EUROPEAN COMMISSION

Reports of the Scientific Committee on Cosmetology (ninth series)

European Commission

REPORTS OF THE SCIENTIFIC COMMITTEE ON COSMETOLOGY (NINTH SERIES)

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server (http://europa.eu.int).

Cataloguing data can be found at the end of this publication.

Luxembourg: Office for Official Publications of the European Communities, 1999

ISBN 92-828-8951-3

Printed in Spain

PRINTED ON WHITE CHLORINE-FREE PAPER

REPORTS OF THE SCC, 9TH SERIES

CONTENTS

49th plenary meeting of 10 February 1992

50th plenary meeting of 2 June 1992

51st plenary meeting of 7 October 1992

52nd plenary meeting of 12 February 1993

53rd plenary meeting of 25 June 1993

54th plenary meeting of 10 Decmber 1993

55th plenary meeting of 9 March 1994

56th plenary meeting of 24 June 1994

57th plenary meeting of 21 October 1994

58th plenary meeting of 3 February 1995

60th plenary meeting of 23 June 1995

62nd plenary meeting of 18 January 1996

65th plenary meeting of 24 Mai 1996

66th plenary meeting of 18 July 1996

68th plenary meeting of 20 December 1996

71st plenary meeting of 24 June 1997

72nd plenary meeting of 14 October 1997

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).

Previous series :

First Series (1982)	EUR 7297
Second Series (1983)	EUR 8634
Third Series (1983)	EUR 8794
Fourth Series (1986)	EUR 10305
Fifth Series (1987)	EUR 11080
Sixth Series (1987)	EUR 11139
Seventh Series (1988)	EUR 11303
Opinion of the SCC period 11/86 – 10/90 (1993)	EUR 14208

Composition of the Scientific Committee on Cosmetology

Members of the SCC

Mr J. Carstensen
Mr L. Celleno
Mr J.-L. Claude
Mrs J. Dony
Mr P. Heistracher
Mr V. Kapoulas
Mr F. Kemper
Mrs A.G.A.C. Knaap
Mr B.A.R. Lina
Mr N. Loprieno
Mrs M.O. Masse
Mr Ph. Masson
Mr L.A. Monteiro Rodrigues
Mr D.P. O'Mahony
Mr J.L. Parra
Mr L. Pons Gimier
Mr C.J. Powell
Mrs E-L. Sainio
Mr A. Somogyi
Mrs M. Tammela
Mr I.R. White

Previous members also contributing to this series

Mr J. Cotte
Mr R. Fielder
Mrs M.F.L. Guedes Bahia
Mr J. Siou

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

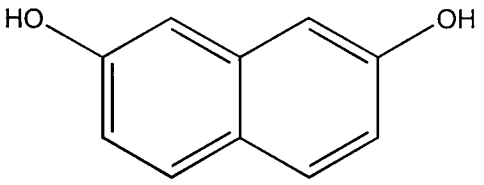
1.3 Trade names and abbreviations

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

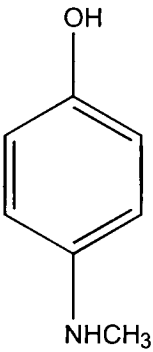
1.3 Trade names and abbreviations

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

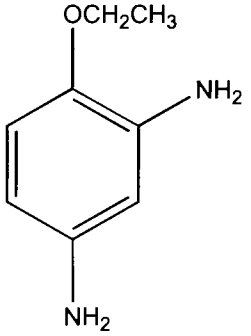
1.3 Trade names and abbreviations

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

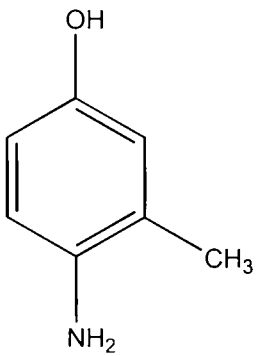
1.3 Trade names and abbreviations

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

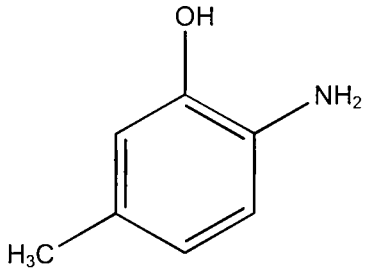
1.3 Trade names and abbreviations

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

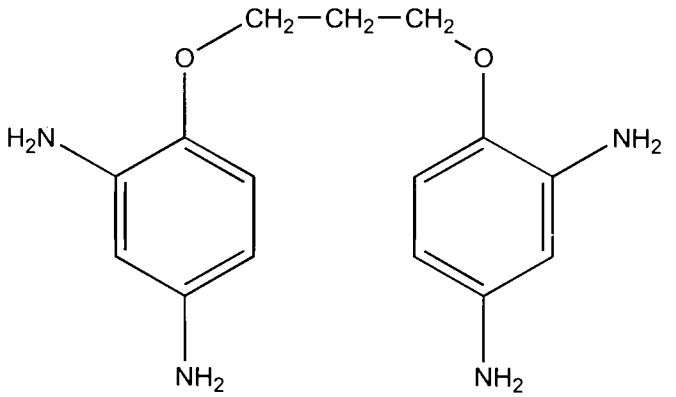
1.3 Trade names and abbreviations

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

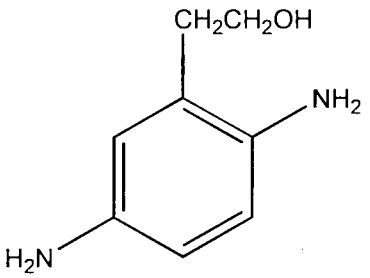
1.3 Trade names and abbreviations

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

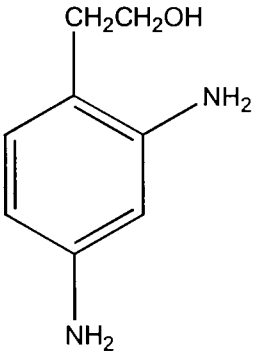
1.2 Chemical names

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, radiochemical 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

