.

Further tests were carried out on the chorio-allantoic membrane of the chick at 10 days incubation. Concentrations of 1 % and 10 % in olive oil were applied. Rinsing was carried out after 20 seconds. The substance is stated by the authors to be “practically non irritant” at these concentrations, but details of the scoring system are not given.

5. Sensitization

Guinea pig: Ten animals were used for the test, and 3 were subjected to challenge only, without induction. The a.i. was dissolved in acetone, and applied to the flank; the same area was used throughout the induction. The first application was of a 50 % solution, and subsequent ones were of 80 %. Nine applications were made over 2 weeks. After a 12 day rest, a challenge application with a 50 % solution was made to the opposite flank. Reading was at 12 hours. There was no evidence of sensitization, or of primary irritation.

Man: A maximisation method was used in 27 male and female subjects; 3 subjects failed to complete the test. The test site was pretreated with aqueous 5 % sodium lauryl sulphate for 24 hours with occlusion. A 25 % solution of a.i. in diethyl phthalate was then applied to the same site, with occlusion, for 48 hours at a time. Five such applications were made. After a two week rest, 5 % lauryl sulphate was applied to a fresh site, with occlusion, for 30 minutes. The challenge applications were the same as those used for induction, and were applied for 48 hours, with occlusion, to the newly prepared site and to a fresh previously untreated site. Control application was of soft paraffin. There were "very few" cases of mild irritation due to the sodium lauryl sulphate. There was no evidence of sensitization or of primary irritation.

Test for capacity to produce photosensitization/photoallergy.

Guinea pig: Preliminary tests on groups of 2 male and 2 female animals were carried out in which the effects of undiluted active ingredient and a gel (active ingredient/water 50/50) were tested. No erythema or oedema were found. The main tests were therefore carried out according to GLP on albino Hartley guinea pigs, using water as the negative control; undiluted active ingredient as the test (note that according to the protocol this application should be slightly irritating, but this could not be achieved because the active ingredient was non-irritant); and a 2 % 3,5,4´-tribromosalicylanilide in dimethylacetamide/acetone/ethanol as the positive control. Three groups of 10 animals (each 5 male and 5 female) were used. All animals were treated identically apart from the applications of test or controls.

An area of skin about 2 cm x 2 cm in the nuchal area was prepared by clipping followed by chemical depilation. These areas were further shaved as required.

The lamps used were computer controlled "Biotronic UV" lamps; this enabled a suberythemogenic dose to be given. It is stated that 3 fluorescent Vilber-Lourmat tubes were used: 1 was rated at 40 W, emitting mainly in the UVB with a peak at 312 nm; the other 2 were also rated at 40 W, with and emission mainly in the UVA with a peak at 365 nm.

Injections of 0.1 ml of Freund´s complete adjuvant, diluted 50 %, were made at each corner of the depilated area in the nuchal region. The appropriate solution was then applied to the area for 30 minutes. After this, 30 minutes of irradiation with both UVA and UVB, at the minimal erythema dose, was given. The above sequence was repeated 5 times in the subsequent 14 days. After a rest period of 14 days, a topical challenge was applied to both flanks. Areas of 2 cm x 2 cm on the flanks were shaved and depilated the day before the challenge. Both sides were treated with the appropriate solutions, and one side was irradiated at 310 to 400 nm at 90 % of the erythemogenic dose, while the opposite flank was protected from radiation. Reading was at 24 and 48 hours. The protocol called for histological examination of the skin in the irradiated area, but if this was done, no account of it was given. There was no reaction of any kind with

the negative control or the active ingredient; the positive control gave grade 1 or 2 erythema only, and no oedema. The protocol called for a more powerful reaction from the positive control, but the investigators concluded that the poor response to the positive control did not invalidate the procedure, considering that the test and the negative control animals showed no reaction of any kind. The animals were weighed at the beginning and the end of the experiment: there was no evidence of any effect of the active ingredient on weight or weight gain. The test was regarded by the authors as negative.

6. Teratogenicity

Test for teratogenic activity and embryotoxicity: Fertile hen eggs were used; a suspension of a.i. in olive oil was injected on day 1 or day 5 of incubation. The doses of a.i. used (µl/egg) were: 0.25, 0.625, 2.5 and 6.25; the control was olive oil.

There was a dose related increase in mortality. The chicks hatched from eggs injected on day 1 of incubation showed no abnormalities; those injected on day 5 showed a significant increase in both absolute and relative weights of the heart, but the absolute increase was small and probably not of biological significance.

7. Toxicokinetics (incl. Percutaneous Absorption)

Man: Two sets of tests are reported in which the technique of photoacoustic spectrometry was used. A 2.5 % concentration of a.i. was applied and the technique was used to follow the disappearance of a.i. from the stratum corneum. It was concluded that all the a.i. had dissappeared from the stratum corneum in 56 hours. No quantitative data were obtained.

An investigation using the stripping technique was carried out in 10 subjects. A gel containing 10.8 % of a.i. was applied to both forearms, for 15 minutes on one and 30 minutes on the other. The areas were stripped 12 times. It was found that about 0.07 mg/cm^2 of a.i. was absorbed into the stratum corneum.

[Rougier et al found that the amount of benzoic acid absorbed in 96 hours could be determined by the stripping method by the use of the formula $y = 1.38 x - 0.52$.

If this is applicable to the a.i., the amount absorbed would be about 100 nmoles/cm^2; extrapolated to 1.6 m^2, this would imply an absorption of about 33 mg/kg b.w.]

Human skin *ex vivo*: An investigation was carried out according to GLP. Human skin was obtained from leg, thumb, breast and abdomen. The epidermis was removed by peeling off following a brief immersion in water at 60° C. The skins were mounted in Franz cells, with an area exposed of 3.14 cm^2. The receptor fluid was physiological saline, continously stirred. The stratum corneum was exposed to the donor solution, and the outer part of the cell was covered with “Parafilm”. A water jacket held the temperature at 32° to 36° C. The integrity of the membranes was determined by the addition of tritiated water to the outer chamber over 60 minutes, the preparation was rejected. This procedure was repeated at the end of the experiment. None of the membranes originally accepted had to be rejected because of the findings at the end of the study.

The active ingredient was applied as a 10 % aqueous solution at a dose of 5µl/cm^2, i.e. approximately 0.5 mg active ingredient /cm^2. As the experiment progressed, aliquots of the receptor fluid were taken and kept for analysis at the following times (hours): 0, 1, 2, 4, 8, 24, 48, 72. After each sampling the volume removed was replaced with physiological saline.

At the end of the experiment, the parafilm, the outer chamber, the skin and the receptor chamber were washed with physiological saline and the amounts of active ingredient measured. Balance studies showed a mean recovery of 102.6 %. The greater part of the active ingredient was found in the epidermal surface.

The results showed that there was a rapid initial flux into the receptor chamber for about 4 hours, followed by a much slower penetration over the following 68 hours. The initial flux was 0.23 µg/cm^2/hr for up to 8 hours and thereafter 0.04 µg/cm^2/hr up to 72 hours in all. The mean total amount permeating over 72 hours was about 4.3 µg/cm^2 (about 0.9 %), of which about one half permeated in the first 8 hours. If these results are extrapolated to use in a sunscreen with exposure of the entire body surface, the percutaneous absorption would represent about 0.25 mg/kg b.w.

The experiments seem to have been well carried out.

8. Mutagenicity

An Ames test was carried out using strains TA 98, 100, 1535 and 1537. There was no evidence of mutagenicity.

A test for capacity to induce chromosomal aberrations *in vitro* was carried out according to GLP. The cells used were cultures of Chinese hamster V79 cells. Metabolic activation was produced by the use of an "Aroclor"-induced rat liver preparation. Tests were carried out in duplicate. The highest concentration, according to the GLP protocol, should be about 10 mM, approximately 12.5 mg/ml and following preliminary tests this was taken as the top dose; however, 13.5 mg/ml was also found to be a usable concentration.

Preliminary testing used doses ranging from 0.1 to 5000 µg/ml; higher concentrations gave acceptable numbers of metaphase, and no undue inhibition of the mitotic index.

The cell cycle of the V79 cells was 13 to 14 hours; therefore, most of the harvests were carried out at 18 hours; a few were also carried out at 28 hours to ensure that aberrations induced during delayed cell cycles were not missed. Exposure to the active ingredient was 18 hours without activation, and 4 hours with activation. Positive controls were ethane methylsulphonate in the absence of activation, and cyclophosphamide in its presence.

In the first experiment, concentrations of active ingredient of 5, 7.5, 10 and 12.5 mg/ml were tested with and without the addition of the S-9 mix, and harvesting was at 18 hours. In the second experiment, without activation, doses of 10.5, 11.5, 12.5 and 13.5 mg/ml were tested; harvesting was at 18 hours. In the third experiment, (a) doses of active ingredient of 7.5, 10 and 12.5 mg/ml were tested, with and without activation, and an 18 hour harvest; and (b) doses of 10 and 12.5 mg/ml with and without activation were tested with a harvest at 28 hours.

Conventional fixation, and examination of metaphases, were carried out; 200 metaphases were counted for each of the test doses, and 100 for the positive controls.

Results: In general, there was no evidence of clastogenicity, and the findings were within the historical controls; the positive controls gave unequivocal results. However, in the first experiment there was a significant increase in aberrations exluding gaps (p less than 0.01) and in exchanges (p less than 0.1) at 12.5 mg/ml active ingredient without activation only; this is slightly outside the historical range for control cultures in the laboratory, but there is no other indication of abnormality in this experiment. In the third experiment, there was a significant increase (p less than 0.01) at 7.5 mg/ml active ingredient with activation. This value is within the historical control levels, and there is no dose relationship; its occurrence is thought to be due to the fact that the negative control in this experiment showed no aberrations, which is an unusual finding. For these reasons the authors do not regard these isolated findings as important, and they regard the results as negative.

Mouse: A micronucleus test was carried out according to GLP standards. The doses used were 2500, 5000 and 10000 mg/kg b.w., given orally. There was no evidence of clastogenic activity.

Tests for photomutagenic activity: A test was carried out according to GLP using the organism *E.coli WP2*. The active ingredient was dissolved in water to form a stock solution which was then filter sterilised; further dilutions were made as required. After exposure to the active ingredient was commenced, the cultures were incubated for 3 days. All tests were carried out in triplicate except for the negative control tests, which were carried out in quintuplicate.

The ultraviolet radiation was derived from an Osram "Vitalux" lamp; the dose of radiation was checked with an Osram "Centra" meter. The doses of ultraviolet radiation were: (UVA/UVB, mJ/cm^2) 5.3/1.7; 11.1/3.6; 230/0; 460/0 (the ultraviolet radiation of the last two exposures were filtered through glass, which cut off all radiation below about 320 nm). Negative and positive controls were included in all experiments: the negative controls were solvent and 8-methoxypsoralen without ultraviolet radiation; the positive controls were 8-methoxypsoralen with ultraviolet radiation and 4-nitroquiniline-1-oxide without ultraviolet radiation.

A range finding study was carried out using the doses (µg/plate) 0, 8, 40, 200, 1000 and 5000. No toxicity was found at any dose, nor any excess of revertants. The first definitive experiment was carried out using the same doses as in the range finding experiment: a second experiment, to study possible effects at higher doses, was carried out using concentrations (µg/plate) of 0, 1000, 2000, 3000, 4000 and 5000. In addition, a set of plates was treated with the active ingredient and kept in the dark throughout. The results showed that there was no evidence of any photomutagenic effect; the positive controls showed large increases in numbers of revertants, and the numbers of revertants in control and test plates were conconant with the historical controls of the laboratory. The test was negative.

A test for chromosomal aberration *in vitro* under the influence of ultraviolet radiation was carried out according to GLP, using Chinese hamster ovary cells in culture. The active ingredient was dissolved in water, and then filter sterilised, after which further dilutions were made as required. The positive controls were 4-nitroquinoline-1-oxide in the absence of light, and 8 methoxypsoralen in the presence of light. The negative controls were solvent and 8-methoxypsoralen in the absence of light. Activation was not used. Cells were cultured for 2 to 3 days before use. Exposure to ultraviolet radiation was carried out not less than 15 minutes or more than 2 hours after the addition of the

active ingredient or the control chemicals. After 2 hours the cultures were re-fed and cultured for a further 18 hours. Colchine was added $1^1/_2$ hours before harvest.

A range finding test, without ultraviolet radiation, was first carried out. The concentrations of active ingredient (µg/ml) were: 78.12; 156.2; 312.5; 625; 1250; 2500; 5000. There was no evidence of mitotic inhibition at any concentration, and 5000 µg/ml was selected as the top dose (although strictly this should have been a dose that caused some mitotic inhibition). The same doses of active ingredient were used for the main study. All cultures were carried out in duplicate.

The lamp used for the production of ultraviolet radiation was an Osram "Vitalux"; the intensity of the radiation was checked with an Osram "Centra" meter. The doses of ultraviolet radiation (mJ/cm^2) were: UVA, 200; UVB, 38; UVA filtered through glass. 700 (the ultraviolet radiation in the last case having a wavelength greater than 320 nm, approximately). Allowance was made for the absorptive properties of the flask material and the culture solution. In the main study, metaphases from the cultures containing the three highest concentrations of active ingredient only were examined. In this study, 200 metaphases were examined.

The results showed no significant increase in chromosomal aberrations in the test compared with the negative controls; the positive controls gave strongly positive findings. The level of aberrations in the test cultures was consonant with the historical negative control findings in the laboratory. The test was negative.

10. Special investigations

Phototoxicity.

Man: Ten subjects were used. Each had 3 applications made to the skin at discrete sites: 10 % aqueous solution of a.i.; 10 % solution of the di-isobutyl ester of diethylaminophthalate; and a control solution. The treated areas were exposed to UV radiation in a stepwise manner to determine the m.e.d. The two compounds were equiactive as sunsreens, and there was no evidence of phototoxicity. The report gives little detail.

11. Conclusions

The compound appears to have low acute and subchronic toxicity. It shows no evidence of being irritant to the skin or the mucous membranes, and tests for sensitization are negative.

A well conducted study in guinea pigs showed no evidence of photo sensitization or photoallergy. Experiments on phototoxicity are poorly reported, but seem to be negative. Tests for clastogenicity *in vivo* and *in vitro*, and for mutagenicity, photomutagenicity and photoclastogenicity *in vitro*, were negative. The method used for testing for teratogenic activity is not a validated one. A test for percutaneous absorption *ex vivo* showed a fairly rapid initial penetration followed by a slow penetration over 72 hours. Over 72 hours, the amount absorbed is found to be about 0.9 % of the amount applied.

Classification: 1

12. Safety evaluation

See next page.

CALCULATION OF SAFETY MARGIN

ETHOXYLATED ETHYL-4-AMINOBENZOATE

S 3

Based on a usage volume of 18 g, containing at maximum 10 %

Maximum amount of ingredient applied:	**I (mg) = 1800 mg**
Typical body weight of human:	**60 kg**
Maximum absorption through the skin:	**A (%) = 0.9 %**
Dermal absorption per treatment:	**I (mg) x A (%) = 1800 mg x 0.9 % = 16.2 mg**
Systemic exposure dose (SED):	**SED (mg)= I (mg) x A (%) / 60 kg = 1800 mg x 0.9 % / 60 kg = 0.27 mg/kg b.w.**
No observed adverse effect level (mg/kg): (rat, oral)	**NOAEL = 1200 mg/kg b.w.**

Margin of Safety: NOAEL / SED = 1200 mg/kg b.w. / 0.27 mg/kg b.w. = 4400

European Commission

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County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 8:12 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Octinoxate HEL Monograph - 9 of 9
Attachments: 79 Kayoko et al 1989 Enhancing effects of cinoxate and methyl sinapate on the frequencies of sister-chromatid exchanges and chromosome aberrations in cultured mammalian cells.docx; 80 Alamar 2017 JAT UV filters migration.pdf; 81 Paredes et al 2014 Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels Isochrysis galbana, Mytilus galloprovincialis.docx; 82 Sanchez Rodrigues et al 2015 Occurrence of eight UV filters in beaches of Gran Canaria Canary Islands. An approach to environmental risk assessment.docx; 83 Sieratowicz et al, 2011. Acute and chronic toxicity of four frequently used UV filter substances for Desmodesmus subspicatus and Daphnia magna.docx; 84 Park et al 2017 Single- and mixture toxicity of three organic UV-filters, ethylhexyl methoxycinnamate, octocrylene, and avobenzone on Daphnia magna.docx; 85 Rehfeld et al Chemical UV Filters Mimic ... Sperm Cells.pdf; 86 Rainieri et al 2016 Occurrence and toxicity of musks and UV filters in the marine environment.docx; 87 Fent et al 2010 Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland.docx

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:
- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

79) Mutat Res. 1989 Jun;212(2):213-21.

Enhancing effects of cinoxate and methyl sinapate on the frequencies of sister-chromatid exchanges and chromosome aberrations in cultured mammalian cells.

Shimoi K1, Nakamura Y, Noro T, Tomita I, Sasaki YF, Imanishi H, Matsumoto K, Shirasu Y.
Author information
1 Laboratory of Health Science, School of Pharmaceutical Sciences, University of Shizuoka, Japan.