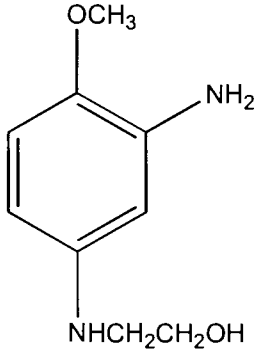
1.2 Chemical names

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

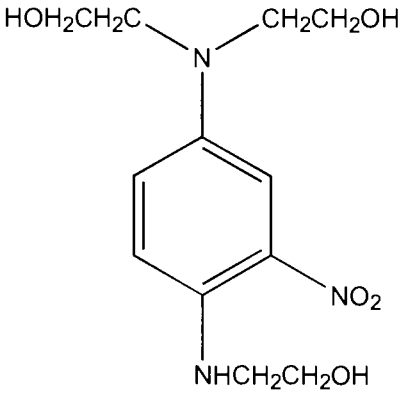
1.3 Trade names and abbreviations

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

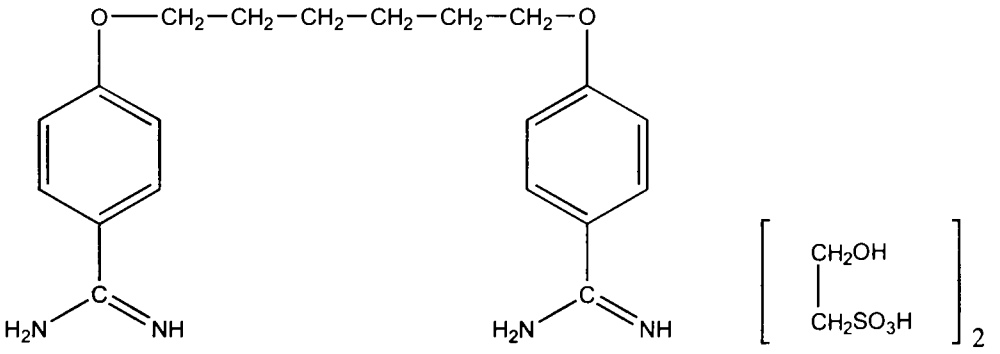
1.1 Primary name

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

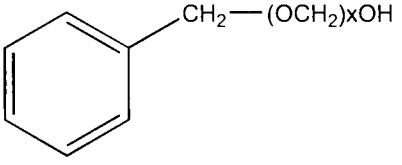
1.2 Chemical names

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

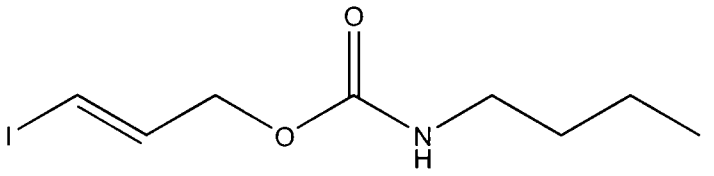
1.2 Chemical names

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

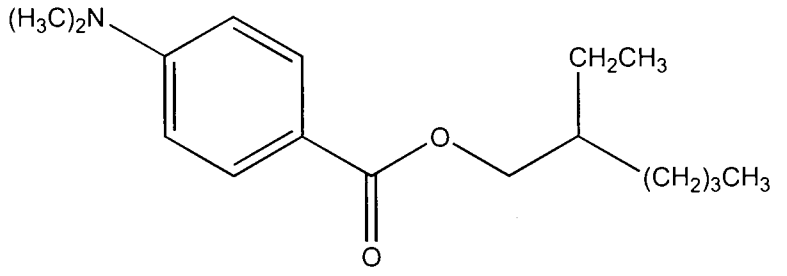
1.2 Chemical names

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

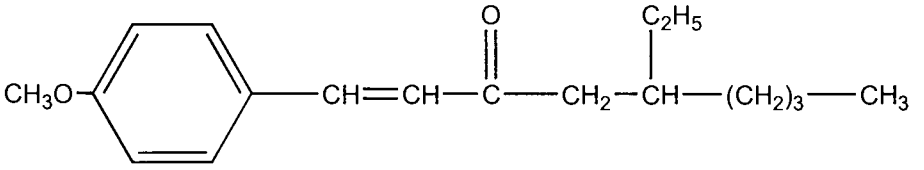
1.2 Chemical names

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

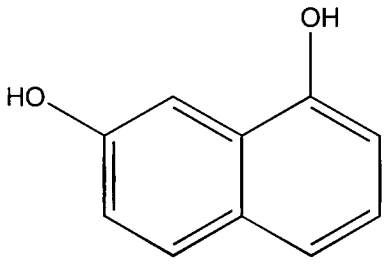
1.3 Trade names and abbreviations

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

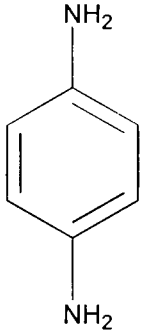
1.1 Primary name

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	+/-	-	$\pm H_2O_2$
	aqueous solution			±non mutagenic
	Aged DMSO		+	couplers
	solution			
	Bandrowski's base		+	
TA 100, 1535	5-1000 µg	+/-	-	No dose response
1537				
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,	5-250 µg/plate	+	-	PB induced S9
100, 1538				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

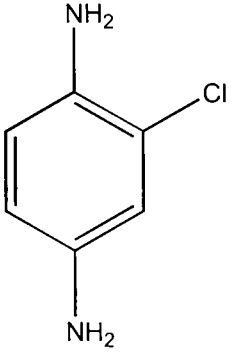
Ursol Brown O

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

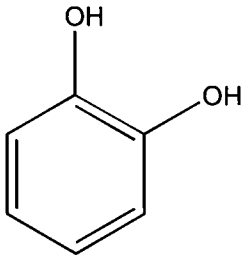
1.3 Trade names and abbreviations

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

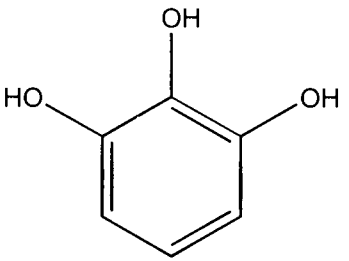
1.3 Trade names and abbreviations

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; $\pm H_2O_2$, 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

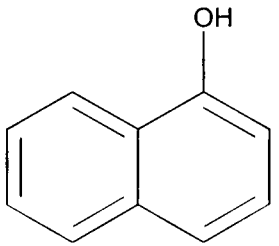
1.2 Chemical names

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

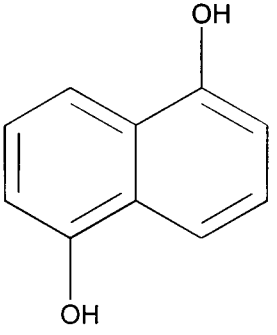
1.2 Chemical names

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/cm², males) and 0.981 % (2.09 µg/cm², 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/cm² (males) and 0.358 µg/cm² (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

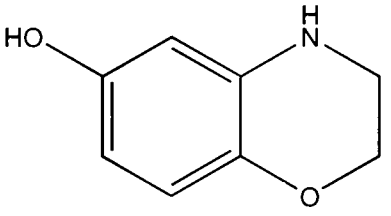
1.2 Chemical names

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

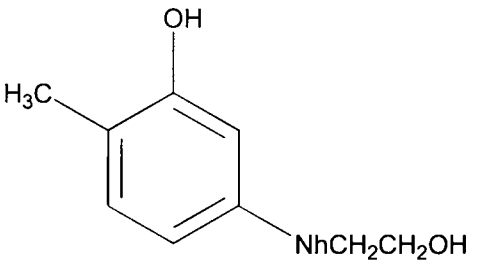
1.3 Trade names and abbreviations

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:	**1 (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

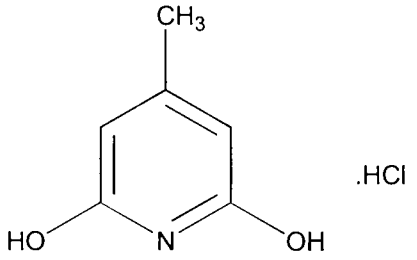
1.2 Chemical names

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* study 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

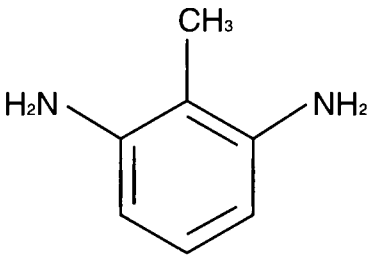
1.2 Chemical names

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

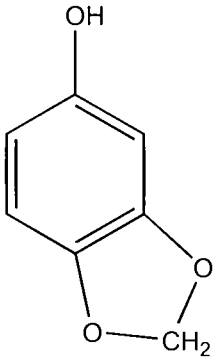
1.1 Primary name

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-(β-HYDROXYETHYLAMINO)-3,4-METHYLENEDIOXY-BENZENE*

1. General

1.1 Primary name

1-(ß-hydroxyethylamino)-3,4-methylenedioxy-benzene

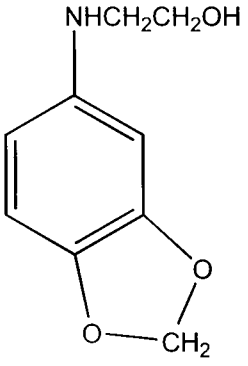
1.2. Chemical names

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

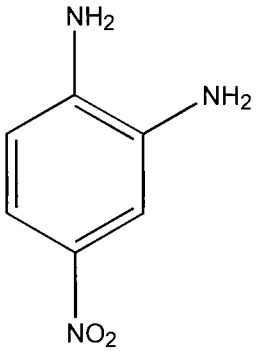
1.2 Chemical names

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

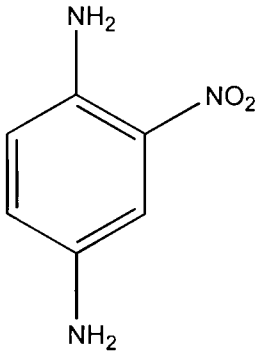
1.2 Chemical names

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

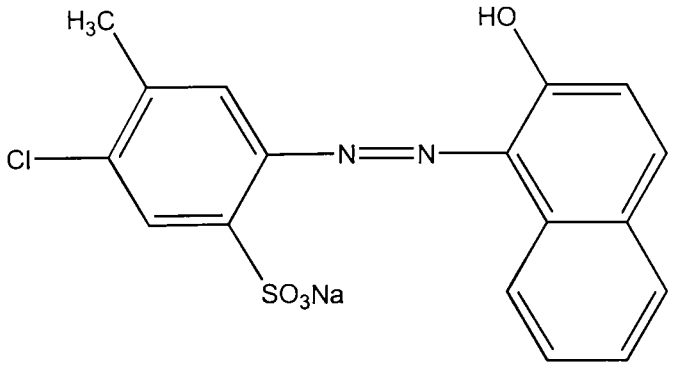
1.2 Chemical names

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

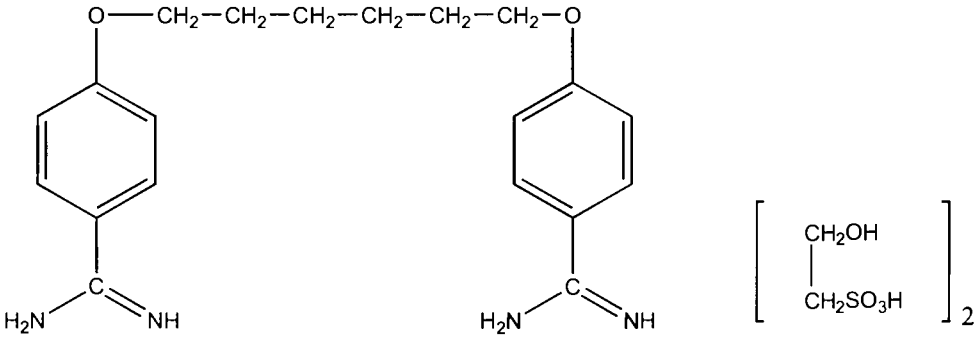
1.1 Primary name

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

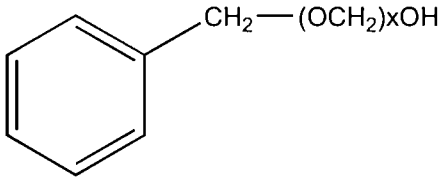
P 21: *BENZYLFORMAL*

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

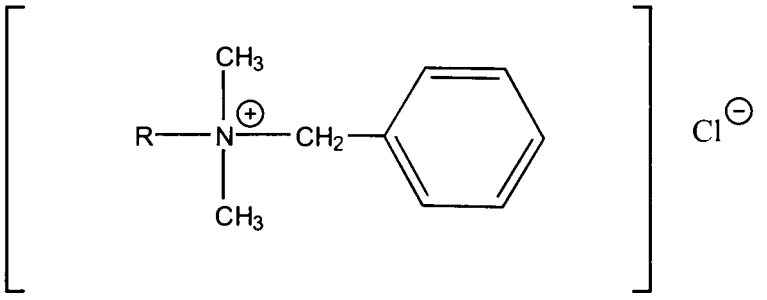
1.2 Chemical names

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

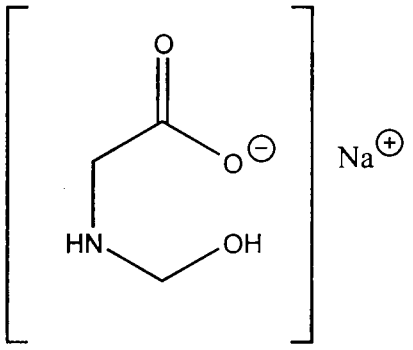
1.1 Primary name

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

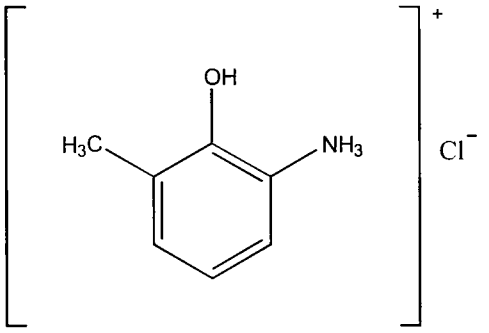
1.1 Primary name

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

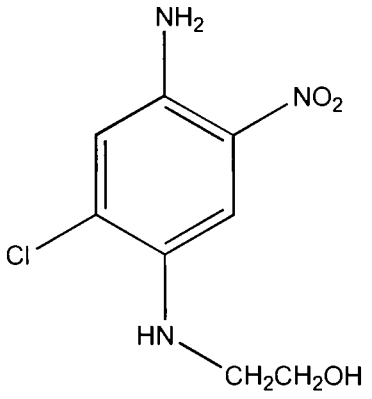
1.3 Trade names and abbreviations

Colipa No. B49

1.4 CAS no.

not given

1.5 Structural formula



1.6 Empirical formula

Emp. formula: $C_{10}H_{10}ClN_3O_3$
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
Species: guinea-pig
Method: Landstein. Draize

Conc.induc.: 0.5 %
Conc.chall.: > 0.0005 %

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
Species: guinea-pig
Method: Magnusson Kligman

Conc.induc.: 3 %
Conc.chall.: 3, 2, 1 %

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	Rot X	HPLC	20	ng/ml		
*urine	Rot X	HPLC	6	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 (1 %). 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

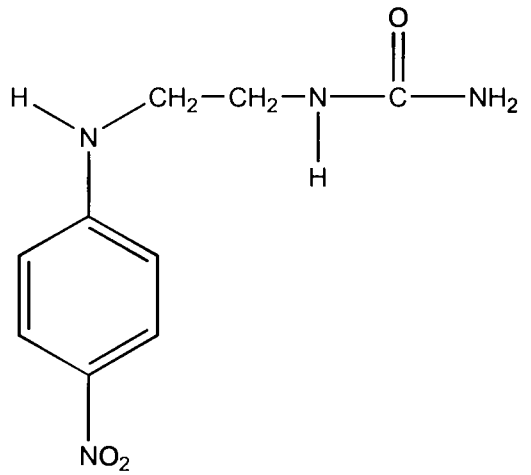
1.2 Chemical names

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 typhinurium* 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

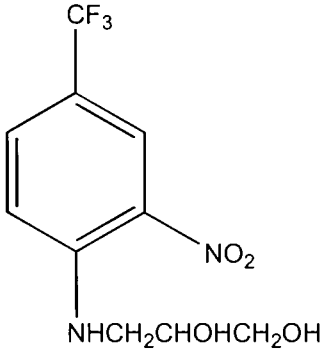
1.3 Trade names and abbreviations

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

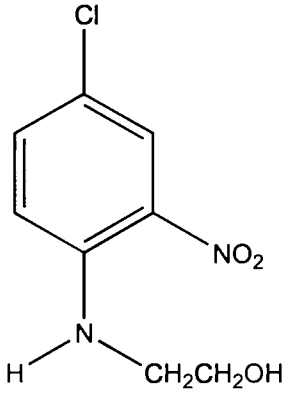
1.2 Chemical names

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: $C_8H_9N_2O_3C_l$
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

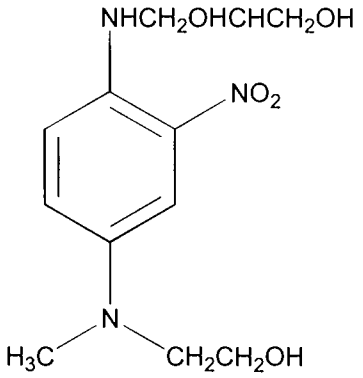
1.2 Chemical names

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: $C_{12}H_{19}N_3O_5$
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

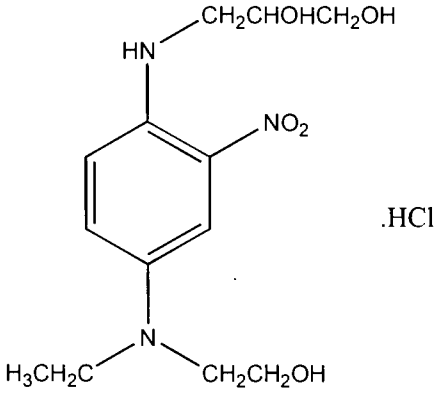
1.3 Trade names and abbreviations

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

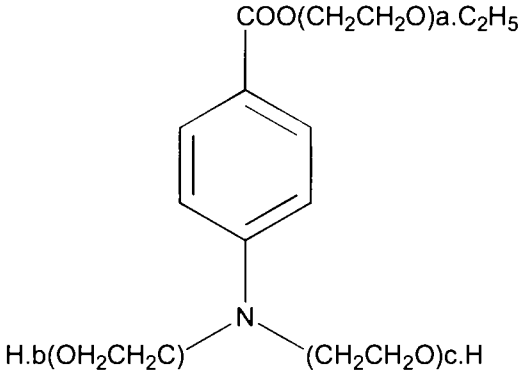
1.1 Primary name

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

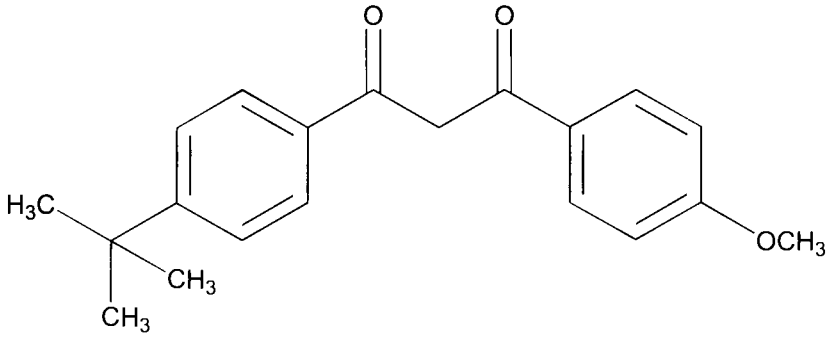
1.2 Chemical names

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

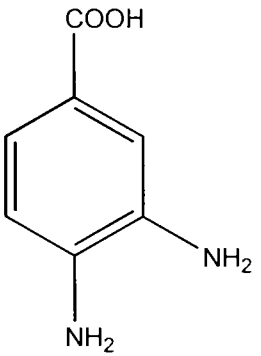
1.1 Primary name

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

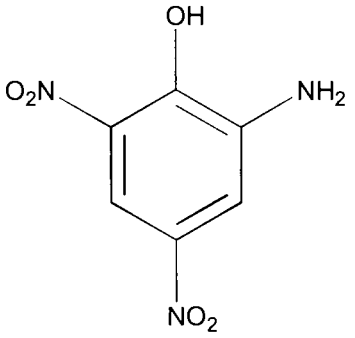
1.1 Primary name

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

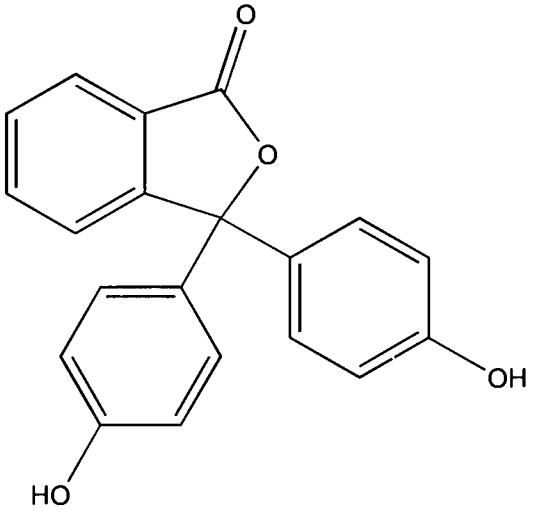
1.1 Primary name

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

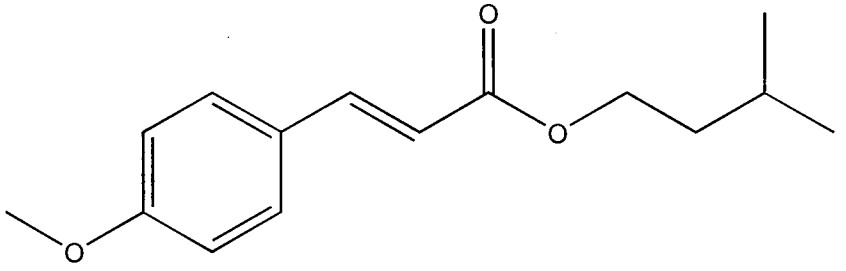
Propenoic acid 3-(4-methoxyphenyl)-3-methylbutyl ester, mixed isomers