Abstract
Sister-chromatid exchanges (SCEs) induced by mitomycin C (MMC), 4-nitroquinoline-1-oxide (4NQO) or UV-light in cultured Chinese hamster ovary cells (CHO K-1 cells) were enhanced by cinoxate (2-ethoxyethyl p-methoxycinnamate) or methyl sinapate (methyl 3,5-dimethoxy 4-hydroxycinnamate). Both substances are cinnamate derivatives and cinoxate is commonly used as a cosmetic UV absorber. Methyl sinapate also increased the frequency of cells with chromosome aberrations in the CHO K-1 cells treated with MMC, 4NQO or UV. These increasing effects of methyl sinapate were critical in the G1 phase of the cell cycle and the decline of the frequencies of UV-induced SCEs and chromosome aberrations during liquid holding was not seen in the presence of methyl sinapate. Both compounds were, however, ineffective in cells treated with X-rays. In cells from a normal human embryo and from a xeroderma pigmentosum (XP) patient, MMC-induced SCEs were also increased by the post-treatment with methyl sinapate. The SCE frequencies in UV-irradiated normal human cells were elevated by methyl sinapate, but no SCE-enhancing effects were observed in UV-irradiated XP cells. Our results suggest that the test substances inhibit DNA excision repair and that the increase in the amount of unrepaired DNA damage might cause the enhancement of induced SCEs and chromosome aberrations.

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RESEARCH ARTICLE

Effects of exposure to six chemical ultraviolet filters commonly used in personal care products on motility of MCF-7 and MDA-MB-231 human breast cancer cells in vitro

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Breast Cancer Research Trust; University of Hafr Albatin (Saudi Arabian Cultural Bureau)

Abstract

Benzophenone (BP)-1, BP-2, BP-3, octylmethoxycinnamate (OMC), 4-methylbenzilidenecamphor and homosalate are added to personal care products to absorb ultraviolet light. Their presence in human milk and their oestrogenic activity suggests a potential to influence breast cancer development. As metastatic tumour spread is the main cause of breast cancer mortality, we have investigated the effects of these compounds on migration and invasion of human breast cancer cell lines. Increased motility of oestrogen-responsive MCF-7 human breast cancer cells was observed after long-term exposure (>20 weeks) to each of the six compounds at $\geq 10^{-7}$ M concentrations using three independent assay systems (scratch assay, live cell imaging, xCELLigence technology) and increased invasive activity was observed through matrigel using the xCELLigence system. Increased motility of oestrogen-unresponsive MDA-MB-231 human breast cancer cells was observed after 15 weeks of exposure to each of the six compounds by live cell imaging and xCELLigence technology, implying the increased migratory activity was not confined to oestrogen-responsive cells. Molecular mechanisms varied between compounds and cell lines. Using MCF-7 cells, reduction in E-cadherin was observed following 24 weeks' exposure to 10^{-5} M BP-1 and 10^{-5} M homosalate, and reduction in β-catenin was noted following 24 weeks' exposure to 10^{-5} M OMC. Using MDA-MB-231 cells, increased levels of matrix metalloproteinase 2 were observed after 15 weeks exposure to 10^{-7} M OMC and 10^{-7} M 4-methylbenzilidenecamphor. Although molecular mechanisms differ, these results demonstrate that exposure to any of these six compounds can increase migration and invasion of human breast cancer cells.

KEYWORDS

Benzophenone, breast cancer, homosalate, invasion, metastasis, methylbenzilidenecamphor, migration, motility, octylmethoxycinnamate, sunscreen, UV filter, xenoestrogen

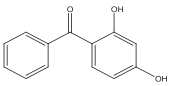
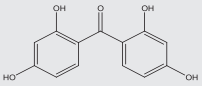
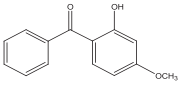
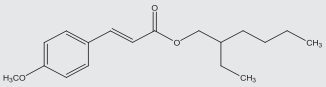
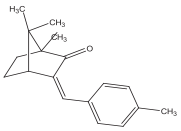
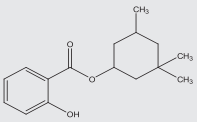
1 | INTRODUCTION

The human population is widely exposed to ultraviolet (UV) filters through their use in personal care products to protect the skin from damage by UV light. In excess of 50 different compounds are approved for use, including benzophenone (BP)-1, BP-2, BP-3, octylmethoxycinnamate (OMC), 4-methylbenzilidenecamphor (4-MBC) and homosalate (HS) (Shaath, 2010) (Table 1). Extensive use of these compounds in consumer products has resulted in measurable levels in waste water (Ramos, Homem, Alves, & Santos, 2016) and, due to their stability, accumulation in the environment in water and soil (Montes-Grajales, Fennix-Agudelo, & Miranda-Castro, 2017; Ramos, Homem, Alves, & Santos, 2015) and in tissues of aquatic organisms (Rainieri, Barranco, Primec, & Langerholc, 2016). Although BP-1 is used less extensively than other UV filters in consumer products (Shaath, 2010) (Table 1), its presence in environmental samples is considered to relate to it being a main metabolite of BP-3 (Kim & Choi, 2014).

Human exposure is mainly from topical application of personal care products, and rapid dermal absorption of BP-3, OMC and 4-MBC has been demonstrated in humans (Janjua et al., 2004; Janjua, Kongshoj, Andersson, & Wulf, 2008). Following whole body topical application of 2 mg cm^{-2} of a formulation with 10% of each sunscreen,

Competing financial interests: The authors have no competing financial interests.

TABLE 1 Chemical structures and chemical abstracts service (CAS) registry numbers for the six chemical ultraviolet filters, which were used in this study

Ultraviolet filter	Chemical name	Chemical structure	Approval for use[a]
Benzophenone-1	2,4-Dihydroxy-benzophenone (CAS 131–56-6)		Japan, South Africa
Benzophenone-2	2,2′,4,4′-Tetrahydroxy-benzophenone (CAS 131–55-5)		Australia, Japan, New Zealand, South Africa
Benzophenone-3	2-Hydroxy-4-methoxy-benzophenone (CAS 131–57-7)		Australia, Canada, European Union, Japan, New Zealand, South Africa, USA
Octyl methoxycinnamate	2-Ethylhexyl-4-methoxycinnamate (CAS 5466–77-3)		Australia, Canada, European Union, Japan, New Zealand, South Africa, USA
3-(4-Methylbenzilidene) camphor	1,7,7-Trimethyl-3-[(4-methylphenyl)methylene]-bicyclo[2,2,1]heptan-2-one (CAS 36861–47-9)		Australia, Canada, European Union, New Zealand, South Africa
Homosalate	2-Hydroxybenzoic acid 3,3,5-trimethylcyclohexyl ester (CAS 118–56-9)		Australia, Canada, European Union, Japan, New Zealand, South Africa, USA

[a]Approval for use as published (Shaath, 2010).

Janjua and colleagues measured dermal uptake of BP-3, OMC and 4-MBC within 1 hour into blood, with maximal levels of 238 ng ml^{-1} BP-3, 16 ng ml^{-1} OMC and 18 ng ml^{-1} 4-MBC after 3 hours: levels of 81 ng ml^{-1} BP-3, 4 ng ml^{-1} 4MBC and 4 ng ml^{-1} OMC were measured after 24 hours in urine (Janjua et al., 2008). Although the source cannot be identified, these compounds are also now widely measurable in human tissues. BP-3 has been measured in ≥95% of human urine samples from the USA (Calafat, Wong, Ye, Reidy, & Needham, 2008; Mortensen et al., 2014), from Belgium (Dewalque, Pirard, Dubois, & Charlier, 2014) and from Puerto Rico (Meeker et al., 2013). BP-1 is found widely in human urine samples due to it being a metabolite of BP-3 (Kunisue et al., 2012). BP-1 has also been detected in all assayed samples of human placenta (Valle-Sistac et al., 2016). In human milk, BP-3, OMC, 4-MBC and HS have been measured at mean values of 52.2, 27.5, 22.1 and 29.4 ng g^{-1} lipid respectively (Schlumpf et al., 2010), and BP-1 has been reported at 0.6 ng ml^{-1} (Rodriguez-Gomez, Zafra-Gomez, Dorival-Garcia, Ballesteros, & Navalon, 2015), which demonstrates their presence in the human breast.

Over recent decades, many environmental chemicals have been shown to possess endocrine disrupting activity (Darbre, 2015), and BP-1, BP-2, BP-3, OMC, 4-MBC and HS have all been shown to possess some degree of oestrogenic activity in assays in vitro and in animal models in vivo (Krause et al., 2012; Schlumpf et al., 2001). All six of these UV filters have been reported to stimulate proliferation of oestrogen-responsive MCF-7 human breast cancer cells in culture (Schlumpf et al., 2001; Schlumpf et al., 2004). Using the in vivo rodent uterotropic assay, increased uterine weight in the prepubertal uterus was reported after exposure to 4-MBC (Schlumpf et al., 2001; Tinwell et al., 2002) and after exposure to BP-1, BP-2, BP-3 and OMC (Schlumpf et al., 2004).

As exposure to oestrogens is an established risk factor for breast cancer (Miller, 1996), it has been suggested that environmental oestrogenic chemicals may also contribute to breast cancer development if they are present in human breast tissue (Darbre, 2015). In addition to the ability of oestrogens to drive sustained proliferation of oestrogen-responsive human breast cancer cells, oestrogens can also act to increase their migratory and invasive activity (Darbre, 2012), which are characteristics essential for the metastatic process (Scheel & Weinberg, 2012). This is significant in the context of breast cancer where the main cause of mortality arises from growth of metastatic tumours (Miller, 1996). Previous work with components of cosmetics has shown that parabens (alkyl esters of *p*-hydroxybenzoic acid), which are measurable in human breast tissue (Barr, Metaxas, Harbach, Savoy, & Darbre, 2012) and which can increase proliferation of oestrogen-responsive cells at tissue concentrations (Charles & Darbre, 2013), can also act to increase migratory and invasive activity of human breast cancer cells (Khanna, Dash, & Darbre, 2014). Aluminium-based salts are used as antiperspirant in underarm cosmetics and aluminium has been measured in human breast tissue (Exley et al., 2007) and breast cyst fluid (Mannello, Tonti, & Darbre, 2009) at higher levels than in blood, and in nipple aspirate fluid at higher levels in samples taken from women with than without breast cancer (Mannello, Tonti, Medda, Simone, & Darbre, 2011). Aluminium is a metalloestrogen (Darbre, 2006) and has been shown to increase

migratory and invasive activity of human breast cancer cells, although as effects were found not only in oestrogen-responsive (Darbre, Bakir, & Iskakova, 2013) but also oestrogen-unresponsive (Bakir & Darbre, 2015) cells, oestrogen-independent mechanisms of action must also exist. In view of the presence of UV filters in human milk and of their intrinsic oestrogenic activity detailed above, we have investigated the effects of exposure to BP-1, BP-2, BP-3, OMC, 4-MBC and HS on migratory and invasive characteristics of oestrogen-responsive (MCF-7) and oestrogen-unresponsive (MDA-MB-231) human breast cancer cells.

2 | MATERIALS AND METHODS

2.1 | Materials

BP-1 (purity 99%), BP-2 (purity 97%), BP-3 (purity 98%), OMC (purity 98%), 4-MBC (purity 98%) and HS (99.9%) were purchased from Sigma-Aldrich (Dorset, UK). 17β-oestradiol was purchased from Steraloids (Croydon, UK).

2.2 | Stock culture of human breast cancer cells

MCF-7 human breast cancer cells were provided by Osborne, Hobbs, and Trent (1987) at passage number 390 from the laboratory of Charles McGrath. These cells remain dependent on oestrogen for proliferation when maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (containing phenol red) (Invitrogen, Paisley, UK) supplemented with 5% (*v*/v) foetal calf serum (FCS) (Invitrogen), 10 μg ml^{-1} insulin (Sigma-Aldrich) and 10^{-8} M 17β-oestradiol (Steraloids) in a humidified atmosphere of 10% CO_2 in air at 37°C (Darbre & Daly, 1989; Shaw, Sadler, Pugazhendhi, & Darbre, 2006). These cells have a classical epithelial-type morphology and proliferate with a sigmoid-shaped growth curve in monolayer culture, taking about 24 hours to adhere after replating and then going through approximately four doublings over a 7 day period in this stock culture medium. Subculturing of the cell stocks was therefore carried out at weekly intervals by suspension with 0.06% trypsin, 0.02% EDTA pH 7.3 and medium was replenished every 3–4 days. In practice, at the start of the week, cells were replated at a density of 0.2×10^5 cells ml^{-1} in 16 ml aliquots in 9 cm tissue culture dishes, medium was replenished after 3–4 days and the cells then subcultured to the same extent again after 7 days.

MDA-MB-231 human breast cancer cells were purchased from the American Tissue Culture Collection at passage number 28. These cells are not considered as responsive to oestrogen for growth (Garcia, DeRocq, Freiss, & Rochefort, 1992). The cells were maintained as monolayer cultures in DMEM (containing phenol red) (Invitrogen) supplemented with 10% (*v*/v) FCS (Invitrogen) in a humidified atmosphere of 10% carbon dioxide in air at 37°C. These cells have a more mesenchymal-type morphology than the MCF-7 cells but also proliferate in a sigmoid-shaped growth curve undergoing approximately four to five doublings over a 7 day period in this stock culture medium. Subculturing of the cell stocks was therefore carried out to the same timetable and extent as for the MCF-7 cells above.

2.3 | Long-term maintenance with ultraviolet filters

As MCF-7 cells are dependent on either oestradiol or insulin for their growth and either component can substitute for the other (Darbre, 2014), it is necessary to use oestrogen-depleted and insulin-depleted medium to study the effects of compounds with oestrogen-mimicking properties such as these UV filters (Schlumpf et al., 2001, 2004). As phenol red possesses oestrogenic activity (Berthois, Katzenellenbogen, & Katzenellenbogen, 1986), phenol-red-free medium is used. Serum is stripped with dextran-charcoal to deplete it of endogenous oestrogens and growth factors (Darbre, Yates, Curtis, & King, 1983). Long-term effects of UV filters were therefore carried out in MCF-7 cells maintained long-term in phenol-red-free DMEM (Invitrogen) containing 5% (*v*/v) dextran-charcoal-stripped FCS (DCFCS) with no further addition (control), with 10^{-8} M 17β-oestradiol added back (control), or with 10^{-7} or 10^{-5} M of each UV filter. UV filters were made as stocks in ethanol and diluted 1/10 000 (*v*/v) into culture medium. Controls contained the same volume of ethanol. Subculturing of the cell stocks was carried out at weekly intervals by suspension with 0.06% trypsin, 0.02% EDTA pH 7.3 and replating the cells at a density of 0.2×10^5 cells ml^{-1} in 16 ml aliquots in 9 cm tissue culture dishes, and medium was replenished after 3–4 days as described for the stock MCF-7 cells. As the growth rate of these stocks varied over time, stocks were maintained by replating weekly at this same density. Over the initial 10 week period, the growth rate depended on the oestrogenic activity of the compound (see Figure 5B) and shows highest growth for cells in the presence of 10^{-8} M oestradiol or 10^{-5} M BP-1 or 10^{-5} M BP-2, and lowest growth for cells in the absence of any addition or with BP-3 or OMC. However, over the longer term (10+ weeks) and in accordance with the wide literature on loss of oestrogen responsive growth in oestrogen-depleted medium (Darbre, 2012), cells without added oestradiol (Shaw et al., 2006) or oestrogenic compound (Khanna et al., 2014) gradually increased their growth eventually to reach the same growth rate without added oestrogen as was initially observed only for cells in the presence of oestradiol. Although the growth rates altered over time, consistency of maintenance was therefore achieved by replating weekly back to the same cell density.

As MDA-MB-231 cells are not considered as oestrogen-responsive (Garcia et al., 1992), these cells were maintained using the stock culture medium of DMEM (containing phenol red) supplemented with 10% (*v*/v) FCS with no further addition or with 10^{-7} M of each UV filter. As the proliferation rate of these cells remained constant throughout, subculturing of the cell stocks was carried out weekly as described above for the stock MDA-MB-231 cells.

2.4 | Scratch assay

For the scratch assay (Liang, Park, & Guan, 2007), cells were seeded in the relevant long-term maintenance medium containing the required concentration of oestradiol, UV filter or ethanol control in 2.5 ml aliquots into six-well tissue culture dishes (Nunc). When visibly confluent, scratches were made down the centre of each well using a sterile pipette tip and the medium was changed to contain mitomycin C (Merck, Darmstadt, Germany) (0.5 μg ml^{-1}) to inhibit cell proliferation. Photographs of the wounds were taken at marked positions after

0 and 24 hours at five positions per well for triplicate wells for each experiment. Analysis of scratch area was quantified using Image J software: the average value per well was calculated, then the average value for triplicate wells of each experiment, and results were collated as the mean ± SE for three independent cell culture experiments. Statistically significant differences were determined using a Student's *t*-test with unequal variances.