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

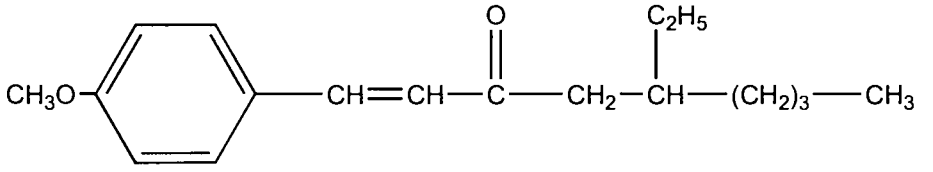
1.2 Chemical names

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

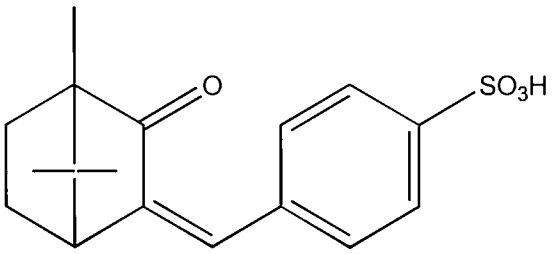
alpha'-(2-oxoborn-3-ylidene)-toluene-4-sulphonic acid

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

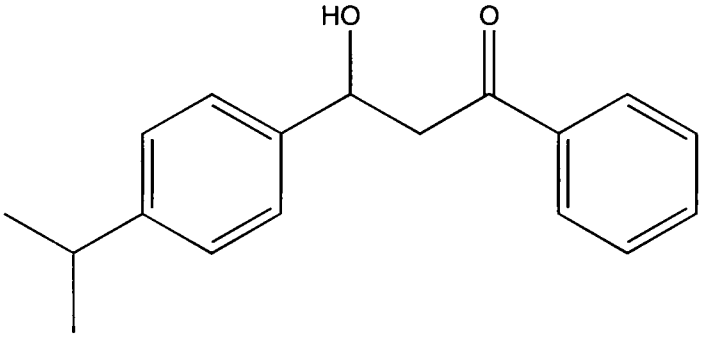
1-p-cumenyl-3-phenylpropane-1,3-dione

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

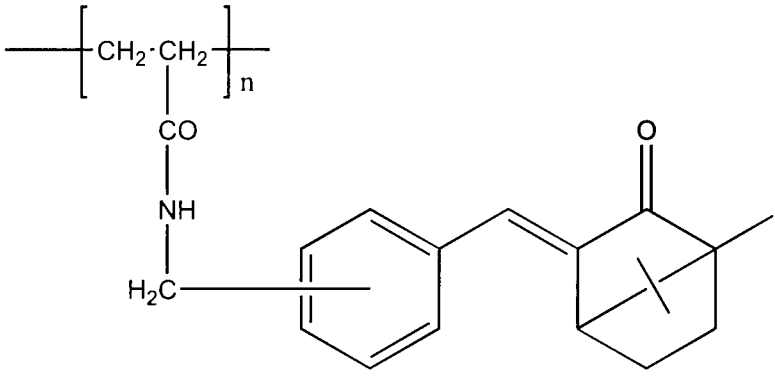
1.1 Primary name

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

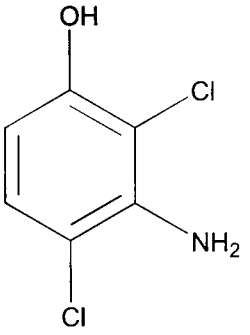
1.3 Trade names and abbreviations

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.

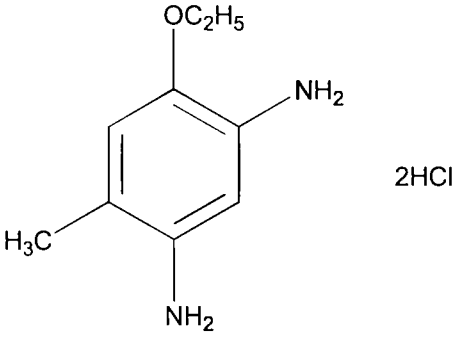
1.2 Chemical names

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 ≥ 717 µg/ml, in the absence of S9-mix ≥ 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

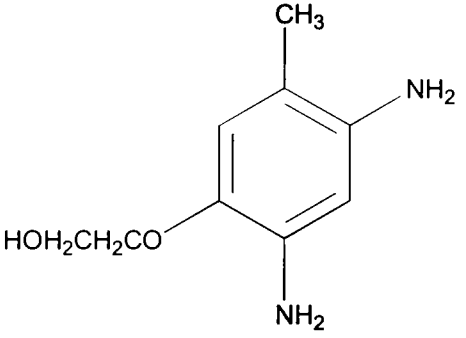
1.1 Primary name

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

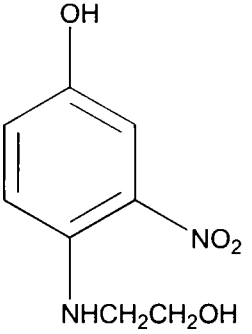
1.1 Primary name

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

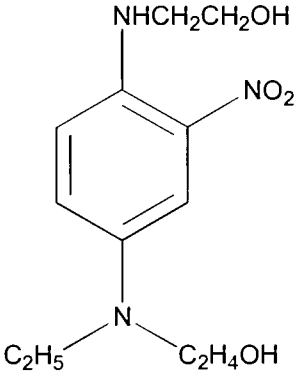
1.1 Primary name

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

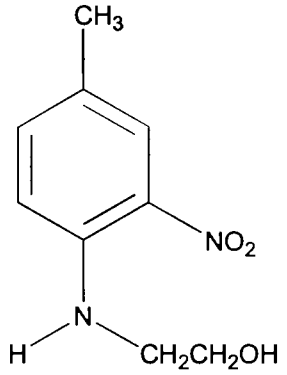
1.2 Chemical names

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

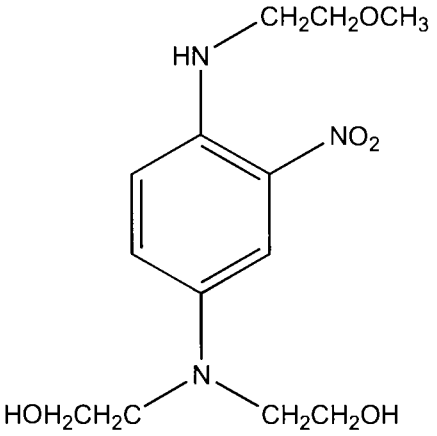
1.3 Trade names and abbreviations

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.

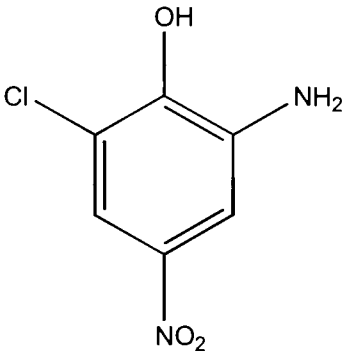
1.2 Chemical names

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

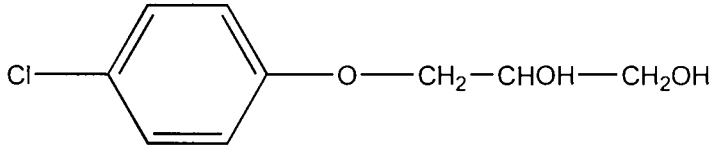
1.2 Chemical names

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

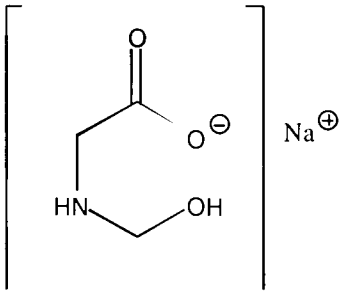
1.1 Primary name

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

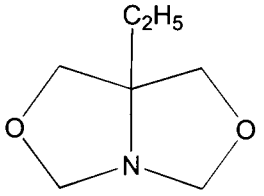
1.1 Primary name

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 ± 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