2.5 | Cell motility assay using live cell imaging

Cells were seeded at 0.2 × 10^5 cells ml^{-1} in the relevant long-term maintenance medium containing the required concentration of oestradiol, UV filter or ethanol control in 0.5 ml aliquots into 12-well tissue culture dishes. After 24 hours, the medium was changed and dishes were placed on a microscope stage in a chamber at 37°C in 5%CO_2/95%air. Cell migration was observed over time using a Nikon EclipseTE200 inverted microscope running NIS-elementsAR2.10 software and equipped with a digital camera. Images were captured every 15 minutes for 24 hours and analysed using Image J software. No mitomycin C was used for these experiments but any cell undergoing division during the 24 hour period was excluded from the assay. At least 10 cells per field of view were tracked for the entire period and the total length travelled by the cell from the start point to the end point (cumulative length) was calculated. This is not a measure of the distance from start to end but rather allows for the totality travelled by varying circuitous routes. For each field of view, the percentage of cells showing any motility versus stationery (% motility) was calculated. Results are presented as mean ± standard error (SE) of the average of all tracked cells per field of view for independent triplicate wells. Statistically significant differences were determined using a Student's *t*-test with unequal variances.

2.6 | MCF-7 cell migration and invasion using xCELLigence technology

The CIM-plate16 contains 16 wells, each a modified Boyden chamber, which can be used independently but simultaneously to measure cell migration in real-time through 8 μm pores of a polyethylene terephthalate membrane on to gold electrodes on the underside of the membrane using the xCELLigence system (ACEA Biosciences, San Diego, CA, USA). Experiments were set up according to the manufacturer's instructions with the membrane uncoated (migration) or coated with growth-factor-reduced-matrigel (invasion) (BD BioSciences, Oxford, UK) (20 μl 1:40 diluted matrigel per well on the upper surface). A chemotactic signal for movement was provided by inoculating the cells in serum-free medium in the upper chamber (phenol-red-free DMEM with relevant concentration of oestradiol, UV filter or ethanol control) and supplying 5% serum in the lower chamber (phenol-red-free DMEM/5%DCFCS with relevant concentration of oestradiol, UV filter or ethanol control). Cell index (electrical impedance) was monitored every 15 minutes for the duration of the experiment. Traces show the average of quadruplicate wells.

2.7 | Western immunoblotting

Cells were grown and lysates prepared as described previously (Shaw et al., 2006) using lysis buffer [50 mM Tris–HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.3% (*v*/*v*) Triton-X-100, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF 0.3 mM), leupeptin (10 μg ml^{-1}) and aprotonin (2 μg ml^{-1})]. Lysates were incubated on ice 30 minutes, passed through needles 19G–25G, run on 10% polyacrylamide-sodium dodecyl sulphate Bio-Rad stain-free gels (25 μg protein per track), and proteins transferred on to Bio-Rad PVDF membranes using the Bio-Rad Trans-Blot-Turbo semi-dry transfer system according to the manufacturer's instructions (Bio-Rad, Watford, UK). Membranes were blocked and immunoblotted as published (Shaw et al., 2006) but using Tris-buffered saline. Primary antibodies to E-cadherin and β-catenin (Cell Signaling, New England Biolabs, Hitchin, UK) were diluted 1:1000; primary antibody to β-actin (Cell Signaling) was diluted 1:5000; horseradish peroxidase-linked secondary antibodies (Cell Signaling) were diluted 1:2000. Bands were detected using enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions and quantitation was performed digitally using the GE ImageQuant LAS4000mini luminescent image analyser. Band signals were normalized relative to digitally quantified total protein using the Bio-Rad stain-free system according to manufacturer's instructions. All results show the average ± SE (*n* = 3) of biological replicates generated from three independent cell cultures and were analysed for statistical significance using a Student's *t*-test with unequal variances.

2.8 | Zymography

Cells were plated at a density of 12.8 × 10^5 cells per 9 cm culture dish (16 ml), and grown for 6 days in stock medium with or without the indicated concentration of oestradiol, UV filter or ethanol control. Medium was then replaced with serum-free medium (DMEM with relevant concentration of oestradiol, UV filter or ethanol control) (4 ml per 9 cm dish) and collected 24 hours later. Cells left on the dishes were counted using a Coulter counter (see below) to provide the cell density at the time of harvest. Conditioned media were concentrated 10-fold using Amicon 10 kDa cut-off spin filters (Merck, Darmstadt, Germany) and run on Bio-Rad 10% polyacrylamide-sodium dodecyl sulphate-gelatin zymogram gels (equivalent of conditioned medium from 10^5 cells per track). Gels were washed for 30 minutes at room temperature in Bio-Rad renaturation buffer, incubated overnight at 37°C in Bio-Rad developing buffer, stained for 30 minutes at room temperature with 0.5% (*w*/*v*) Coomassie Blue R-250 and destained in 4 × 30 minute washes in Bio-Rad destaining buffer. Areas of protease activity appeared as clear bands against a dark blue background and were quantified using the Bio-Rad Gel Doc Imager (white light plate). All results show the average ± SE (*n* = 3) of biological replicates generated from three independent cell cultures.

2.9 | Expression of ERE-LUC reporter gene in MCF-7 cells

This study used a clone of the MCF-7 cells as described above, which had been stably transfected with an oestrogen-inducible oestrogen response element (ERE)-LUC reporter gene (Shaw et al., 2006). The

reporter gene consisted of the ERE-containing nucleotide sequence (5′-CTAGAAAGTCAGGTCACAGTGACCTGATCAAT-3′) cloned into the multiple cloning site of the pGL3promoter vector containing the coding sequence for firefly luciferase (Promega, Southampton, Hants, UK). Transfected cells were selected as G418 sulphate resistant and stocks were maintained in the stock MCF-7 cell medium as above containing in addition to 100 μg ml^{-1} G418 sulphate (Invitrogen).

In the experimental assay of luciferase expression, cells were added to the required volume of phenol red-free DMEM containing 5% DCFCS at a concentration of 0.8×10^5 cells ml^{-1} and plated in monolayer in 0.5 ml aliquots into two replicate 24-well plastic tissue culture dishes. After 6 days in the oestrogen-depleted medium, the medium was changed to phenol red-free DMEM supplemented with 5% DCFCS with or without 17β-oestradiol or UV filter at the required concentrations giving three wells per treatment per plate. After 24 hours, one of the replicate dishes was used to count the cells (see below) and the other replicate dish was used for luciferase assays, which were performed using commercial kits according to the manufacturer's instructions (Promega). Results were calculated as average units of luminescence per 10 000 cells with an SE for triplicate wells.

2.10 | Assay of cell proliferation using a coulter counter

Cells were added to the required volume of phenol red-free DMEM containing 5% DCFCS at a concentration of 0.2×10^5 cells ml^{-1} and plated in a monolayer in 0.5 ml aliquots into 24-well plastic tissue culture dishes. After 24 hours, the medium was changed to phenol red-free DMEM supplemented with 5% DCFCS with or without 17β-oestradiol or UV filter as required. Culture medium was changed routinely every 3–4 days. Cell counts were performed by counting released nuclei on a model ZBI Coulter Counter, as described previously (Shaw et al., 2006). All cell counts were done in triplicate on triplicate wells. The doubling time of the cells was calculated from cell numbers in three wells at day 0 and three wells at the final time point as a function of the slope, m ($\log_{10}2/m$). The number of cell doublings was calculated as the mean ± SE of all nine values for triplicate estimates of cell numbers at day 0 and the final time point. P values were calculated using a two-tailed Student t-test, two sample assuming unequal variance.

3 | RESULTS

3.1 | Experimental strategy

The effects of exposure to BP-1, BP-2, BP-3, OMC, 4-MBC and HS were studied using MCF-7 and MDA-MB-231 human breast cancer cell lines on the basis that the MCF-7 cells are dependent on oestrogen for their proliferation (Darbre & Daly, 1989; Shaw et al., 2006) but MDA-MB-231 cells are unresponsive to oestrogen for their growth (Garcia et al., 1992). The effects were studied over a long-term period of weeks of exposure on the basis that the effects on migration and invasion were only noted previously in MCF-7 cells after long-term exposure to parabens (20 ± 2 weeks) (Khanna et al., 2014) or to aluminium (32–37 weeks) (Darbre et al., 2013) and in MDA-MB-231 cells to aluminium (20–25 weeks) (Bakir & Darbre, 2015). For the long-term exposures, separate stock cultures of MCF-7 or MDA-MB-231 cells were maintained under the same conditions to the same passage number in parallel with the difference only of addition or not of oestradiol or UV filter. Concentrations for study were chosen on the basis that 10^{-5} M was the concentration that gave the maximal proliferative response (Schlumpf et al., 2001) and 10^{-7} M was within the upper range of measurement in human milk (Schlumpf et al., 2010).

3.2 | Effect of ultraviolet filters on motility of MCF-7 cells as determined by scratch assay

The scratch assay (Liang et al., 2007) is a measure of collective migration of cells, which is calculated as a reduction in the scratch area with time as the cells move to fill in the space created by the scratch. Long-term exposure (21 weeks) of MCF-7 human breast cancer cells to 10^{-7} M concentrations of each of the six chemical UV filters was observed to increase scratch closure to a significantly ($P < 0.05$) greater extent than in unexposed cells cultured for the same length of time (Figure 1A) and images of one representative experiment are shown in Figure 1(B). Increase in exposure concentrations to 10^{-5} M further increased scratch closure for BP-3 and 4-MBC but not for the other chemicals (Figure 1A). In line with previous publications (Khanna et al., 2014), 10^{-8} M 17β-oestradiol did not increase scratch closure after long-term exposure (Figure 1A, track 2). Our previously published work with these MCF-7 cells has shown that over short periods of 7 days the cells give a greater scratch closure in the presence of 10^{-8} M 17β-oestradiol than in its absence, but after longer periods of 20 weeks growth with and without oestradiol, the scratch closure was no longer greater in the presence of oestradiol (Khanna et al., 2014).

Following a shorter exposure time of 7–8 weeks, increased scratch closure was noted but only after exposure to 10^{-5} M concentrations of the UV filters. Percentage of wound healing in the untreated cells was 14.3 ± 3.1, which increased after treatment to 39.3 ± 5.6 ($P = 0.03$) with BP-1, to 44.3 ± 7.6 ($P = 0.04$) with BP-2, to 30.6 ± 2.1 ($P = 0.02$) with OMC, to 47.0 ± 3.1 ($P = 0.002$) with 4-MBC, and to 37.1 ± 4.7 ($P = 0.03$) with HS. The smaller increase to 20.4 ± 1.3 with BP-3 did not reach significance ($P = 0.16$).

3.3 | Effect of ultraviolet filters on motility of MCF-7 cells as determined by live cell imaging

To confirm the results of the scratch assay, live cell imaging was used to measure movement of individual MCF-7 cells. This assay measures the total length (cumulative length) moved by each cell on its entire circuitous route from start to end over a 24 hour period. Average cumulative length moved by cells in 24 hours was increased significantly ($P < 0.05$) following 21 weeks of previous exposure to 10^{-7} M concentrations of BP-2, BP-3 and HS (Figure 2A). As MCF-7 cells are not very motile, a further assessment was carried out of the percentage of the cells that were motile compared to those that did not move during the 24 hour period. This showed a significant ($P < 0.05$) increase

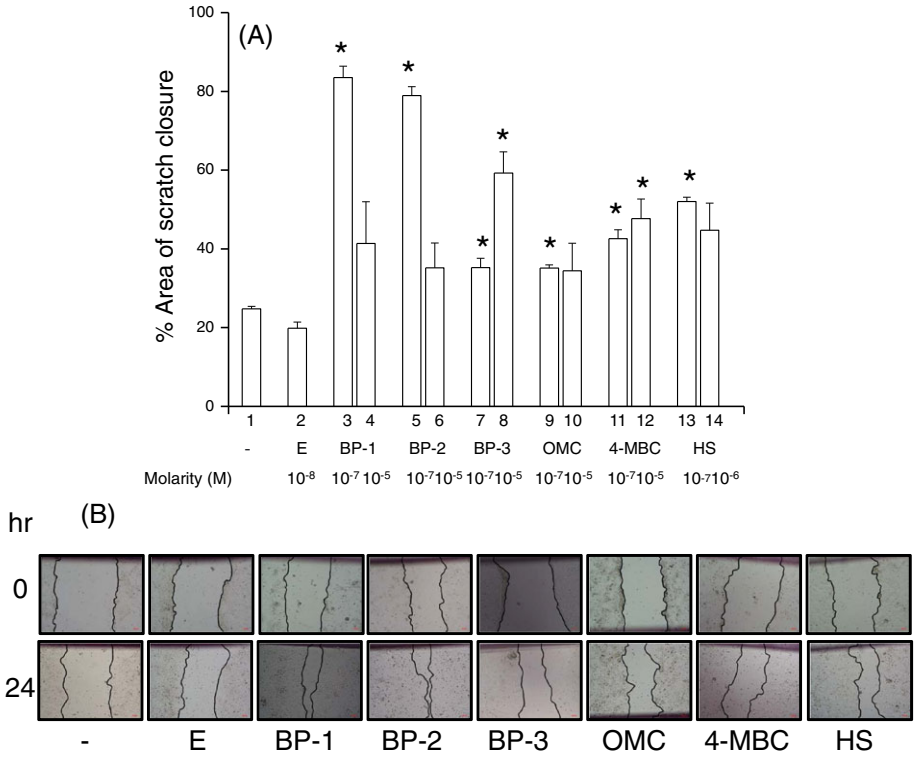


FIGURE 1 Effect of six chemical ultraviolet filters on motility of MCF-7 human breast cancer cells as determined using a scratch assay. No addition (–), 10^{-8} M 17β-oestradiol, 10^{-7} M, 10^{-6} M or 10^{-5} M of each ultraviolet filter as indicated after 21 weeks of exposure. (A) wound area was measured from 12 images at each time point (0, 24 hours): Calculated percentages of wound area (mean ± SE) are shown for three independent cell culture experiments. *$P < 0.05$ By *t*-test compared to control with no addition. (B) representative photographs of wound areas at 0 and 24 hours for no addition, 10^{-8} M 17β-oestradiol and 10^{-7} M concentrations of each of the six chemical ultraviolet filters. E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; OMC, octylmethoxycinnamate

in percentage motile cells following previous exposure to 10^{-7} M concentrations of BP-1, BP-2, BP-3, 4-MBC and HS (Figure 2B).

10^{-5} and 10^{-7} M concentrations of each UV filter were investigated on cumulative length and percentage motile cells after a shorter exposure time of 2 weeks. However, most of the effects were too small to reach significance and the only two conditions that reached a significant *P* value of <0.05 were for 10^{-5} M OMC where untreated cells gave percentage motile cells of 5.0 ± 2.1, which increased after treatment to 14.3 ± 1.2 ($P = 0.03$), and for 10^{-5} M 4-MBC where untreated cells gave a cumulative length of 19.5 ± 12.3, which increased after treatment to 75.5 ± 17.4.

3.4 | Effect of ultraviolet filters on migration and invasion of MCF-7 cells using xCELLigence technology

The effects on the migratory properties of MCF-7 cells were investigated using xCELLigence technology following exposure to 10^{-7}, 10^{-6} and 10^{-5} M concentrations of the chemical UV filters after 2, 10 and 23 weeks, and all results have been presented in a PhD thesis (Alamer, 2017). The effects after 23 weeks at 10^{-5} M concentrations are shown in Figure **3**(A). Time courses over 18 hours showed the rate of cell migration through uncoated membranes increased following previous exposure to the UV filters, with the greatest effect observed for OMC (Figure **3**A). The effects on the invasive properties of MCF-7 cells were studied using matrigel-coated membranes, and results following exposure to 10^{-5} M concentrations of the UV filters after 23 weeks are shown in Figure **3**(B). The rate of cell invasion was observed to increase most markedly following exposure to OMC and HS (Figure **3**B).

3.5 | Effect of ultraviolet filters on levels of E-cadherin and β-catenin in MCF-7 cells

One of the molecular mechanisms associated with increased cell motility is known to involve downregulation of the transmembrane glycoprotein E-cadherin followed by alterations to catenin signalling (Scheel & Weinberg, 2012). Therefore, western immunoblotting was used to investigate the effects of long-term exposure to UV filters on levels of E-cadherin or β-catenin in the MCF-7 cells using 10^{-5} M concentrations of the chemicals after a period of 24–25 weeks, a period similar to that where effects were seen on cell migration (Figures 1 and 3). E-cadherin and β-catenin were identified as bands of 135 and 92 kDa respectively on the polyacrylamide gel against molecular weight markers. Figure 4(B) shows that levels of E-cadherin were reduced in cells following 24–25 weeks of exposure to 10^{-5} M concentrations of BP-1 and HS. Figure 4(D) shows a reduction in levels of β-catenin following 24–25 weeks of exposure to 10^{-5} M OMC.