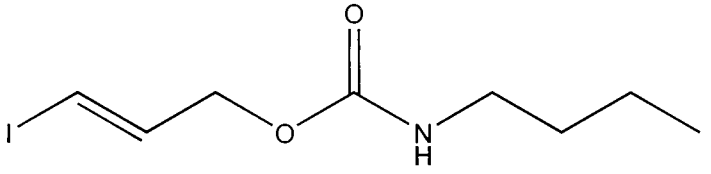
1.2 Chemical names

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

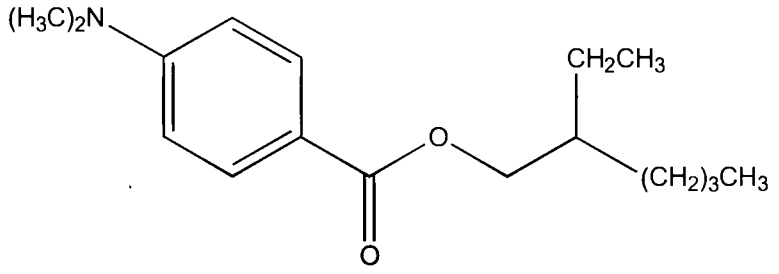
1.2 Chemical names

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

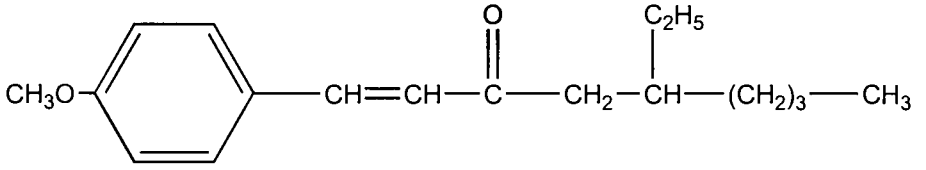
1.2 Chemical names

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 $\mu g/cm^2$. 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.

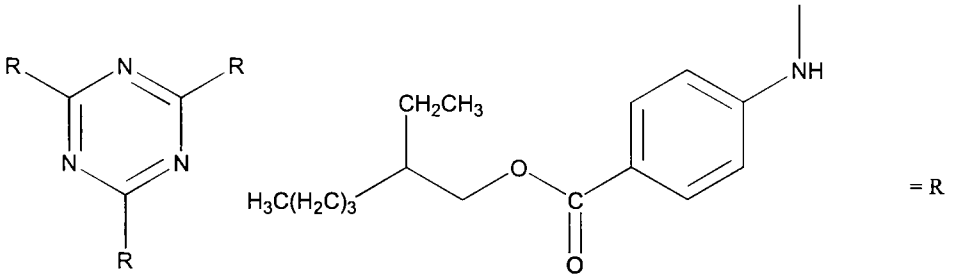
S 69: *2,4,6-TRIANILINO-(P-CARBO-2'-ETHYLHEXYL-1'-OXI) 1,3,5-TRIAZINE*

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

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

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_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 0.21 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 $SrC_{12}6H_2O$ 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

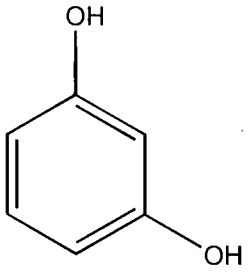
1.2 Chemical names

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

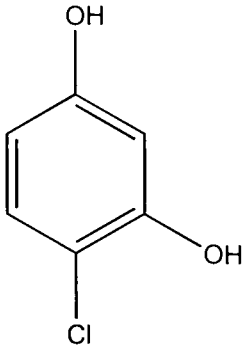
1.2 Chemical names

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

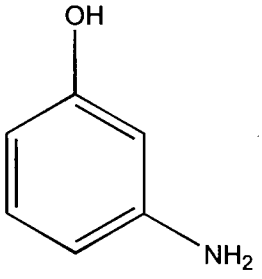
1.2 Chemical names

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

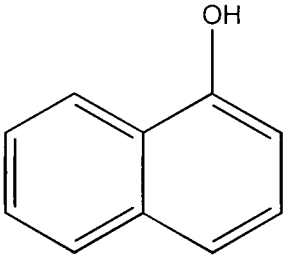
1.2 Chemical names

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

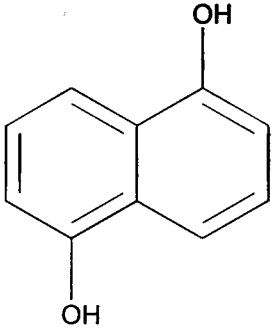
1.3 Trade names and abbreviations

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

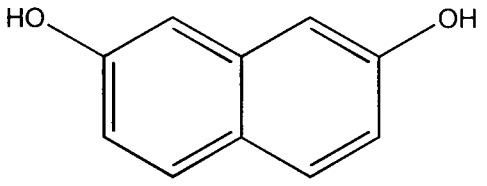
1.3 Trade names and abbreviations

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

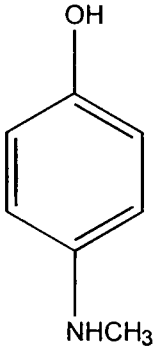
1.3 Trade names and abbreviations

IFG 62/78

1.4 CAS no.

150-75-4

1.5 Structural formula



1.6 Empirical formula

Emp. formula: C_7H_9NO
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

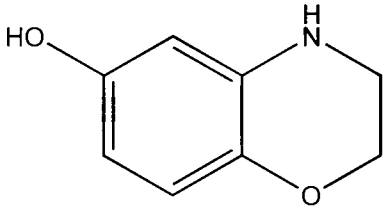
1.3. Trade names and abbreviations

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) = 1 (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

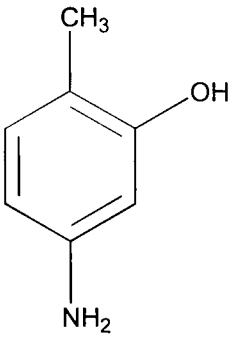
1.2 Chemical names

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

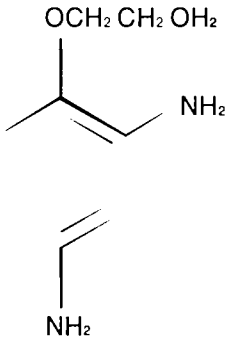
1.2 Chemical names

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

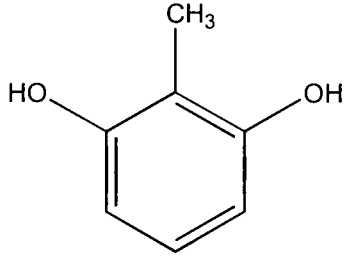
1.3. Trade names and abbreviations

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-methylresorcinol 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 42: *2,4-DIAMINOPHENOXYETHANOL DIHYDROCHLORIDE*

1. General

1.1 Primary name

2,4-diaminophenoxyethanol dihydrochloride

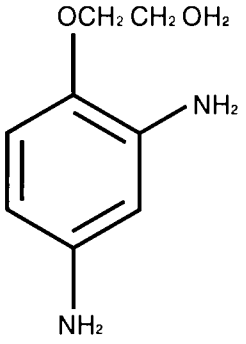
1.2 Chemical names

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} 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

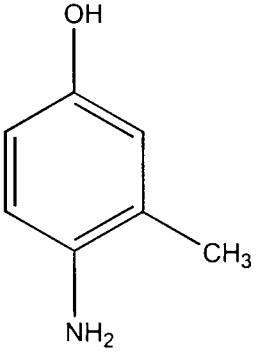
1.3 Trade names and abbreviations

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

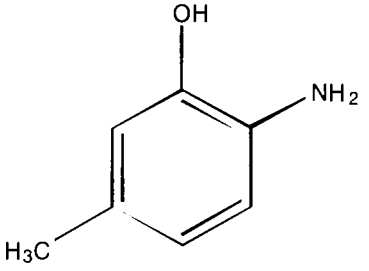
1.3 Trade names and abbreviations

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

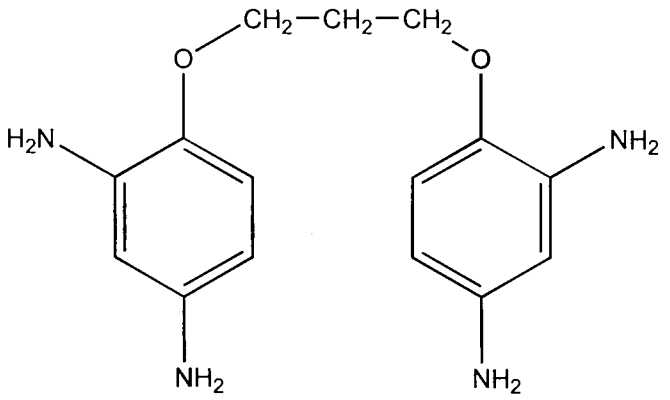
1.3 Trade names and abbreviations

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

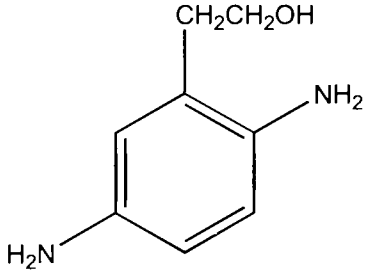
1.3 Trade names and abbreviations

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

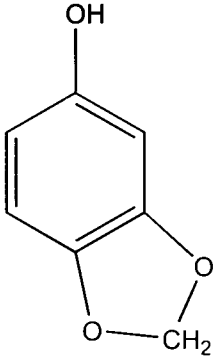
1-hydroxy-3,4-methylenedioxybenzene

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

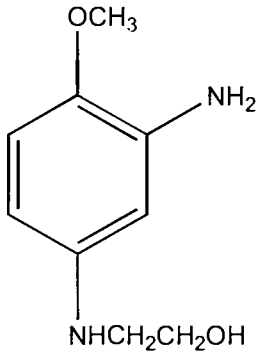
1.2 Chemical names

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)