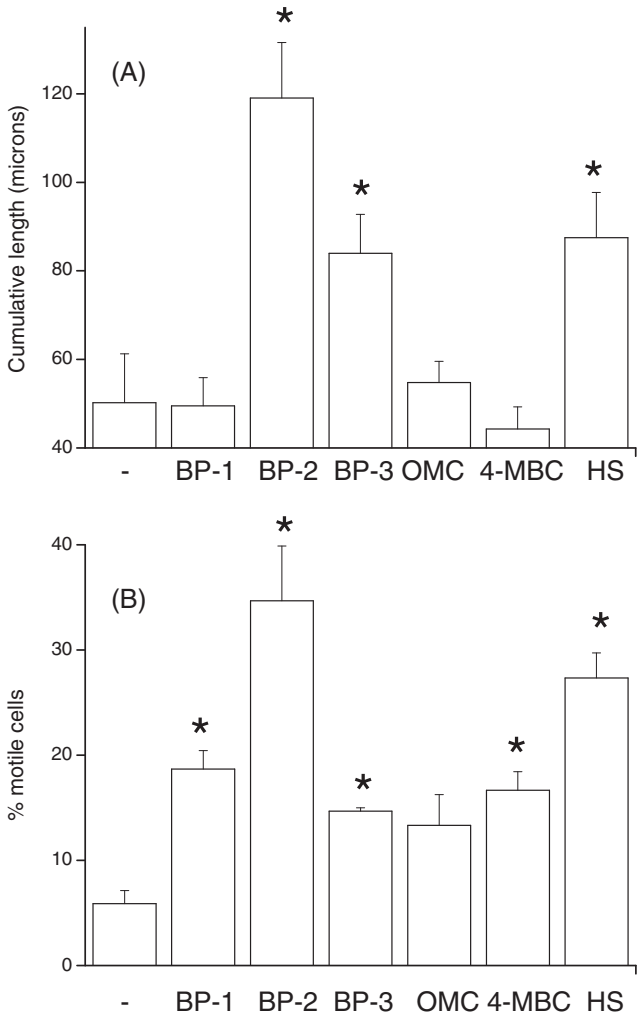


FIGURE 2 Effect of six chemical ultraviolet filters on motility of MCF-7 human breast cancer cells determined using live cell imaging. Results are presented as the cumulative length moved by individual cells (A) and percentage of cells showing any motility (B). No addition (−), 10^{-7} M of each ultraviolet filter as indicated after 21 weeks of exposure. For each experiment, cumulative length was measured for each of 10 cells per field of view over 24 hours for each of three wells per experiment (A) and for each field of view the percentage of cells showing any motility versus those stationery was measured (B): Results are shown (mean ± SE) for three independent experiments. *$P < 0.05$ By *t*-test compared to control with no addition. E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; OMC, octylmethoxycinnamate

3.6 | Effect of ultraviolet filters on oestrogen-regulated gene expression and proliferation in MCF-7 cells

Established protocols for identifying oestrogenic activity of a chemical include identifying the ability of the compound to bind to oestrogen receptors leading to effects on gene expression (genomic action) and increasing proliferation of a cell line (such as MCF-7), which is dependent on oestrogen for growth (Darbre, 2015). Although varying degrees of oestrogenic activity have been previously reported in MCF-7 cells for these six chemical UV filters using these assay protocols (Krause et al., 2012; Schlumpf et al., 2001), we felt it was important to confirm relative effects of these compounds using the MCF-7 cell line in our laboratory. For this, the relative effects of 10^{-7} and 10^{-5} M concentrations of each of the six chemical UV filters were investigated on expression of a stably transfected oestrogen-responsive ERE-LUC gene in the MCF-7 cells using the established protocol where cells are deprived of oestrogen for 6 days and then treated with the test compound for 24 hours (Darbre, 2015; Shaw et al., 2006). Effects were also investigated on proliferation of the oestrogen-responsive MCF-7 cells used in our laboratory over a 14 day established protocol (Darbre, 2015). All six UV filters were able to increase expression of the ERE-LUC gene at 10^{-5} M concentrations while BP-2, 4-MBC and HS were also effective at 10^{-7} M concentrations (Figure 5A). Proliferation of MCF-7 cells was increased by BP-1, BP-2, 4-MBC and HS in a dose-dependent manner for 10^{-7} and 10^{-5} M concentrations, while BP-3 and OMC had no effect at these same concentrations (Figure 5B).

3.7 | Effect of ultraviolet filters on migration of MDA-MB-231 cells

To determine whether UV filters could influence the motility of oestrogen unresponsive human breast cancer cells, the MDA-MB-231 cell line was used. This cell line has been reported as unresponsive to oestrogen for proliferation (Garcia et al., 1992) and we have never observed any increase in proliferation in response to 17β-oestradiol in these cells in our laboratory. The MDA-MB-231 cells are generally more motile than the MCF-7 cells, and using live cell imaging, overall cumulative length moved over a 24 hour period was observed to be greater in the MDA-MB-231 cells (Figure 6A) compared to the MCF-7 cells (Figure 2A). Following maintenance of the MDA-MB-231 cells as stock cultures with 10^{-7} M concentrations of the six UV filters, effects on cell motility were monitored using xCELLigence technology, and no alterations were observed after 1 or 2 weeks exposure: only after 8 weeks of maintenance were increases in cell migration found on the cell index traces (data not shown). Live cell imaging was performed after 15 weeks of exposure to 10^{-7} M concentrations of the six UV filters and results are shown in Figure 6(A). For each of the six UV filters, cumulative length moved by the cells was significantly increased compared to control unexposed cells maintained for the same length of time in culture ($P < 0.05$) (Figure 6A).

3.8 | Effect of ultraviolet filters on levels of matrix metalloproteinases in MDA-MB-231 cells

The MDA-MB-231 cells did not have E-cadherin that could be detected by western immunoblotting even following 15 weeks of exposure to the chemical UV filters. Therefore, in search of molecular mechanisms of the increase in cell motility, matrix metalloproteinases were considered. Increased production of matrix metalloproteinases (MMPs) may also be involved in increasing cell migration and invasion (Kessenbrock, Plaks, & Werb, 2010) and previous work has shown that long-term exposure of these cells to aluminium-based antiperspirant salts resulted in an increase in secretion of enzymatically active

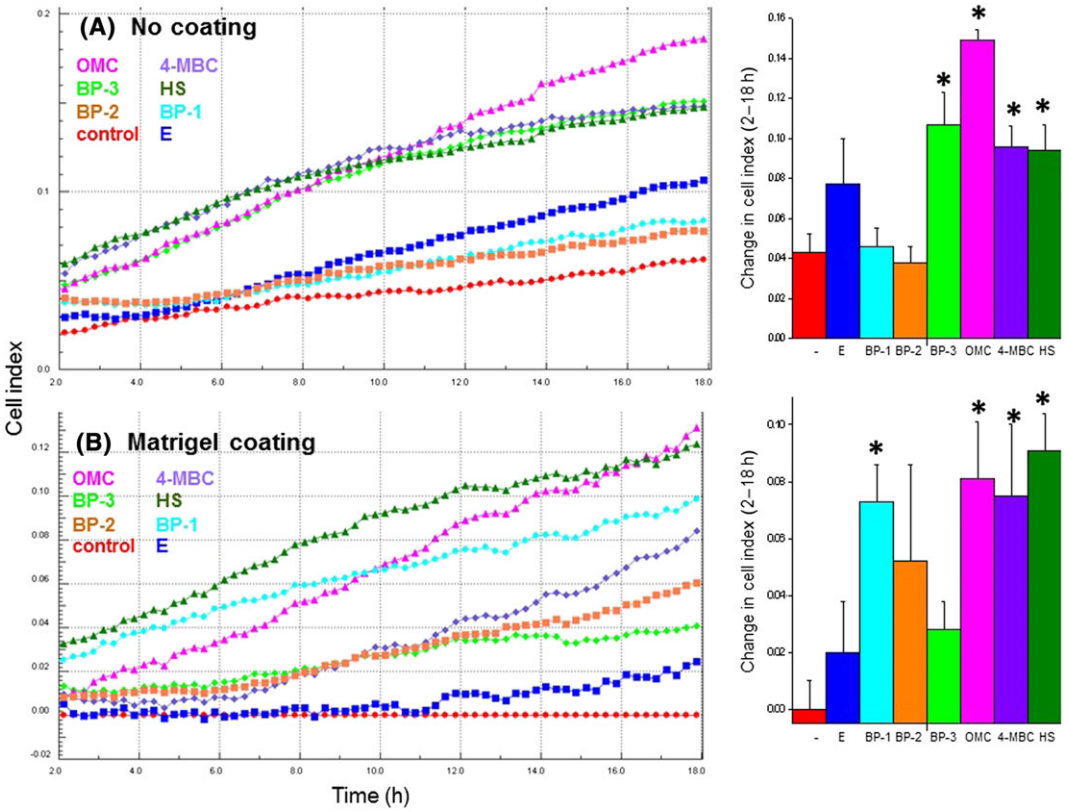


FIGURE 3 Effect of six chemical ultraviolet filters on migratory (A) and invasive (B) properties of MCF-7 human breast cancer cells as determined using xCELLigence technology with CIM-16 plates uncoated (A) or coated with matrigel (B). No addition (–), 10^{-8} M 17β-oestradiol, 10^{-5} M of each ultraviolet filter as indicated after 23 weeks of exposure. Results are presented on the left as the trace over time (2–18 h) for the average of quadruplicate wells, and on the right as bar charts showing the mean ± SE for the change in cell index from 2 to 18 hours for the quadruplicate wells. E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; OMC, octylmethoxycinnamate

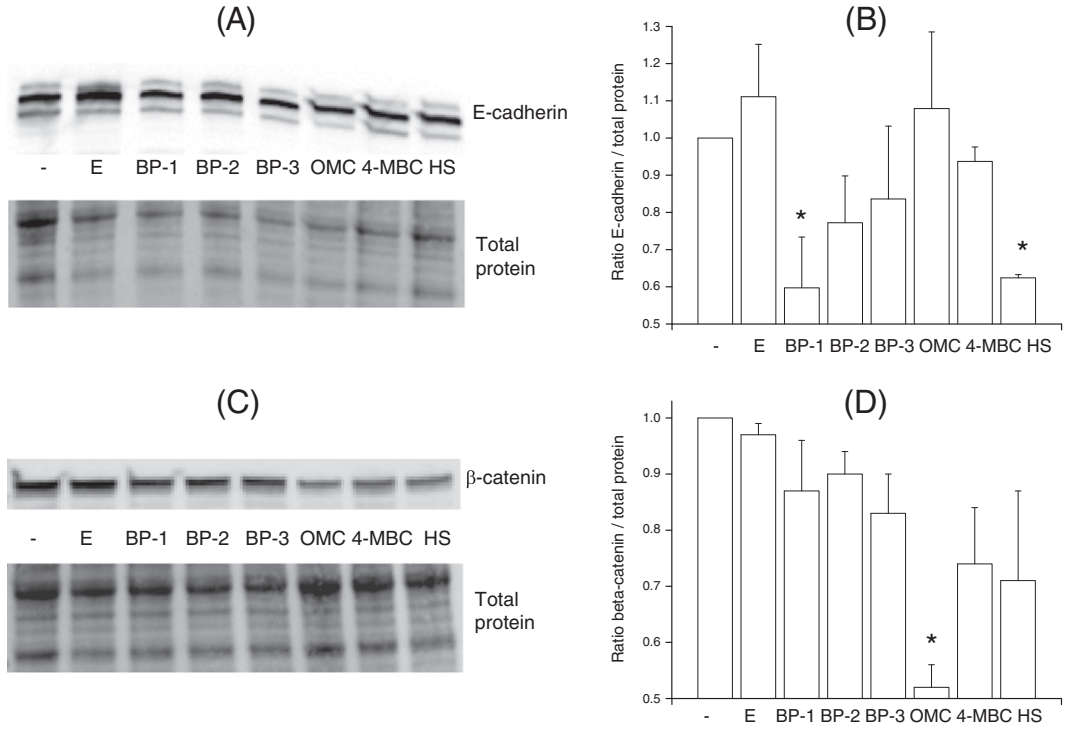


FIGURE 4 Levels of E-cadherin (A,B) and β-catenin (C,D) after exposure to six chemical ultraviolet filters for 24–25 weeks in MCF-7 human breast cancer cells as determined using western immunoblotting. No addition (–), 10^{-8} M 17β-oestradiol, 10^{-5} M of each ultraviolet screen as indicated. Immunoblots show one representative experiment (A,C); bar charts show calculated mean ratios of band intensity normalized to total protein ± SE for three independent cell culture experiments (B,D). E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; OMC, octylmethoxycinnamate

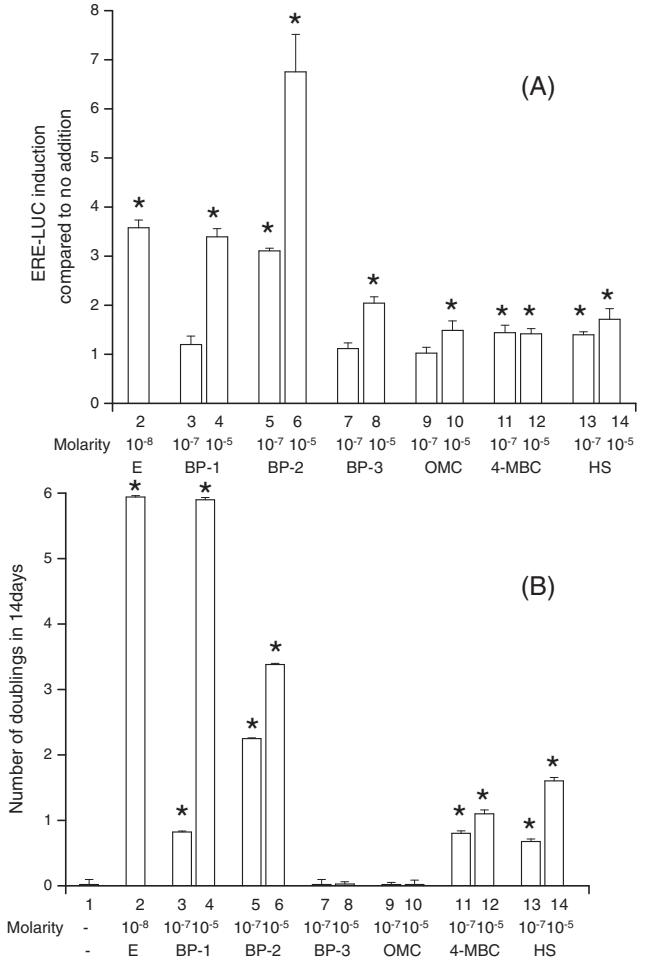


FIGURE 5 Oestrogenic activity of six chemical ultraviolet filters: Effects on luciferase gene expression from a stably transfected ERE-LUC gene (A) and proliferation (B) in MCF-7 human breast cancer cells. (A) cells were maintained in phenol-red-free Dulbecco's modified Eagle's medium/5% dextran-charcoal-stripped foetal calf serum for 6 days followed by treatment for 24 hours with the same medium containing no addition (−), 10^{-8} M 17β-oestradiol, 10^{-7} M or 10^{-5} M of each ultraviolet filter as indicated. Results are presented as a ratio to the control without any addition. (B) cells were grown for 14 days in phenol-red-free Dulbecco's modified Eagle's medium/5% dextran-charcoal-stripped foetal calf serum with no addition (−), 10^{-8} M 17β-oestradiol, 10^{-7} M or 10^{-5} M of each ultraviolet filter as indicated. Results are presented as the number of doublings in 14 days calculated from all nine values for triplicate wells of cell numbers at day 0 and 14. Error bars are the standard error of triplicate wells. *$P < 0.05$ compared to control with no addition. E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; OMC, octylmethoxycinnamate

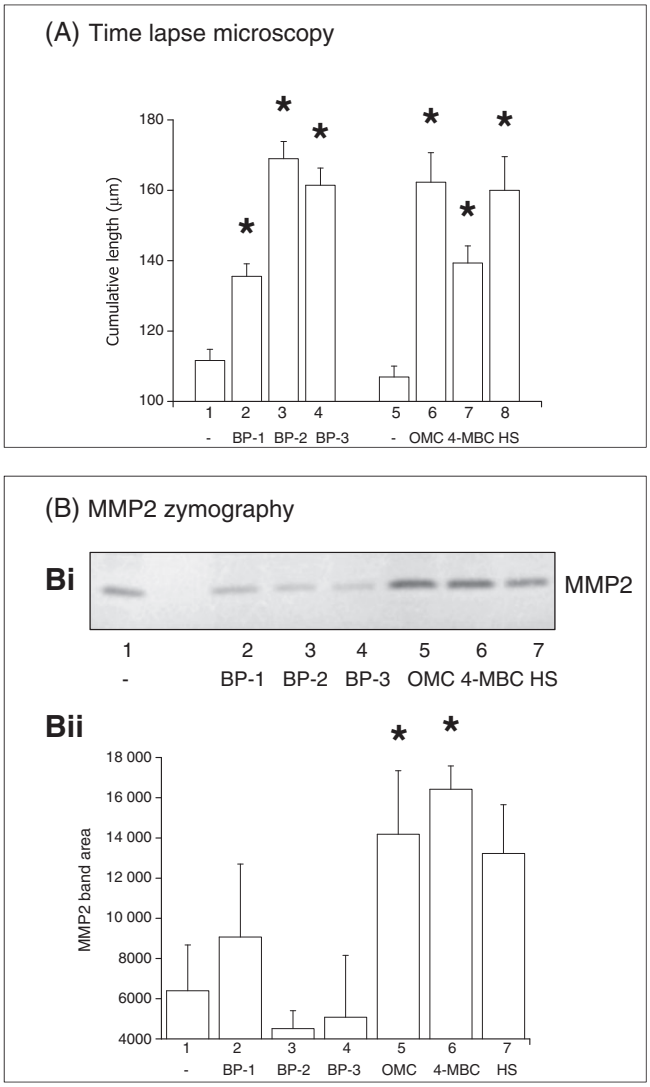


FIGURE 6 Effects of six chemical ultraviolet filters on motility of MDA-MB-231 human breast cancer cells as determined using live cell imaging (A) and alterations to MMPs as determined by zymography (B). No addition (−), 10^{-7} M of each ultraviolet filter as indicated after 15 weeks of exposure. (A) for each experiment, cumulative length was measured for each of 10 cells per field of view over 24 hours for each of three wells per experiment and results are shown (mean ± SE) for three independent experiments. (B, Bi) picture of one representative zymogram; (Bii) bar charts show calculated areas of the bands equivalent to conditioned medium from 10^5 cells, presented as mean ± SE for three independent cell culture experiments. *$P < 0.05$ *t*-test compared to control with no addition. E, 17β-oestradiol; BP, benzophenone; HS, homosalate; 4-MBC, 4-methylbenzilidenecamphor; MMPs, matrix metalloproteinases; OMC, octylmethoxycinnamate

MMP9 as determined by gelatin zymography. Gelatin zymography was used to identify secreted enzymatically active MMP2 and MMP9 as bands of 72 and 92 kDa respectively against coloured molecular weight markers. The zymography did not show any alterations to MMP9 (data not shown) but there was an increase in secretion of enzymatically active MMP2 following long-term exposure (15 weeks) to OMC, 4-MBC and HS at 10^{-7} M concentrations (Figure 6B).

4 | DISCUSSION

These results have shown that long-term exposure to the UV filters BP-1, BP-2, BP-3, OMC, 4-MBC or HS can increase the migratory and invasive properties of two human breast cancer cell lines in vitro. The MCF-7 cells possess intrinsic low motility, but increased motility was observed after exposure to the UV filters using three independent assay systems (scratch assay, live cell imaging and xCELLigence

technology) and increased invasive activity was demonstrated from the ability of the cells to invade through matrigel in the xCELLigence system. The MDA-MB-231 cells are generally more motile cells, but cell motility was further increased after exposure to the UV filters using two independent assay systems (live cell imaging and xCELLigence technology): scratch assays were not performed because these cells do not form an ordered monolayer. Therefore exposure to these UV filters resulted in increased motility of the breast cancer cells irrespective of the intrinsic motility of the cell line. Previous publications have reported an increased wound closure in a scratch assay following short times of exposure to 10^{-5} M BP-1 in MCF-7 cells (48–72 hours) (In, Kim, Go, Hwang, & Choi, 2015) and to 10^{-6} M BP-1 in ovarian cancer cells (48–96 hours) (Shin et al., 2016), but to our knowledge, this is the first time that BP-2, BP-3, OMC, 4-MBC and HS have been shown to increase cell migration and invasion. It is also the first report of effects after longer term exposures of weeks rather than hours, which is of environmental relevance where these compounds are present long-term in human tissues. Furthermore, from an environmental perspective, it is interesting that the lower concentrations of 10^{-7} M used in these studies took longer to generate an effect than the higher 10^{-5} M concentrations.

Increased migration was observed following exposure to these UV filters in both oestrogen responsive (MCF-7) and oestrogen unresponsive (MDA-MB-231) human breast cancer cells, implying that their ability to increase cell motility is not confined to oestrogen-responsive cells. Previous work has demonstrated that these six compounds all possess intrinsic oestrogenic activity (Krause et al., 2012; Schlumpf et al., 2001), and we have confirmed here that all six of these compounds can induce expression of an oestrogen-responsive ERE-LUC gene in the MCF-7 cells used in our laboratory. However, despite their intrinsic oestrogenic activity in gene expression assays, they were not all able to increase proliferation of MCF-7 cells and we did not observe any increase in MCF-7 cell proliferation in response to BP-3 or OMC. The intrinsic oestrogenic activity of a compound is therefore not always correlated with a proliferative response in the MCF-7 cells, and furthermore, neither does the proliferative response appear to be necessarily correlated with motility response, despite the known ability of oestrogen to increase cell motility (Li, et al., 2010; Planas-Silva & Waltz, 2007; Sanchez et al., 2010). A similar disparity has been previously reported for aluminium salts, which despite their metalloestrogenic activity (Darbre, 2006), also increased motility of the oestrogen unresponsive (MDA-MB-231) cells (Bakir & Darbre, 2015) as well as the oestrogen-responsive (MCF-7) cells (Darbre et al., 2013).

Many molecular mechanisms have been implicated in increased migration and motility of cells. One mechanism is through epithelial to mesenchymal transition and this has been associated with downregulation of E-cadherin followed by alterations to catenin signalling (Scheel & Weinberg, 2012). In these studies, reduction in levels of E-cadherin were observed in the MCF-7 cells following long-term exposure to BP-1 and HS, and reduction in β-catenin levels after exposure to OMC, which suggests that molecular mechanisms of action of these six compounds differ. MMPs are another mechanism by which cancer cells may achieve the matrix degradation necessary for cell migration and invasion (Kessenbrock et al., 2010), and in the MDA-MB-231 cells, increased secretion of MMP2 was observed after long-term exposure to OMC and 4-MBC. There is no a priori reason to expect these different compounds to act by similar mechanisms or indeed to act by similar mechanisms in different breast cell lines, and their combined presence in human milk may be indicative of a more complex set of interactions on human breast epithelial cells in vivo. Previous work on other cosmetic chemicals has demonstrated that reduced E-cadherin levels were associated with increased motility of MCF-7 cells following exposure to parabens (Khanna et al., 2014) but not after exposure to aluminium salts (Darbre et al., 2013). Using MDA-MB-231 cells, aluminium was found to increase levels of MMP9 and MMP14 (Bakir & Darbre, 2015) rather than MMP2 as found here for OMC and 4-MBC. As both parabens (Barr et al., 2012) and aluminium (Exley et al., 2007) have been measured in human breast tissue, this implies again a potential for complex interactions of mixtures of cosmetic chemicals in the human breast in vivo.

In the absence of any published measurements of these UV filters in human breast tissue, physiological relevance can only be assessed by comparison to measurements in human milk (Schlumpf et al., 2010). Mean and range values in terms of ng g^{-1} lipid in milk have been published by Schlumpf et al. (2010) as 52.2 (range 7.3–121.4) for BP-3, 27.5 (range 2.1–79.9) for OMC, 22.1 (range 6.7–48.4) for 4-MBC and 29.4 (range 11.4–61.2) for HS. Assuming that human milk has 3–5% fat by weight, an average value of 4% fat by weight would imply 1 g of lipid is dispersed in a 25 ml volume of milk. Based on this, the values reported in human milk equate to BP-3 of 0.1–2.1 10^{-8} M, OMC of 0.03–1.0 × 10^{-8} M, 4-MBC of 0.1–0.8 × 10^{-8} M and HS of 0.2–0.9 × 10^{-8} M. Therefore, the studies carried out here at 10^{-7} M are close to concentrations that can be measured in human milk. Further studies at 10- and 100-fold lower concentrations would be useful. However, additive effects of the UV filters need also to be taken into consideration in assessing functional significance in vivo, because not one but five of these UV filters have been measured in human milk samples (see above), which opens the possibility that the UV filters could act together at lower concentrations than required for each individually as has been reported for effects of the parabens on cell proliferation (Charles & Darbre, 2013).

The acquisition of migratory and invasive activity by epithelial cells is one of the hallmarks of cancer and required for metastasis (Hanahan & Weinberg, 2011), and these results demonstrate that exposure to any one of these six UV filters at concentrations within levels measureable in human milk can enable this hallmark of cancer to develop in breast epithelial cells. It has been previously suggested that absorption of dermally applied cosmetic chemicals at low doses over the long term might contribute to the rising incidence of breast cancer (Darbre, 2001, 2003), and as mortality from breast cancer arises from metastatic cancers rather than growth of the primary tumour in the breast itself (Miller, 1996), the ability of exposure to cosmetic chemicals to increase cell motility is poignant. In total, 12 chemicals, namely five paraben esters (Khanna et al., 2014), aluminium chlorohydrate (Bakir & Darbre, 2015; Darbre et al., 2013) and six UV filters have all now been shown to increase motility of breast cells individually at concentrations measurable in the human breast. Studies are now needed to investigate the effects of mixtures of these chemicals and whether there might be additive effects enabling each individual

chemical to act at lower concentrations when part of a mixture. In view of the results here showing effects developing more quickly at the higher 10^{-5} M concentrations than the lower 10^{-7} M concentrations, studies are now needed to investigate the effects of mixing low doses over the long term. Evidence has been recently reviewed showing the potential for low doses of environmental chemicals to impact over the long term on cancer development (Goodson et al., 2015), and if cosmetic chemicals can act in this way, then reduction in exposure to cosmetic products could offer a preventative strategy against breast cancer by reducing chemical pollutant burdens in the human breast.

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CONFLICT OF INTEREST

The authors did not report any conflict of interest.

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81) Chemosphere. 2014 Jun;104:44-50. doi: 10.1016/j.chemosphere.2013.10.053. Epub 2013 Dec 19.

Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels Isochrysis galbana, Mytilus galloprovincialis, Paracentrotus lividus, and Siriella armata.

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Abstract
Due to the concern about the negative effects of exposure to sunlight, combinations of UV filters like 4-Methylbenzylidene-camphor (4-MBC), Benzophenone-3 (BP-3), Benzophenone-4 (BP-4) and 2-Ethylhexyl-4-methoxycinnamate (EHMC) are being introduced in all kind of cosmetic formulas. These chemicals are acquiring a concerning status due to their increasingly common use and the potential risk for the environment. The aim of this study is to assess the behaviour of these compounds in seawater, the toxicity to marine organisms from three trophic levels including autotrophs (Isochrysis galbana), herbivores (Mytilus galloprovincialis and Paracentrotus lividus) and carnivores (Siriella armata), and set a preliminary assessment of potential ecological risk of UV filters in coastal ecosystems. In general, EC50 results show that both EHMC and 4-MBC are the most toxic for our test species, followed by BP-3 and finally BP-4. The most affected species by the presence of these UV filters are the microalgae I. galbana, which showed toxicity thresholds in the range of μg L(-1) units, followed by S. armata>P. Lividus>M. galloprovincialis. The UV filter concentrations measured in the sampled beach water were in the range of tens or even hundreds of ng L(-1). The resulting risk quotients showed appreciable environmental risk in coastal environments for BP-3 and 4-MBC.

82) Chemosphere. 2015 Jul;131:85-90. doi: 10.1016/j.chemosphere.2015.02.054. Epub 2015 Mar 17.

Occurrence of eight UV filters in beaches of Gran Canaria (Canary Islands). An approach to environmental risk assessment.

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Abstract
Due to the growing concern about human health effects of ultraviolet (UV) radiation, the use of UV filters has increased in recent decades. Unfortunately, some common UV filters are bioaccumulated in aquatic organisms and show a potential for estrogenic activity. The aim of the present study is to determine the presence of some UV filters in the coastal waters of six beaches around Gran Canaria Island as consequence of recreational seaside activities. Eight commonly used UV filters: benzophenone-3 (BP-3), octocrylene (OC), octyl-dimethyl-PABA (OD-PABA), ethylhexyl methoxy cinnamate (EHMC), homosalate (HMS), butyl methoxydibenzoyl methane (BMDBM), 4-methylbenzylidene camphor (4-MBC) and diethylamino hydroxybenzoyl hexyl benzoate (DHHB), were monitored and, with the exception of OD-PABA, all were detected in the samples collected. 99% of the samples showed some UV filters and concentration levels reached up to 3316.7 ng/L for BP-3. Environmental risk assessment (ERA) approach showed risk quotients (RQ) higher than 10, which means that there is a significant potential for adverse effects, for 4-MBC and EHMC for those samples with highest levels of UV filters.

83) J Environ Sci Health A Tox Hazard Subst Environ Eng. 2011;46(12):1311-9. doi: 10.1080/10934529.2011.602936.

Acute and chronic toxicity of four frequently used UV filter substances for Desmodesmus subspicatus and Daphnia magna.

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Abstract
As a consequence of growing public concern about UV radiation effects on human health chemical and physical UV filters are increasingly used in personal care and other products. The release of these lipophilic and often persistent compounds into surface waters may pose a risk for aquatic organisms. The aim of the study was to determine effects of four frequently used UV filters on primary aquatic producers and consumers, the green alga Desmodesmus subspicatus and the crustacean Daphnia magna. Exposure to benzophenone 3 (BP3), ethylhexyl methoxycinnamate (EHMC), 3-benzylidene camphor (3-BC) and 3-(4'-methylbenzylidene)-camphor (4-MBC) resulted in growth inhibition of D. subspicatus with 72 h IC(10) values of 0.56 mg/L (BP 3), 0.24 mg/L (EHMC), 0.27 mg/L (3-BC) and 0.21 mg/L (4-MBC). EC(50) concentrations in the acute test with D. magna were 1.67, 0.57, 3.61 and 0.80 mg/L for BP3, EHMC, 3-BC and 4-MBC, respectively. Chronic exposure of D. magna resulted in NOECs of 0.04 mg/L (EHMC) and 0.1 mg/L (3-BC and 4-MBC). BP 3 showed no effects on neonate production or the length of adults. Rapid dissipation of these substances from the water phase was observed indicating the need for more frequent test medium renewal in chronic tests or the use of flow-through test systems.

84) Ecotoxicol Environ Saf. 2017 Mar;137:57-63. doi: 10.1016/j.ecoenv.2016.11.017. Epub 2016 Dec 19.

Single- and mixture toxicity of three organic UV-filters, ethylhexyl methoxycinnamate, octocrylene, and avobenzone on Daphnia magna.

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Abstract
In freshwater environments, aquatic organisms are generally exposed to mixtures of various chemical substances. In this study, we tested the toxicity of three organic UV-filters (ethylhexyl methoxycinnamate, octocrylene, and avobenzone) to Daphnia magna in order to evaluate the combined toxicity of these substances when in they occur in a mixture. The values of effective concentrations (ECx) for each UV-filter were calculated by concentration-response curves; concentration-combinations of three different UV-filters in a mixture were determined by the fraction of components based on EC25 values predicted by concentration addition (CA) model. The interaction between the UV-filters were also assessed by model deviation ratio (MDR) using observed and predicted toxicity values obtained from mixture-exposure tests and CA model. The results from this study indicated that observed ECxmix (e.g., EC10mix, EC25mix, or EC50mix) values obtained from mixture-exposure tests were higher than predicted ECxmix (e.g., EC10mix, EC25mix, or EC50mix) values calculated by CA model. MDR values were also less than a factor of 1.0 in a mixtures of three different UV-filters. Based on these results, we suggest for the first time a reduction of toxic effects in the mixtures of three UV-filters, caused by antagonistic action of the components. Our findings from this study will provide important information for hazard or risk assessment of organic UV-filters, when they existed together in the aquatic environment. To better understand the mixture toxicity and the interaction of components in a mixture, further studies for various combinations of mixture components are also required.

Chemical UV Filters Mimic the Effect of Progesterone on Ca^{2+} Signaling in Human Sperm Cells

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Progesterone released by cumulus cells surrounding the egg induces a Ca^{2+} influx into human sperm cells via the cationic channel of sperm (CatSper) Ca^{2+} channel and controls multiple Ca^{2+}-dependent responses essential for fertilization. We hypothesized that chemical UV filters may mimic the physiological action of progesterone on CatSper, thus affecting Ca^{2+} signaling in human sperm cells. We examined 29 UV filters allowed in sunscreens in the United States and/or the European Union for their ability to induce Ca^{2+} signals in human sperm by applying measurements of the intracellular free Ca^{2+} concentration. We found that 13 UV filters induced a significant Ca^{2+} signal at 10 μM. Nine UV filters induced Ca^{2+} signals primarily by activating the CatSper channel. The UV filters 3-benzylidene camphor (3-BC) and benzylidene camphor sulfonic acid competitively inhibited progesterone-induced Ca^{2+} signals. Dose-response relations for the UV filters showed that the Ca^{2+} signal-inducing effects began in the nanomolar-micromolar range. Single-cell Ca^{2+} measurements showed a Ca^{2+} signal-inducing effect of the most potent UV filter, 3-BC, at 10 nM. Finally, we demonstrated that the 13 UV filters acted additively in low-dose mixtures to induce Ca^{2+} signals. In conclusion, 13 of 29 examined UV filters (44%) induced Ca^{2+} signals in human sperm. Nine UV filters primarily activated CatSper and thereby mimicked the effect of progesterone. The UV filters 3-BC and benzylidene camphor sulfonic acid competitively inhibited progesterone-induced Ca^{2+} signals. In vivo exposure studies are needed to investigate whether UV filter exposure affects human fertility. (***Endocrinology*** **157: 4297–4308, 2016)**

Human male infertility is widespread and fertility rates are declining globally (1). Sperm cell defects or dysfunction is a common cause of infertility, although the etiology in many cases is not known (1). The use of intracytoplasmic sperm injection, a method developed to treat male infertility due to poor sperm function, is rising both in the United States (2) and Europe (3).