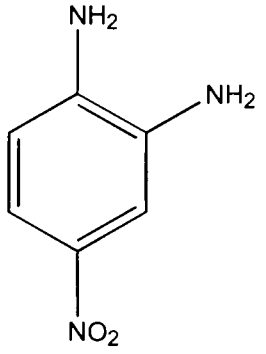
1.2 Chemical names

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 ^3H present in the excreta and carcasses of the animals 48 hours after application. The results showed that 2.2 $\mu g/cm^2$ (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

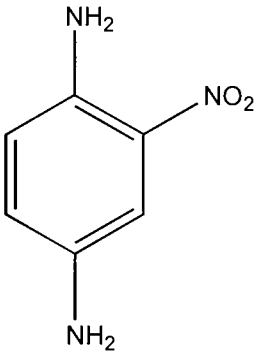
o-nitro-p-phenylenediamine

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. [$^{14-C}$]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)

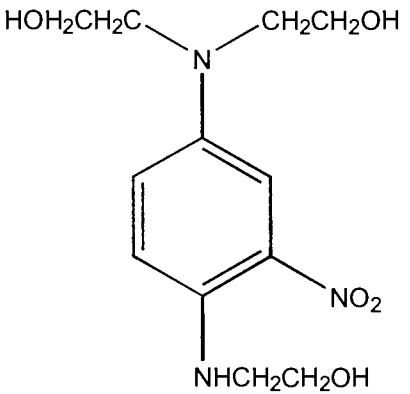
1.3 Trade names and abbreviations

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/cm² (after 5 min), 0.07 µg/cm² (after 10 min), 0.08 µg/cm² (after 20 min) or 0.10 µg/cm² (after 30 min) in rat; and 0.04 µg/cm² 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/cm², 0.02 µg/cm², 0.10 µg/cm² and 0.12 µg/cm² 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/cm² (single), 0.23 mg/cm² (2 applications), 0.60 mg/cm² (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 cm²), 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/cm² for clipped skin or 0.03 mg/cm² 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 Benzylhemiformal/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 11th 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.	3.80	4.56
	9453	10	0.22	2.60	3.01	3.31	2.	3.01	3.31
	9589	4	0.01	2.97	2.98	3.00	2.	2.98	3.00
	9590	9	0.12	2.92	3.07	3.32	2.	3.06	3.32
	9591	5	0.12	2.77	2.94	3.09	2.	2.98	3.09
	9592	5	0.07	3.00	3.10	3.18	3.	3.11	3.18
	TOTAL	42	0.40	2.60	3.20	4.56	2.	3.08	4.52
Pituitary Weight (mg)	8109	9	5.63	14.00	22.22	30.00	14	20.00	30.00
	9453	10	9.20	13.00	27.70	42.00	13	24.00	42.00
	9589	4	13.60	25.00	41.25	56.00	25	42.00	56.00
	9590	9	8.79	23.00	33.22	51.00	23	32.00	51.00
	9591	5	7.79	35.00	44.80	56.00	35	43.00	56.00
	9592	5	8.94	29.00	41.00	53.00	29	41.00	53.00
	TOTAL	42	11.43	13.00	32.62	56.00	13	31.00	56.00
Relative Pituitary Weight (mg/100g b.w.)	8109	9	0.17	0.34	0.59	0.85	0.	0.55	0.85
	9453	10	0.29	0.39	0.92	1.33	0.	0.89	1.33
	9589	4	0.46	0.84	1.38	1.87	0.	1.41	1.87
	9590	9	0.28	0.75	1.08	1.63	0.	0.96	1.63
	9591	5	0.23	1.17	1.52	1.81	1.	1.52	1.81
	9592	5	0.28	0.94	1.32	1.70	0.	1.37	1.70
	TOTAL	42	0.41	0.34	1.05	1.87	0.	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

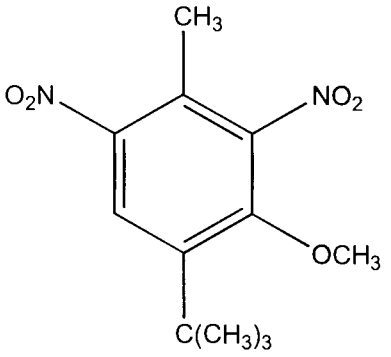
1.2 Chemical names

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

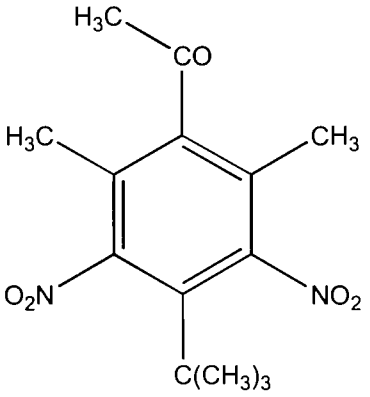
1.2 Chemical names

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®(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

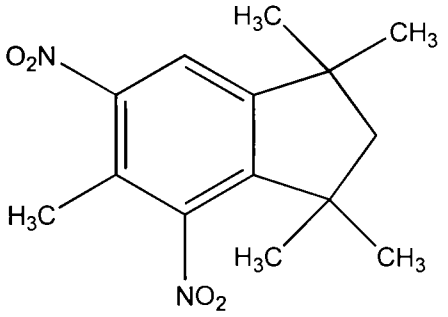
1.2 Chemical names

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

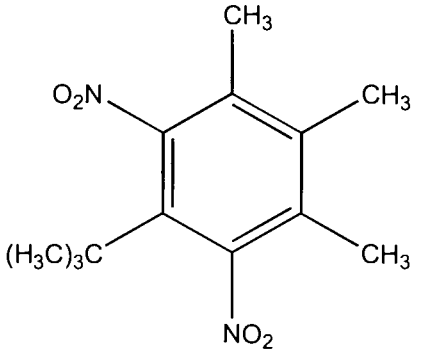
1.2 Chemical names

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

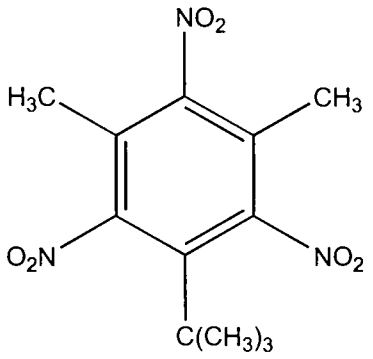
1.2 Chemical names

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®(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. It 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

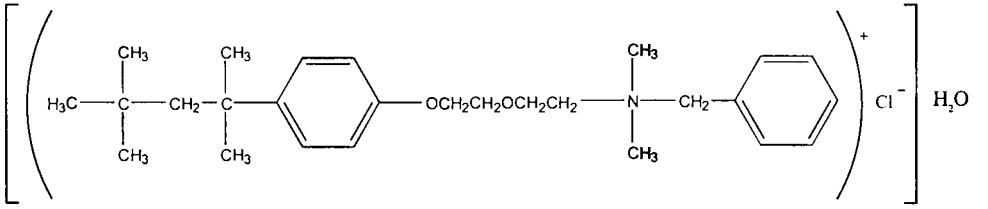
1.1 Primary name

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/cm²/hr) with 4.56 µg/cm in the stratum corneum in 30 minutes. Percutaneous penetration rate was calculated as 51 µg/cm²/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/cm²/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

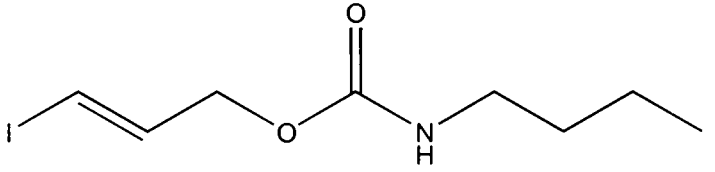
1.2 Chemical names

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

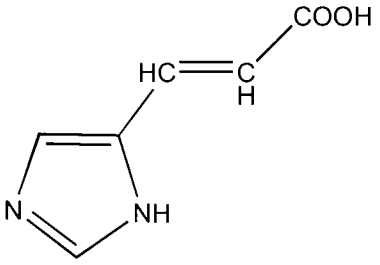
Urocanic acid

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 x 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 x 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-1$^+$ 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-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 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 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 x 10^{-3}; UVB 5.4 x 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

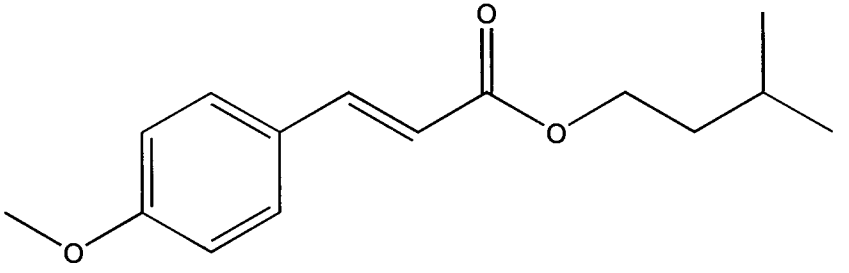
1.1 Primary name

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 1 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).