Successful natural fertilization of the egg by a sperm cell depends on the precise control of diverse sperm functions during its journey through the female reproductive tract (4, 5). Almost every sperm function is controlled via the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), including sperm motility, chemotaxis, and acrosome reaction, and $[Ca^{2+}]_i$ also has important roles in sperm capacitation (4, 6), a maturation process during which sperm cells acquire the capacity to fertilize the egg. It is crucial that these individual $[Ca^{2+}]_i$-controlled sperm functions are triggered at the correct time and in the correct order for fertility to occur (4). In human sperm cells, the cationic channel of sperm (CatSper) channels located in the plasma membrane of the flagellum are the principal facilitators of channel-mediated Ca^{2+} influx (7). CatSper is activated by the natural ligands progesterone and prostaglandins (8, 9), leading to a rapid Ca^{2+}-influx into the sperm cell. Pro-

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Abbreviations: ABHD2, abhydrolase domain containing 2; AM, acetoxymethyl ester; BCECF, 2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein; BCSA, benzylidene camphor sulfonic acid; BP-3, benzophenone-3; CatSper, cationic channel of sperm; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DHHB, diethylamino hydroxybenzoyl hexyl benzoate; DMSO, dimethylsulfoxide; HTF^+, human tubular fluid; OD-PABA, ethylhexyl dimethyl PABA; 3-BC, 3-benzylidene camphor.

gesterone is released by the cumulus cells surrounding the egg, and the progesterone-induced Ca^{2+}-influx has been shown to mediate chemotaxis toward the egg (5, 10) to control sperm motility (11, 12) and to stimulate the acrosome reaction (13). Consequently, several authors have found that a suboptimal progesterone-induced Ca^{2+} influx is associated with reduced male fertility (14–19), in line with other studies demonstrating that CatSper function is absolutely essential for male fertility (18, 20–25).

Our recent pilot study showed that 33 of 96 diverse endocrine-disrupting chemicals induced Ca^{2+} signals in human sperm cells and that this affected sperm motility and acrosome reaction (26), raising the concern that exposure to endocrine-disrupting chemicals could adversely be affecting male fertility. Because some chemical UV filters were among the most potent Ca^{2+} signal-inducing chemicals in our pilot study (26) and because some chemical UV filters have been measured in body fluids after topical application (27), we decided to examine all chemical UV filters allowed in sunscreens in the European Union and/or the United States for their ability to induce Ca^{2+} signals in human sperm cells.

Materials and Methods

Reagents and chemical UV filters

Our objective was to test all 31 chemical UV filters allowed in sunscreens in the European Union and/or the United States. We obtained 30 of these chemical UV filters from various chemical providers: 4-methylbenzylidene camphor was purchased from Merck. 3-Benzylidene camphor was purchased from Induchem AG. Polysilicone-15 was purchased from VWR. EG-25 PABA was purchased from Boc Sciences. Benzylidene camphor sulfonic acid was ordered by custom synthesis from Life Chemicals. All remaining UV filters were purchased from Sigma-Aldrich. We were unable to obtain the UV filter polyacrylamidomethyl benzylidene camphor from any known distributor. The UV filters polysilicone-15, ethylhexyl triazone, drometrizole trisiloxane, and diethylamino hydroxybenzoyl hexyl benzoate were dissolved in ethanol at a concentration of 10 mM. Bis-ethylhexyloxyphenol methoxyphenyl triazine was dissolved in ethanol at a concentration of 1 mM. The UV filter methylene bis-benzotriazolyl tetramethylbutylphenol could not be dissolved in dimethylsulfoxide (DMSO) or ethanol. All remaining UV filters were dissolved in DMSO at a concentration of 10 mM. Progesterone, ionomycin, and EGTA were obtained from Sigma-Aldrich. MDL 12330A hydrochloride was purchased from Tocris R&D Systems. Fluo-4 acetoxymethyl ester (AM), fura-2 AM, and 2′,7′-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (BCECF) AM were purchased from Invitrogen. Human serum albumin was obtained from Irvine Scientific.

Semen samples

All semen samples were produced by masturbation and ejaculated into clean, wide-mouthed plastic containers on the same day as the experiment. After ejaculation, the samples were allowed to liquefy for 15–30 minutes at 37°C.

Purification of motile sperm cells via swim-up

Motile spermatozoa were recovered from raw ejaculates by swim-up separation in human tubular fluid (HTF^+) medium containing 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 4 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH as described elsewhere (26). After 1 hour at 37°C, the swim-up fraction was removed carefully and sperm concentration was determined by image cytometry as described in (28). After two washes, the sperm samples were adjusted to 10×10^6/mL in HTF^+ with human serum albumin (3 mg/mL). and the sperm cells were incubated for at least 1 hour at 37°C.

For the experiments with capacitated sperm cells, the semen samples were adjusted to 10×10^6/mL in a capacitating medium containing 72.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 25 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. Human serum albumin (3 mg/mL) was added to the capacitating medium, and the sperm cells were incubated for at least 3 hours at 37°C in a 10% CO_2 atmosphere.

Measurement of changes in $[Ca^{2+}]_i$

Changes in the free $[Ca^{2+}]_i$ in human sperm cells were measured in 384 multiwell plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech) at 30°C as reported elsewhere (26). Sperm cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 (10 μM) for 45 minutes at 37°C. After incubation, excess dye was removed by centrifugation ($700 \times g$, 10 minutes, room temperature). The sperm pellet was resuspended in HTF^+ to $5 \cdot 10^6$ sperm/mL. Aliquots of 50 μL were loaded to the wells of the multiwell plate. Fluorescence was excited at 480 nm and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after injection of 25 μL (1:3 dilution) of the chemical UV filters, negative control (buffer with vehicle), positive control (progesterone, 5 μM final concentration) manually with an electronic multichannel pipette to duplicate wells. The stock solutions of the chemical UV filters in DMSO or ethanol were diluted in HTF^+ to 3× the desired final concentration before being added to the wells. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (percentage), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of UV filters, positive control, and negative control. In contrast to our pilot study (26), we loaded the sperm cells with Fluo-4 without the use of pluronic because we wanted to keep the sperm cells in as natural conditions as possible and because we found that this did not significantly affect the mean relative maximal Ca^{2+} signals of the Ca^{2+} signal-inducing UV filters (Supplemental Table 1).

The measurements of the free $[Ca^{2+}]_i$ of sperm cells in a low Ca^{2+} HTF^+ medium was done by resuspending Fluo-4-loaded sperm cells to $5 \cdot 10^6$ sperm/mL in low Ca^{2+} HTF^+ medium, which was formulated as the HTF^+, without the addition of $CaCl_2$ and supplemented with 5 μM EGTA.

The Ca^{2+} measurements on single sperm cells were done similar to those described elsewhere (29) but on noncapacitated

sperm cells. One milliliter of the swim-up purified sperm cells ($10 \cdot 10^6$ sperm/mL) was added onto polylysine-coated chambers to minimize movement of the sperm cells (Lab-Tek; Nalge Nunc International) and loaded with the fluorescent Ca^{2+} indicator fura-2 (3 μM) for 30 minutes at 37°C. After incubation, the supernatant containing excess dye and nonadhered sperm cells was removed, and the remaining cells were carefully resuspended in 1 mL HTF^+. A Zeiss Axiovert 135 microscope equipped with a Zeiss Achrostigmat 40 × 1.3 NA objective was used to acquire images from fura-2. Excitation was obtained by a Polychrome Villuminator from Till Photonics, and images were acquired using a Cool Snap charge-coupled device camera (Photometrics) from Robert Scientific. Fluorescence was excited at 338 and 380 nm (dual excitation) and emission was recorded above 510 nm using a cutoff filter. Regions of interest were drawn around the head and neck region as in a recent study (30). Background fluorescence was subtracted and changes in the ratio of fura-2 fluorescence between the 338 and 380 nm excitation are shown as $\Delta R/R_0$ (%), indicating the percentage change in the ratio of fluorescence between the two modes of excitation (ΔR) with respect to the mean basal ratio of fluorescence between the two modes of excitation (R_0) before addition of 3-benzylidene camphor (3-BC) (10 nM) and later the positive control (progesterone, 5 μM). Based on the presence of a progesterone induced Ca^{2+} signal in the end of the experiment, we selected 19 sperm cells for analysis from three experiments.

Measurement of changes in pH_i

Changes in pH_i in human sperm cells were measured in 384 multiwell plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech) at 30°C as reported elsewhere (26). Sperm cells were loaded with the fluorescent pH indicator BCECF (10 μM) for 15 minutes at 37°C. After incubation, excess dye was removed by centrifugation (700 × *g*, 10 min, room temperature). The sperm pellet was resuspended in HTF^+ to $5 \cdot 10^6$ sperm/mL. Aliquots of 50 μL were loaded to the wells of the multiwell plate. Fluorescence was excited at 440 and 480 nm (dual excitation) and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after injection of 25 μL (1:3 dilution) of the chemical UV filters (50 μM final concentration), negative control (buffer with vehicle), and positive control (NH_4CL, 30 mM final concentration) manually with an electronic multichannel pipette to duplicate wells. The stock solutions of chemical UV filters in DMSO or ethanol were diluted in HTF^+ to 3× the desired final concentration before being added to the wells. Changes in the ratio of BCECF fluorescence between the 440 and 480 nm excitation are shown as $\Delta R/R_0$ (percentage), indicating the percentage change in the ratio of fluorescence between the two modes of excitation (ΔR) with respect to the mean basal ratio of fluorescence between the two modes of excitation (R_0) before addition of UV filters, positive control, and negative control.

Assessment of dose-response relations

For generation of dose-response curves, 10 serial dilutions with a fixed ratio were performed from a high concentration of the UV filter or progesterone, which induced a saturating response. These 11 concentrations of the UV filter were added to the sperm cells together with a negative control. The change in $\Delta F/F_0$ of the negative control was subtracted from the other $\Delta F/F_0$ to remove the dilution and pipetting artifacts, and the dose-response curves were calculated from the maximal values of the first $\Delta F/F_0$ peaks, using the log(agonist) vs response-variable slope (four parameters) nonlinear regression analysis of GraphPad Prism 6.

Ethical approval

Human semen samples were obtained from healthy volunteers with their prior consent. The donors were recruited from the semen donor corps, which is routinely donating samples for semen quality control analyses at the Department of Growth and Reproduction. After delivery, the samples were fully anonymized and no data on the fertility status or general health of donors are provided. Each donor received a fee of 500 DKK (about 75 UD dollars) per sample for their inconvenience. All samples were analyzed on the same day of delivery and destroyed immediately after the laboratory analyses. Because of the full anonymization of the samples and the destruction of the samples immediately after the laboratory analyses, no ethical approval was needed for this work, according to the regional scientific ethical committee of the Capital Region of Denmark.

Results

Chemical UV filters induce Ca^{2+} signals in human sperm cells

Using a recently developed Ca^{2+} fluorimetric assay (26), we investigated 29 of the 31 chemical UV filters allowed in sunscreens in the European Union and/or the United States for their ability to induce Ca^{2+} signals in human sperm cells (Table 1). The UV filters were tested at 10 μM along with a positive control (progesterone, 5 μM) and negative control (HTF^+ with vehicle). We tested the UV filters at 10 μM because this was the concentration at which the positive hits were best distinguished from negative hits and buffer controls in our pilot study (26). The induced Ca^{2+} signals were recorded at up to 232 seconds after addition of the chemicals, buffer, and positive control (Figure 1). To compare results from different experiments, we calculated the relative maximally induced Ca^{2+} signal from a given experiment by dividing the maximal Ca^{2+} signals with that of the paired positive control. Thirteen of the 29 chemical UV filters tested induced a mean relative maximal Ca^{2+} signal in human sperm cells larger than the induced mean relative maximal Ca^{2+} signal of negative controls (HTF^+ with vehicle) ± 3 SD (0.298% ± 3 × 2.624%, giving a maximal value of 8.17%) (Table 1). These 13 chemical UV filter chemicals (Supplemental Figure 1) were categorized as positive hits and investigated further.

The sperm cells for these experiments were incubated using a noncapacitating HTF^+ medium. To test whether ca-

Table 1. UV Filters Ranked According to the Mean Relative Maximal Ca^{2+} Signal Induced at 10 μM, ie, the Maximal Ca^{2+} Signal Induced by the UV Filter at 10 μM Divided by the Maximal Ca^{2+} Signal Induced by Progesterone at 5 μM in the Same Experiment

Rank	INCI Name	CAS Number	Abbreviation	Allowance in Sunscreens in the European Union (EU) and United States (US)		Mean Relative Maximal Ca^{2+} Signal at 10 μM, % (n = 3)
Positive hits						
1	4-Methylbenzylidene camphor	36861-47-9/ 38102-62-4	4-MBC	EU, 4%		97.07
2	3-Benzylidene camphor	15087-24-8	3-BC	EU, 2%		92.70
3	Methyl anthranilate	134-09-8	Meradimate		US, 5%	79.81
4	Isoamyl P-methoxycinnamate	71617-10-2	Amiloxate	EU, 10%		62.89
5	Ethylhexyl salicylate	118-60-5	Octisalate	EU, 5%	US, 5%	53.39
6	Benzylidene camphor sulfonic acid	56039-58-8	BCSA	EU, 6%		52.69
7	Homosalate	118-56-9	HMS	EU, 10%	US, 15%	47.56
8	Ethylhexyl dimethyl PABA	21245-02-3	OD-PABA	EU, 8%	US, 8%	46.01
9	Benzophenone-3	131-57-7	BP-3	EU, 10%	US, 6%	38.19
10	Ethylhexyl methoxycinnamate	5466-77-3	Octinoxate	EU, 10%	US, 7.5%	29.35
11	Octocrylene	6197-30-4	Octocrylene	EU, 10%	US, 10%	24.23
12	Butyl methoxydibenzoylmethane	70356-09-1	Avobenzone	EU, 5%	US, 3%	22.54
13	Diethylamino hydroxybenzoyl hexyl benzoate	302776-68-7	DHHB	EU, 10%		10.68
Negative hits						
14	Benzophenone-8	131-53-3	Dioxybenzone		US, 3%	4.03
15	Camphor benzalkonium methosulfate	52793-97-2	CBM	EU, 6%		4.01
16	Polysilicone-15	207574-74-1	POLYSILICONE-15	EU, 10%		3.55
17	Drometrizole trisiloxane	155633-54-8	Drometrizole trisolane	EU, 15%		3.28
18	Benzophenone-4	4065-45-6	BP-4	EU, 5%	US, 10%	1.23
19	Diethylhexyl butamidotriazone	154702-15-5	Iscotrizinol	EU, 10%		0.98
20	Ethylhexyl triazone	88122-99-0	Ethylhexyl triazone	EU, 5%		0.86
21	Cinoxate	104-28-9	Cinoxate		US, 3%	0.13
22	PEG-25 PABA	116242-27-4	PEG-25 PABA	EU, 10%		0.04
23	Bis-ethylhexyloxyphenol methoxyphenyl triazine	187393-00-6	Bemotrizinol	EU, 10%		−0.34
24	TEA-salicylate	2174-16-5	TEA salicylate		US, 12%	−0.56
25	Phenylbenzimidazole sulfonic acid	27503-81-7	Ensulizole	EU, 8%	US, 4%	−3.08
26	PABA	150-13-0	PABA		US, 15%	−3.09
27	Disodium phenyl dibenzimidazole tetrasulfonate	180898-37-7	Bisdisulizole	EU, 10%		−3.42
28	Benzophenone-5	6628-37-1	BP-5	EU, 5%		−3.45
29	Terephthalylidene dicamphor sulfonic acid	92761-26-7 / 90457-82-2	Ecamsule	EU, 10%		−3.54

Based on their ability to induce Ca^{2+} signals, the UV filters are categorized into positive hits, which had values above that of the negative controls (HTF^+ with vehicle) ± 3 SD (0.298% ± 3 · 2624%, giving a maximal value of 8.17%), and negative hits. INCI name, CAS number, abbreviation, and allowance in sunscreens in the EU and US are also listed in the table.

pacitation of the sperm cells could alter the results, we tested 10 chemical UV filters on sperm cells from the same donors, which were either incubated using a noncapacitating HTF^+ medium or using a capacitating HTF^+ medium (n = 3). The difference in the maximally induced Ca^{2+} signals recorded up to 232 seconds after the addition of the chemicals from the capacitated and noncapacitated sperm cells were not statistically significant ($P > 0.25$, from paired T-tests).

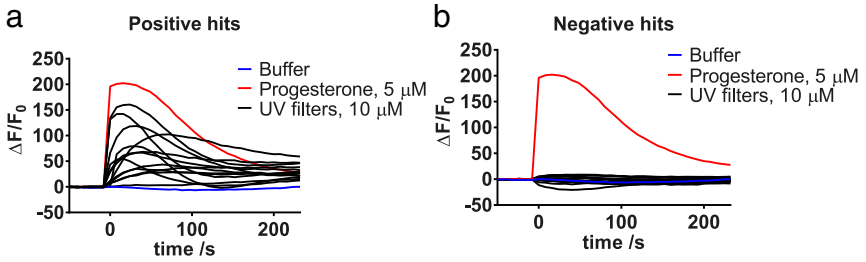

Figure 1. a, Ca^{2+} signals induced by addition of buffer, progesterone, 5 μM, and the positive-hit UV filters, 10 μM. b, Ca^{2+} signals induced by the addition of buffer, progesterone, 5 μM, and the negative-hit UV filters, 10 μM.

Source of Ca^{2+}

To examine the mode of action of the positive-hit UV filters, we tested whether the induced Ca^{2+} signals reflected an influx of extracellular Ca^{2+} or a release of Ca^{2+} from internal stores. We did this by repeating the Ca^{2+} measurements of the positive-hit UV filters at 10 μM on sperm cells resuspended in a low Ca^{2+} HTF^{+} medium with 5 μM EGTA (n = 3). The UV filters were added to the sperm cells less than 5 minutes after the resuspension in the low Ca^{2+} HTF^{+} medium, similar to the procedure in a recent study (30). We then compared the maximally induced Ca^{2+} signal with those obtained from paired experiments on sperm cells resuspended in normal HTF^{+} medium. Similar to progesterone, the maximally induced Ca^{2+} signals for these 13 UV filters were greatly reduced when using low Ca^{2+} HTF^{+} medium (Figure 2, mean reduction in signal ± SD, n = 3). The Ca^{2+} signals induced by UV filters diethylamino hydroxybenzoyl hexyl benzoate (DHHB) and Avobenzone were, however, inhibited to a lesser extent than the other UV filters.

Shape of curves of induced Ca^{2+} signals

To further investigate the mode of action of the positive-hit UV filters, we compared the shape of the induced Ca^{2+} signal of the UV filters, 10 μM (n = 3), with that of progesterone, 5 μM (n = 12) (Figure 3). Most of the UV filters induce transient Ca^{2+} signals at 10 μM, resembling the transient Ca^{2+} signals induced by progesterone. The four UV filters, octinoxate, octocrylene, avobenzone and DHHB, however, induce sustained or slowly rising Ca^{2+} signals at 10 μM, which did not resemble the Ca^{2+} signal induced by progesterone.

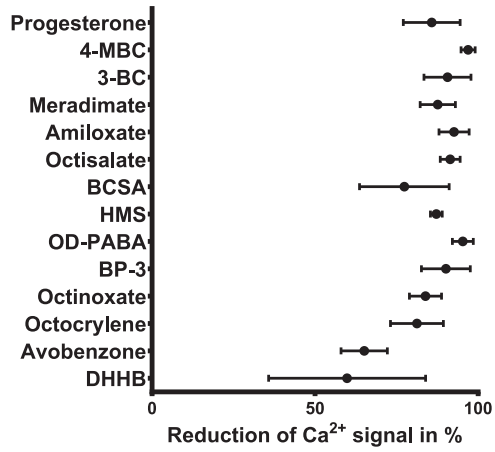

Figure 2. Reduction of maximally induced Ca^{2+} signals (mean ± SD, n = 3) in sperm cells kept in a low Ca^{2+} HTF^{+} medium compared with sperm cells kept in normal HTF^{+} medium.

Effect on CatSper

To test whether the positive-hit UV filters affected influx of extracellular Ca^{2+} through CatSper, we used the CatSper inhibitor MDL 12330A (31). We compared the Ca^{2+} signals induced by the chemical UV filters at 50 μM and progesterone at 5 μM in the presence or absence of 20 μM MDL 12330A (Figure 4, mean inhibition of signal ± SD, n = 3). We found that all UV filters, except ethylhexyl dimethyl (OD)-PABA, octocrylene, avobenzone and DHHB, were, similar to progesterone, highly inhibited with the CatSper inhibitor MDL 12330A. These findings show that these UV filters, at least in part, induce the Ca^{2+} signals via CatSper. The CatSper channel can be activated by increased $pH_{(i)}$. We therefore tested whether the positive-hit UV filters induced changes in $pH_{(i)}$ and found no effect of the positive-hit UV filters on $pH_{(i)}$ at 50 μM (n = 4) (Figure 5). This suggests that the effect of the positive-hit UV filters on CatSper is either direct through interaction with the binding pockets of progesterone and prostaglandins or indirect through some other, unknown mechanism, not involving changes in $pH_{(i)}$.

Inhibition of progesterone signal

To investigate whether some of the UV filters interfered with the progesterone-induced Ca^{2+} signals, we performed inhibitor studies with 10 μM of 3-BC, benzylidene camphor sulfonic acid (BCSA), or benzophenone-3 (BP-3) on progesterone dose-response curves (n = 3) (Figure 6). 3-BC, BCSA, and BP-3 were chosen because the Ca^{2+} signals induced by these UV filters were highly inhibited by MDL 12330A and similar in shape to the Ca^{2+} signals induced by progesterone. Sperm cells were preincubated for 5 minutes with or without the UV filters before the addition of progesterone. Our results showed that none of the UV filters affected the maximal $\Delta F/F_0$ of the progesterone dose-response curves or the kinetics of the progesterone-induced Ca^{2+} signals. Furthermore, we found that BCSA and 3-BC shifted the EC_{50} of progesterone toward higher values, indicating competitive inhibition by these two UV filters on the progesterone effect.

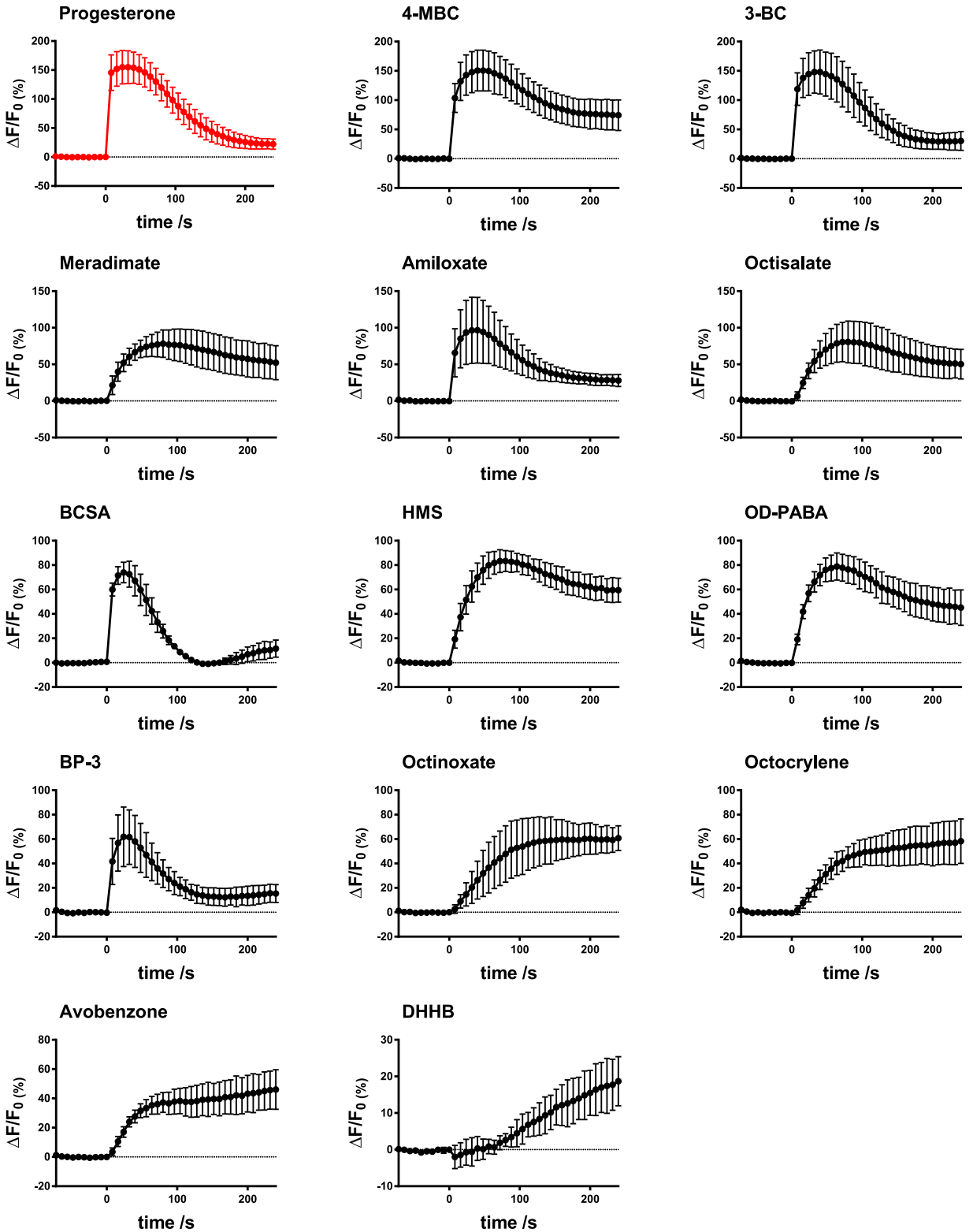

Figure 3. Induced Ca^{2+} signals (mean ± SD) of the UV filters, 10 μM [n = 3] and progesterone, 5 μM [n = 12]). Note the decrease in the y-axis range from the upper left corner (progesterone) toward the lower right corner (DHHB).

Dose-response relationship for Ca^{2+}signal inducing effect of the positive-hit UV filters

To examine whether the positive-hit UV filters induced Ca^{2+} signals at physiologically relevant levels, dose-response relations were assessed for these 13 UV filters. All except DHHB, produced saturating dose-response curves, with mean EC_{50} values within the concentration range 0.5–6.7 μM (Table 2 and Figure 7). Because some doses

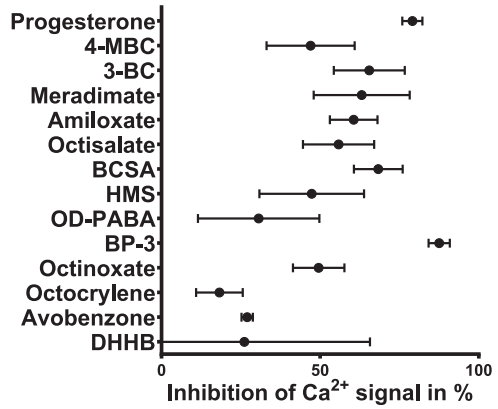

Figure 4. Inhibition of Ca^{2+} signals induced by the UV filters, 50 μM, and progesterone, 5 μM, in the presence 20 μM MDL12330A (mean inhibition of signal ± SD, n = 3).

of some of the UV filters induced sustained or slowly rising Ca^{2+} signals, the earliest Ca^{2+} signal peak recorded at up to 232 seconds after the addition of the chemicals was used to determine the dose-response curves.

Effects of low dose 3-BC on single sperm cells

To test the accuracy of the lowest estimated EC_{02} of the UV filters, we tested the ability of 10 nM 3-BC to induce Ca^{2+} signals in single sperm cells (Figure 8). Using single-cell Ca^{2+} imaging microscopy (29), we investigated the Ca^{2+} signals induced in 19 individual sperm cells from three experiments after the addition of 10 nM 3-BC. Figure 8 shows that Ca^{2+} signals are induced in some but not all sperm cells. Only sperm cells that generated a subsequent Ca^{2+} signal in response to the addition of 5 μM of progesterone (not shown in figure) were included in the analysis. It should be noted that we registered changes only in $[Ca^{2+}]_i$ at the head and neck region of these cells, whereas the $[Ca^{2+}]_i$ data from sperm cells in suspension are based on the total fluorescence from all sperm cells of each well.

Mixture effect of UV filters

Rather than being exposed to single chemicals, sperm cells are in vivo presumably exposed to a complex mixture of chemicals. Therefore, we tested whether the Ca^{2+} signal-inducing effect of UV filters was found to be additive at low levels. We found that a mixture of the 13 UV filters

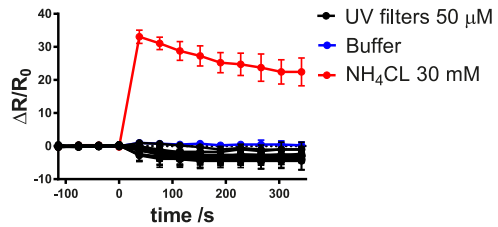

Figure 5. Induced changes in $pH_{(i)}$ (mean ± SD, n = 4) by UV filters, 50 μM, buffer, and positive control (NH_4CL, 30 mM).

at 100 nM each induced a small but consistent Ca^{2+} signal, which were larger than the Ca^{2+} signals induced by the single chemicals at 100 nM (n = 3) (Figure 9A). The maximal Ca^{2+} signal induced by the mixture is in the same range as that induced by pM concentrations of progesterone (Figure 9B).

Discussion

We found that 13 of 29 tested chemical UV filters (44%) could induce Ca^{2+} signals in human sperm cells. Our findings confirm the results for the eight UV filters tested before in our pilot study (26), the seven positive hits Octocrylene, OD-PABA, Octinoxate, 4-MBC, 3-BC, BP-3, and HMS and the negative hit BP-4.

The induced Ca^{2+} signals were greatly reduced when the sperm cells were kept in a low Ca^{2+} HTF^+ medium, showing that the UV filters induce Ca^{2+} signals by affecting the uptake of extracellular Ca^{2+}. The Ca^{2+} signals induced by UV filters DHHB and Avobenzone were, however, inhibited to a lesser extent than the other UV filters. The Ca^{2+} signals induced by all UV filters, except Octinoxate, Octocrylene, Avobenzone, and DHHB, were fast and transient and resembled the Ca^{2+} signal induced by progesterone, suggesting a similar mode of action between these UV filters and progesterone. We found that Ca^{2+} signals induced by all UV filters, except OD-PABA, Octocrylene, Avobenzone, and DHHB, were highly inhibited with the CatSper inhibitor MDL 12330A. This indicates that these UV filters at least in part induce Ca^{2+} signals via CatSper, similar to progesterone. The Ca^{2+} signals induced by OD-PABA, Octocrylene, Avobenzone, and DHHB were also inhibited by MDL 12330A, although to a much lesser degree, indicating that these UV filters primarily affect Ca^{2+} signaling by a mechanism separate from CatSper, at least at the high concentration of 50 μM. Three of the UV filters that resisted blocking by MDL, Octocrylene, Avobenzone, and DHHB also induced slowly rising or sustained Ca^{2+} signals, which differed from the transient Ca^{2+} signals induced by progesterone and the UV filters inhibited to a higher extent by MDL 12330A. In addition, the Ca^{2+} signals induced by Avobenzone and DHHB were inhibited to a lesser extent in the low Ca^{2+} medium. Together these findings indicate that Octocrylene, Avobenzone, and DHHB might induce Ca^{2+} signals via release of Ca^{2+} from internal Ca^{2+} stores in contrast to the other UV filters, which primarily induce Ca^{2+} signals via CatSper.

In our pilot study, we found a similar strong inhibition with MDL 12330A for 4-MBC, 3-BC, HMS, and BP-3, whereas OD-PABA, in contrast to in our study, was also

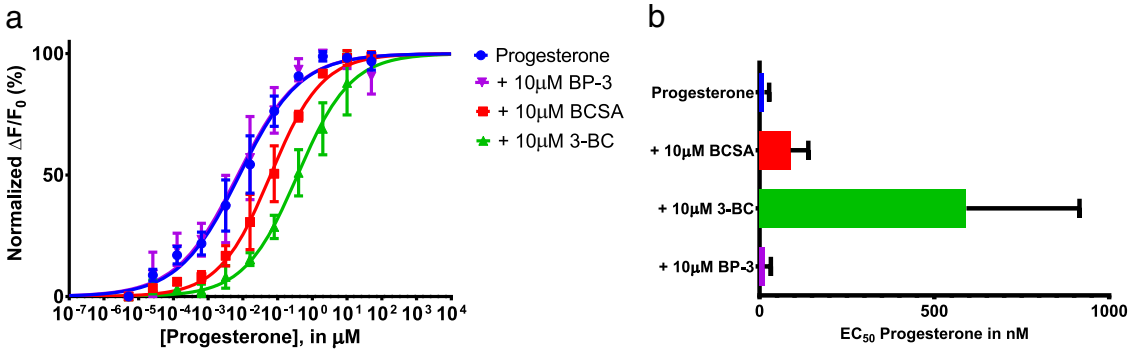

Figure 6. a, Normalized dose-response curves (mean ± SD, n = 3) for progesterone without any preincubation and after 5 minutes of preincubation with the UV filters, BP-3, BCSA, and 3-BC at 10 μM. The mean R^2 value of the 12 individual fitted curves was 0.99. b, EC_{50} for the dose response curves (mean ± SD, n = 3) for progesterone without any preincubation and after 5 minutes of preincubation with the UV filters, BP-3, BCSA, and 3-BC at 10 μM.

found to be highly inhibited (26). In the present study, we used the inhibitor MDL 12330A at 20 μM and the UV filters at 50 μM, whereas in our pilot study, the inhibitor MDL 12330A was used at 100 μM and the UV filters at 3–30 μM, which might explain the observed difference for OD-PABA. We showed that the induction of Ca^{2+} signals is independent of changes in $pH_{(i)}$, suggesting that the UV filters, which primarily induced Ca^{2+} signals via an effect on CatSper, either act agonistically on the binding pockets of progesterone or prostaglandin or affect CatSper through another hitherto unknown mechanism.