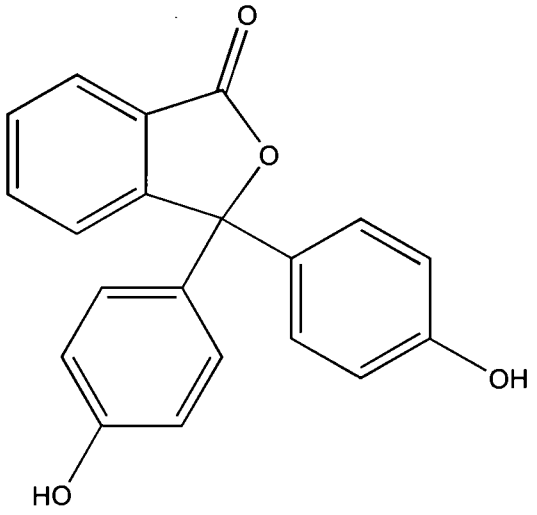
1.1 Primary name

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

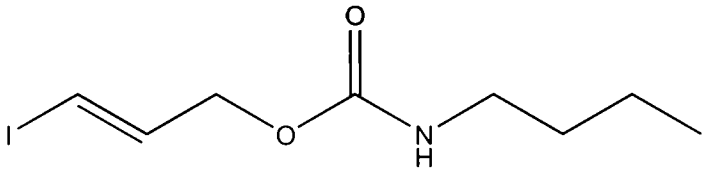
1.2 Chemical names

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 bygiene 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

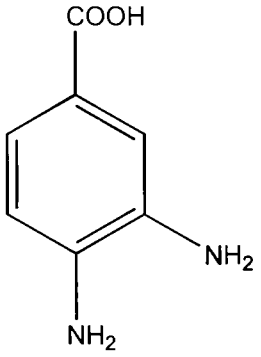
MARGIN OF SAFETY NOAEL/SED = 300

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

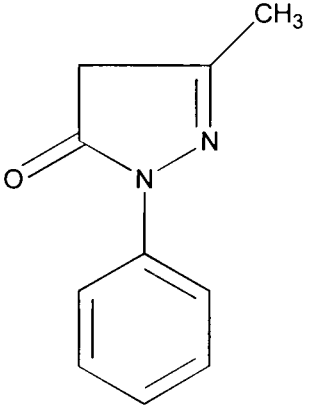
1.3 Trade names and abbreviations

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

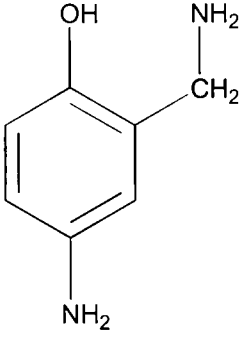
1.3 Trade names and abbreviations

Colipa No.: A112

1.4 CAS no.

79352-72-0

1.5 Structural formula



2HCl

1.6 Empirical formula

Emp. formula: $C_7 H_{10} N_2 O$. 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

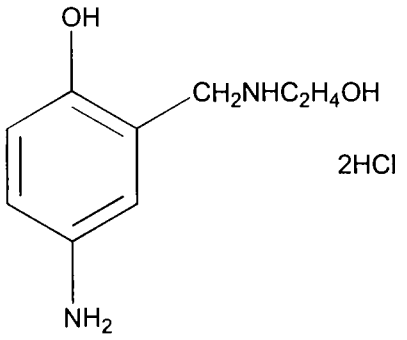
1.2 Chemical names

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: $C_9 H_{14} N_2 O_2 \cdot 2$ 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

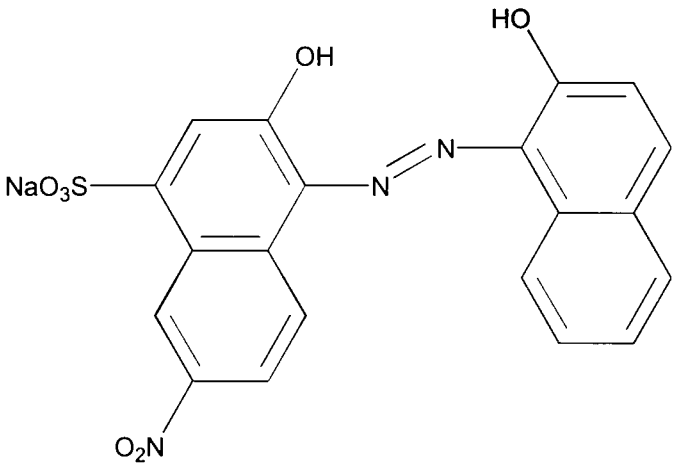
1.3 Trade names and abbreviations

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
Species: rat
Subst.: sA

Exposure: 90 days
Recov.p.: 4 weeks

DWE: 45 mg/kg b.w.
LED:100 mg/kg b.w.

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

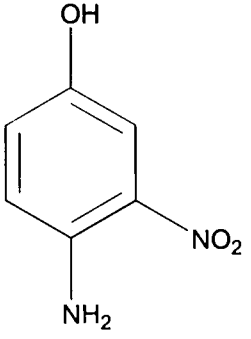
1.3 Trade names and abbreviations

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

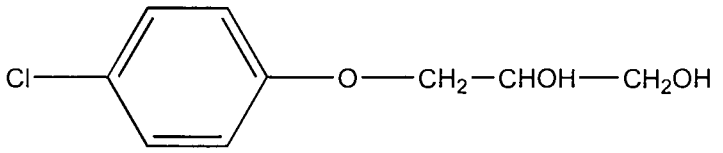
1.2 Chemical names

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

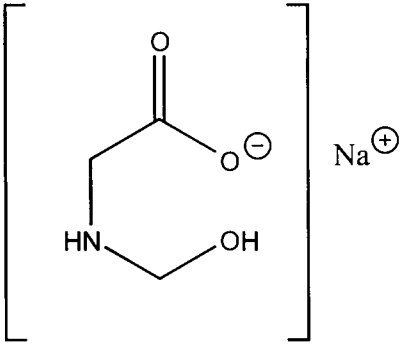
1.2 Chemical names

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

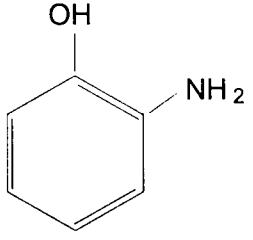
Colipa no.: A 14

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 $\mu g/cm^2$) 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

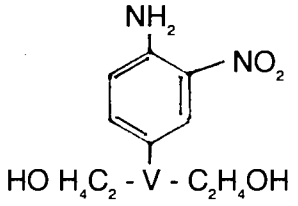
1.2 Chemical names

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

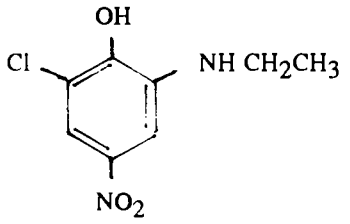
1.3 Trade names and abbreviations

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	≥ 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.I 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

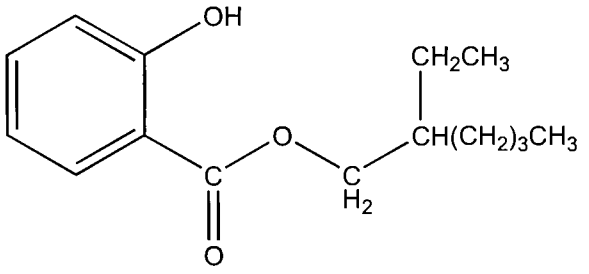
1.3 Trade names and abbreviations

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

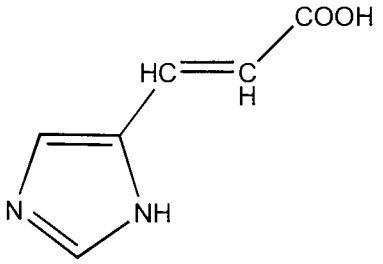
1.1 Primary name

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 $\mu g/cm^2$). 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 $Thy\text{-}1^+$ 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 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

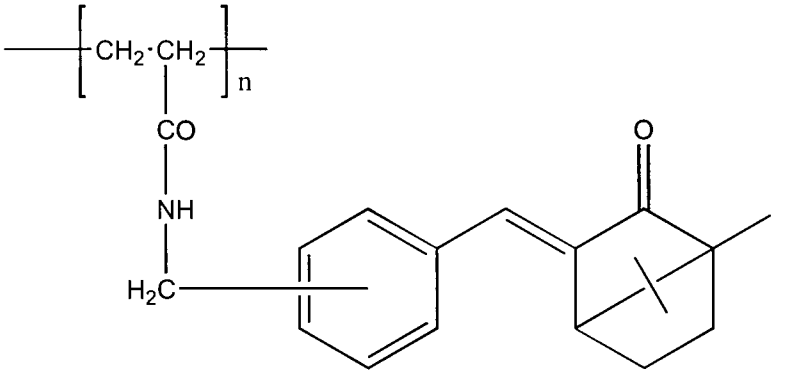
1.1 Primary name

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 III 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	$21.44.10^{1}$	3.10^{1}	2.10^{1}	10.10^{1}	16.10^{1}	
Repartition and/or rinse off coefficient	%				10 %	100 %	
Daily exposure/ products	mg/kg/d	$21.44.10^{1}$	3.10^{1}	2.10^{1}	1.10^{1}	$0.16.10^{1}$	$27.6.10^{1}$
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

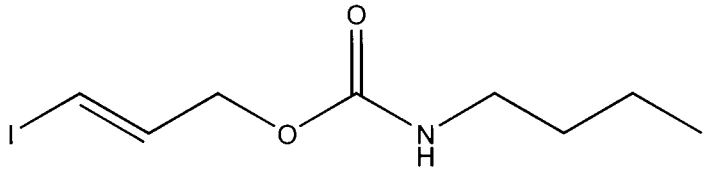
3-iodo-2-propynyl butylcarbamate

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 1 % 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 1 % 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 ± (barely perceptible) reaction). However, the three grade 1 responses were not re-elicited after rechallenge (maximum grades of ± 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 (± SD; n = 24) skin penetration (the sum of radioactivity recovered in the dermis and receptor fluid) was 54 ± 12 % (38 ± 5 %, 54 ± 10 %, 55 ± 3 % and 68 ± 6 % for the respective individual donors). Peak penetration into the receptor fluid occurred within 2-8 hours of application for all donors. 14 ± 3 % of the applied radioactivity evaporated from the skin surface during the 24-hour experimental period. Overall recovery of radioactivity was 87 ± 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

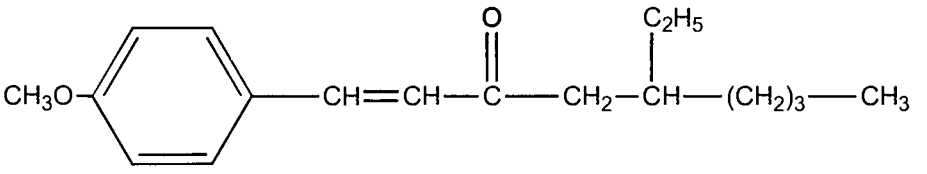
1.2 Chemical names

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 $\mu g/cm^2$. 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 photomutagenic 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:

$$24/1.286 = \mathbf{19}$$

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:

$$1.286 \text{ mg/kg x } 0.5 \text{ \% absorption} = \mathbf{0.00643 \text{ mg/kg b.w.}}$$

taking a NOAEL of 24 mg/kg into account, the safety margin would be:

$$24 : 0.00643 = \mathbf{3732}$$

- The calculated daily rat body load by percutaneous absorption would be:

$$1.286 \text{ mg/kg x } 20 \text{ \% absorption} = 0.2572 \text{ mg/kg b.w.}$$

taking the same NOAEL of 24 mg/kg into account, the safety margin would then be:

$$24 : 0.2572 = \mathbf{93}$$

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.

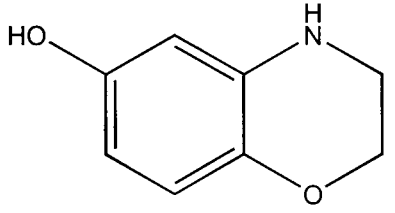
1.3 Trade names and abbreviations

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/cm²/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/cm²/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

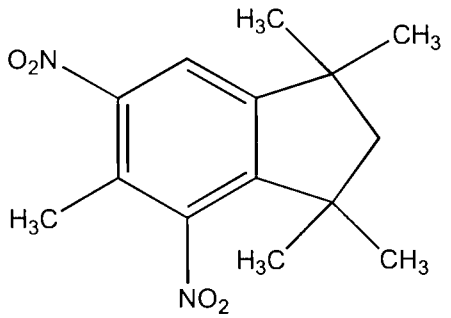
1.2 Chemical names

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 µl/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

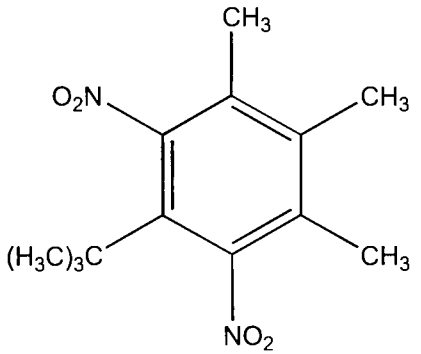
1.2 Chemical names

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

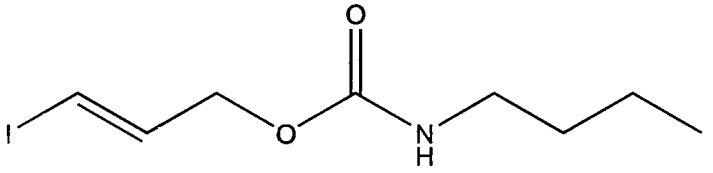
1.2 Chemical names

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

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 1 % 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 1 % 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 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 ± (barely perceptible) reaction). However, the three grade 1 responses were not re-elicited after rechallenge (maximum grades of ± 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 ≤ 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

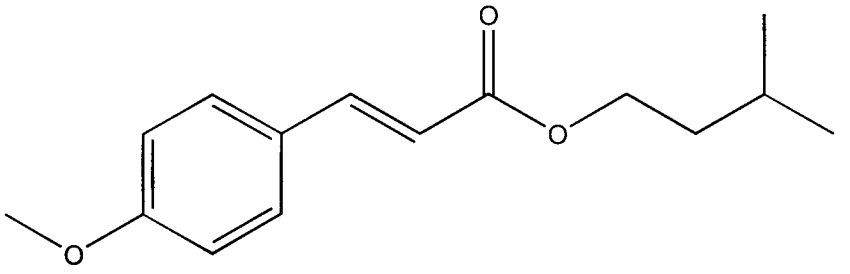
1.3 Trade names and abbreviations

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

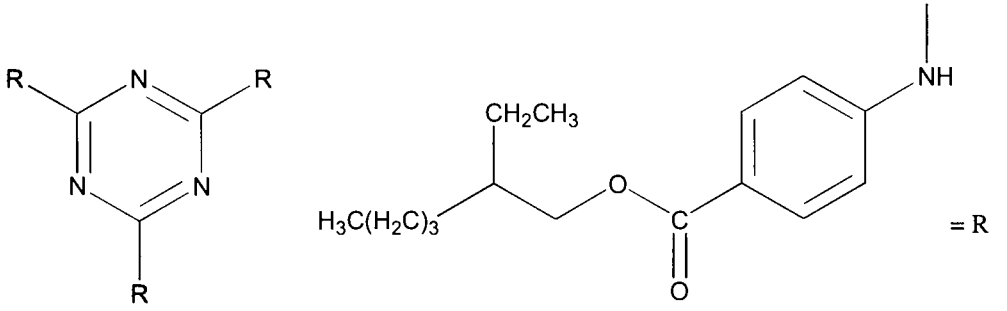
S 69: *ISOCTYLTRIAZONE*

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)

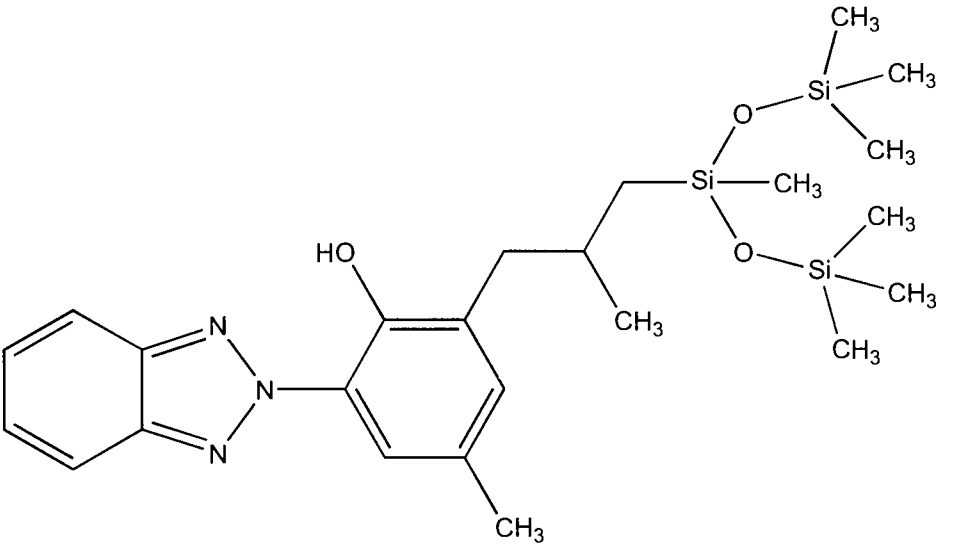
1.2 Chemical names

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: $C_{24}H_{36}N_3O_3Si_3$
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 (Icolbm: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 1½ 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

Reports of the Scientific Committee on Cosmetology, 9th Series

Luxembourg: Office for Official Publications of the European Communities

1999 — 1008 pp. — 16.2 x 22.9 cm

ISBN 92-828-8951-3

15

ND-27-99-249-EN-C

OFFICE FOR OFFICIAL PUBLICATIONS
OF THE EUROPEAN COMMUNITIES

L-2985 Luxembourg

ISBN 92-828-8951-3