To investigate whether the UV filters targeted the progesterone binding pocket, we performed progesterone dose-response curves with and without 5 minutes of preincubation with 10 μM of 3-BC, BCSA, or BP-3. The $\Delta F/F_0$ values used for the progesterone dose response curves were normalized to compensate for different, but optimal gain settings for each individual experiment. In a separate experiment we found that, at the same gain setting, the maximal $\Delta F/F_0$ of the progesterone dose-response curves was always the same and independent of the presence of inhibitors. Our results showed that the UV filters 3-BC and BCSA can inhibit the binding of progesterone in a competitive fashion, suggesting that these two UV filters target areas of the progesterone binding pocket. 4-MBC has previously been shown to competitively inhibit progesterone-induced Ca^{2+} signals (26), hinting that the structurally similar uncharged camphor-derived UV filters, such as 3-BC, 4-MBC, and BCSA (Supplemental Figure 1), might share this ability. Progesterone acts as a strong chemoattractant in the picomolar range (32). Exposure to these UV filters might inhibit this progesterone-mediated chemotaxis because the Ca^{2+} signals induced by progesterone at picomolar concentrations are almost abolished when preincubating with 10 μM of BCSA or 3-BC.

We showed that the Ca^{2+} signals induced by all positive-hit UV filters, except DHHB, can be used to form saturating dose-response curves, with EC_{50} values ranging from 0.51 to 7.33 μM and the lowest effective dose values

Table 2. EC_{50}, EC_{02}, and R^2 of the Fit for the Dose-Response Curves (Mean and SD, n = 3–4) of All Positive-Hit UV Filters, Except DBBH, Which Did Not Produce a Saturating Dose-Response Curve

	EC_{50}, μM		EC_{02}, μM		R^2 of Fit		
	Mean	SD	Mean	SD	Mean	SD	n
4-MBC	0.5189	0.3154	0.0142	0.0069	0.9759	0.0284	3
3-BC	0.5241	0.2927	0.0063	0.0073	0.9947	0.0028	4
Meradimate	2.1319	0.8656	0.0736	0.0538	0.9763	0.0238	4
Amiloxate	2.7173	1.0874	0.2275	0.0407	0.9878	0.0079	3
Octisalate	1.8907	0.5720	0.0545	0.0409	0.9891	0.0060	3
BCSA	1.4867	0.5722	0.1015	0.0442	0.9957	0.0007	3
HMS	1.3961	1.0978	0.0629	0.0585	0.9851	0.0086	3
OD-PABA	4.9530	1.2951	0.0683	0.0675	0.9904	0.0062	3
BP-3	4.6773	0.6977	0.2990	0.0478	0.9975	0.0010	3
Octinoxate	7.3305	4.1781	0.4254	0.2616	0.9895	0.0105	4
Octocrylene	2.3423	0.5693	0.0921	0.0579	0.9861	0.0085	3
Avobenzone	7.0793	0.5395	0.2030	0.1049	0.9797	0.0092	3
DBBH			Nonsaturating dose-response curve				3

Abbreviations are as in Table 1.

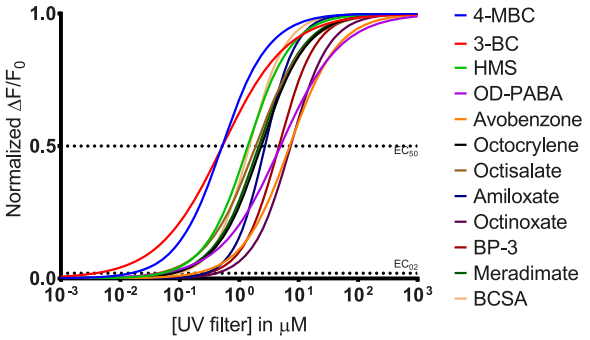

Figure 7. Normalized dose-response curves for the 12 UV filters with saturating responses. The curves were generated using the mean EC_{50} and hill slope values and normalized between 0 and 1.

(EC_{02}) ranging from 6.2 to 425.4 nM. Although we find lower estimates of both EC_{50} and EC_{02} values for 4-MBC, 3-BC, HMS, and BP-3 and slightly higher values for OD-PABA than in our pilot study (26), the EC_{50} values are still within the micromolar range and the EC_{02} values within the nanomolar range in both studies. We performed our dose-response experiments on noncapacitated sperm cells. It has been shown that capacitation shifts the EC_{50} value of progesterone toward lower concentrations (8), hinting that the estimated EC_{50} values for the UV filters, BCSA and 3-BC, which target areas of the progesterone binding pocket, might also be lower in capacitated sperm cells.

We showed that 10 nM of 3-BC can induce Ca^{2+} signals in the head-neck region of some sperm cells, confirming that our estimated EC_{02} for this UV filter is effective for inducing Ca^{2+} signals. In addition, we showed that the UV filters can act additively in low-dose mixtures of 100 nM each to induce Ca^{2+} signals, similar in amplitude to the Ca^{2+} signals induced by picomolar concentrations of progesterone. In our pilot study, we similarly found that other Ca^{2+} signal-inducing chemicals could act additively (26). This additive effect is noteworthy because progesterone in picomolar concentrations can act as a chemoattractant (32), prime sperm cells for acrosome reaction, and even induce acrosome reaction in some sperm subpopulations (33). We speculate that the small Ca^{2+} signals induced by the low-dose mixture of UV filters, or other Ca^{2+} signal-

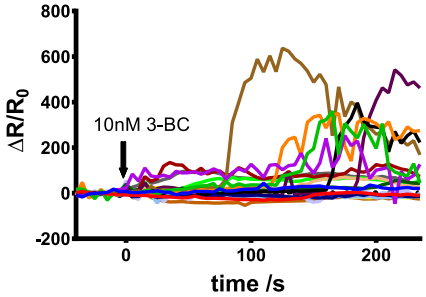

Figure 8. Ca^{2+} signals induced in the head-neck region of 19 individual sperm cells from three experiments after addition of 10 nM 3-BC.

inducing chemicals, which are comparable with the Ca^{2+} signals induced by progesterone in the picomolar range, might act similarly on the sperm cells, thus interfering with, for example, chemotaxis.

Few data on the UV filter concentration in plasma exist (27). Only the levels of 4-MBC, BP-3, and Octinoxate have been measured in plasma after repeated whole-body topical application (34, 35), in which the maximally measured levels for 4-MBC (440 nM) and BP-3 (3.51 μM) were much higher than our estimated EC_{02} values, whereas the maximally measured level for Octinoxate (330 nM) was slightly lower than our estimated EC_{02} value (34). Interestingly, the highest levels for each of these UV filters were found in female subject, whereas the maximal levels in males were slightly lower (34). However, all of the positive-hit UV filters have molecular weights below 500 Da, suggesting that they could all have the ability to penetrate the human skin (36). The first metabolite of OD-PABA has been measured in semen after topical application of sunscreen (37), showing that OD-PABA, similar to 4-MBC, BP-3, and Octinoxate, can penetrate the human skin. Few data exist on the metabolic pathways of UV filters in humans because this has been investigated only for 4-MBC, BP-3, and OD-PABA (27). 4-MBC has been found to have a half-life of 9 hours after topical application (38) and BP-3 to be metabolized slowly during the first 24 hours after topical application (39). For OD-PABA the rate of metabolism is not known (27). For some UV filters, it might be relevant to investigate the effect of their metabolites also, eg, OD-PABA, for which the first metabolite rather than OD-PABA itself was measured in semen (37).

Sperm cells are quiescent in the male reproductive tract and first become motile upon ejaculation (40). After ejaculation the sperm cells acquire some of their key molecules for Ca^{2+} signaling from fusion with proteasomes, which are extracellular vesicles released by the prostate gland, just as they enter the female reproductive tract (41). During the journey of a sperm cell through the female reproductive tract, triggering of $[Ca^{2+}]_i$ controlled sperm functions at the correct time and in the correct order is necessary for successful natural fertilization of the egg (4). Together this suggests that the potential disruptive effects of the UV filters on sperm Ca^{2+} signaling and thus sperm function takes place within the female reproductive tract. However, because the sensitivity of CatSper to progesterone arises early during spermiogenesis (21), there might also be effects of the UV filters on sperm cells in the male reproductive tract, although the role of sperm Ca^{2+} signaling in the male reproductive tract is unclear (42). Interestingly, studies have found evidence for the bioaccumulation of BP-3 and its metabolites as well as the

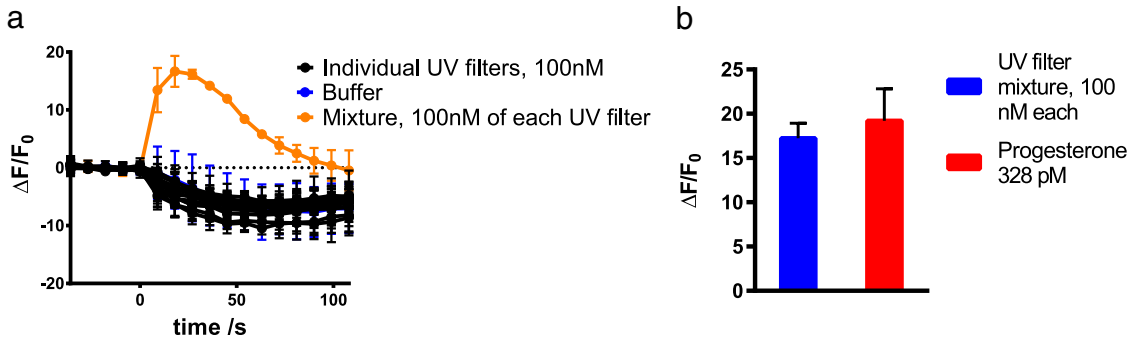

Figure 9. a, Ca^{2+} signals (mean ± SD) induced by the individual UV filters, 100 nM, buffer, and the UV filters in mixture, 100 nM each. b, Comparison of the Ca^{2+} signal (mean ± SD) induced by the UV filters in mixture, 100 nM each, and 328 pM of progesterone.

metabolites of OD-PABA in semen after topical application of sunscreen (37, 43), suggesting that the sperm cells might already be highly exposed to these chemicals upon entering the female reproductive tract.

The female reproductive tract is not protected by blood-tissue barriers (44), and UV filters in plasma and within the female reproductive tract would thus be expected to be found in similar concentrations. In contrast to this, the developing follicles in the ovaries and parts of the male reproductive tract are protected from substances in the blood by the blood-follicle barrier (45), blood-testis barrier, and the blood-epididymis barrier, respectively (46), which are blood-tissue barriers formed mainly by tight junctions between epithelial cells. The follicular fluid enters the Fallopian tube together with the egg after ovulation (47). The concentrations of UV filters in follicular fluid has not been measured, but because many of the positive-hit UV filters have high lipid solubility (48) and all of them have molecular masses below 500 Da, they theoretically fulfill the criteria for crossing a blood-tissue barrier (49) such as the blood-follicle barrier. In support of this, 3-BC has been found in the brain of rats after dermal application (50) and BP-3 has been measured in several human placenta samples (51), showing that these UV filters can cross the rat blood-brain barrier and the human blood-placental barrier, respectively. Taken together, there is strong evidence that at least some of the UV filters can cross blood-tissue barriers, such as the blood-follicle barrier, blood-testis barrier, and blood-epididymis barrier.

Our data raise further concern that some chemical UV filters have endocrine-disrupting effects (52). The four UV filters, BP-3, 3-BC, 4-MBC, and HMS, have previously been shown to have estrogenic effects and to act antagonistic on progesterone receptor bioassays (53), indicating that our findings on effects on Ca^{2+} signaling in human sperm cells could be proxies for other endocrine disrupting effects elsewhere. In line with this, we have recently shown that Steviol can both induce Ca^{2+} signals via CatSper and affect several other parts of progesterone signaling (54). Progesterone has recently been shown to activate CatSper through the receptor abhydrolase domain containing 2 (ABHD2), a progesterone-dependent lipid hydrolase that depletes the endocannabinoid 2-arachidnoylglycerol from the plasma membrane, thereby activating CatSper (55). ABHD2 is widely expressed in other cells and tissues, hinting that some of the positive-hit UV filters from our study could have effects via ABHD2 elsewhere.

We are all exposed to complex mixtures of chemicals, which is why it can be difficult to see effects of single chemicals in epidemiological studies, if not correcting for all other chemicals with effects on the endpoint of interest. A recent epidemiological study investigated the effect of the benzophenone-type UV filters, BP-1, BP-2, BP-3, BP-8, and 4-hydroxybenzophenone, on time to pregnancy in couples trying to conceive (56). BP-3 was the only of these compounds included in our study, and it was unexpectedly found to have no effect on time to pregnancy, whereas BP-2, which was found not induce Ca^{2+} signals in our pilot study (26), did have an effect on time to pregnancy (56). Our study add additional substances to the growing list of chemicals, with the ability to interfere with Ca^{2+} signaling in human sperm cells (26, 30, 31, 52, 57, 58). Because $[Ca^{2+}]_i$ controls sperm motility, chemotaxis, and acrosome reaction and participates in sperm capacitation (4, 6), any disturbance of Ca^{2+} signaling in human sperm cells, regardless of the mechanism, might adversely affect fertility, eg, by inducing premature acrosome reaction (59). Furthermore, a part of the chemicals that induce Ca^{2+} signals via the CatSper, might inhibit the normal progesterone induced Ca^{2+} signaling, as shown for 3-BC and BCSA in our study and 4-MBC in our pilot study (26) and thus lead to impaired fertility because suboptimal progesterone-induced Ca^{2+} influx is associated with reduced male fertility (14–19).

In conclusion, we investigated 29 of the 31 chemical UV filters allowed in sunscreens in the European Union and/or the United States, for their ability to induce Ca^{2+} signals

in human sperm cells and showed that 13 UV filters induced Ca^{2+} influxes in human sperms (44%) at physiologically relevant doses. Nine of the UV filters induced Ca^{2+} signals primarily through activation of CatSper, thereby mimicking the effect of progesterone on human sperm cells. Two UV filters, 3-BC and BCSA, were found to competitively inhibit the progesterone-induced Ca^{2+} signals, indicating that these UV filters target areas of the progesterone binding pocket. Because Ca^{2+} signaling controls important sperm functions, including chemotaxis, motility, capacitation, and acrosome reaction, all of which are essential for fertilization, the effect of these UV filters on $[Ca^{2+}]_i$, either via CatSper or by another mechanism, might interfere with the normal human fertilization process and thus impair fertility. Our experiments have been carried out in vitro, and human exposure studies would be needed to examine the effects of exposure to chemical UV filters on fertility in vivo.

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Occurrence and toxicity of musks and UV filters in the marine environment.

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Abstract
Emerging chemical contaminants in the marine ecosystem represent a threat to the environment and also to human health due to insufficient knowledge about their toxicity and bioaccumulation in the food chain. Consequently, many of them are not regulated. In this review we focus on musks and organic UV filters. For both groups of compounds we describe occurrence in the marine environment, toxic effects identified so far and methods used to identify such effects. The final objective of this work is to identify gaps in the understanding of their toxicology.

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Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland.

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Abstract
We performed a trace analytical study covering nine hormonally active UV-filters by LC-MS/MS and GC-MS in river water and biota. Water was analysed at 10 sites above and below wastewater treatment plants in the river Glatt using polar organic chemical integrative samplers (POCIS). Four UV-filters occurred in the following order of decreasing concentrations; benzophenone-4 (BP-4) > benzophenone-3 (BP-3) > 3-(4-methyl)benzylidene-camphor (4-MBC) > 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC). BP-4 ranged from 0.27 to 24.0 microg/POCIS, BP-3, 4-MBC and EHMC up to 0.1 microg/POCIS. Wastewater was the most important source. Levels decreased with higher river water flow. No significant in-stream removal occurred. BP-3, 4-MBC and EHMC were between 6 and 68 ng/L in river water. EHMC was accumulated in biota. In all 48 macroinvertebrate and fish samples from six rivers lipid-weighted EHMC occurred up to 337 ng/g, and up to 701 ng/g in 5 cormorants, suggesting food-chain accumulation. UV-filters are found to be ubiquitous in aquatic systems.