EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON

4-NITRO-O-PHENYLENEDIAMINE

COLIPA N° B24

Scientific Committees
- on consumer products
- on emerging and newly identified health risks
- on health and environmental risk

The SCCP adopted this opinion at its 10th plenary on 19 December 2006

About the Scientific Committees
Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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Opinion to be cited as: Opinion of the SCCP on 4-nitro-o-phenylenediamine (COLIPA n° B24)

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1. BACKGROUND

Submission I for 4-Nitro-*o*-phenylenediamine was submitted in December 1979 by COLIPA [1, 2].

Submission II for this substance was submitted in December 1981 by COLIPA.

Submission III for this substance was submitted in June 1985 by COLIPA.

Submission IV for this substance was submitted in February 1986 by COLIPA.

Submission V for this substance was submitted in November 1987 by COLIPA.

The Scientific Committee on Cosmetology (SCC) in its 48th plenary meeting of 4 October 1991 adopted an opinion (CSC/857/91) on 4-Nitro-*o*-phenylenediamine with the conclusion that:

"*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*,"

The SCC adopted on its 54th plenary meeting of 10 December 1993 a revision of the opinion on 4-Nitro-*o*-phenylenediamine. In this opinion, under general, it is cited:

"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 13 December 1985: EUR 8634, p.1, 1980).

Revision 17 October 1986
-Banned in Italy and Denmark; recommended for banning in Federal Republic of Germany.
-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."

And with the conclusion:
"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

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

[2] According to records of COLIPA

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"

Submission VI for this substance was submitted by COLIPA in July 2005.
According to this submission the substance is used in oxidative hair dyes to a maximum concentration of 1.0%. After mixing 1:1 with hydrogen peroxide, the concentration on the scalp is 0.5%.
Submission VI presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 4-Nitro-o-phenylenediamine safe for use as an oxidative hair dye with an on-head concentration of maximum 0.5 % taken into account the scientific data provided?*

2. *Does the SCCP recommend any further restrictions with regard to the use 4-Nitro-o-phenylenediamine in oxidative hair dye formulations?*

3. OPINION

3.1. Chemical and Physical Specifications

All physico-chemical properties relate to 4-Nitro-o-phenylenediamine,free base.

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

4-Nitro-o-phenylenediamine

3.1.1.2.	Chemical names

1,2-Diamino-4 nitrobenzene
2-Amino-4-nitroaniline
4-Nitro-1,2-diaminobenzene
4-Nitro-1,2-phenylenediamine
p-Nitro-o-phenylenediamine

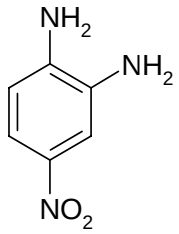
3.1.1.3. Trade names and abbreviations

CI 76020
COLIPA n° B24

3.1.1.4. CAS / EINECS number

	Free base	*Sulphate*	*Dihydrochloride*
CAS:	99-56-9	68239-82-7	6219-77-8
EINECS:	202-766-3	269-476-7	228-293-2

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_6H_7N_3O_2$

3.1.2. Physical form

Orange-red powder

3.1.3. Molecular weight

Molecular weight: 153.14

3.1.4. Purity, composition and substance codes

Purity and impurities in various batches of 4-nitro-o-phenylenediamine

Description	**Batch**		
	A9366	**Rodol 4JP Lot No. I 36465**	**Rodol 4JP Lot No. I 31327***
Chemical Characterisation	NMR, GC-MS, UV-Vis, elemental analysis		NMR, GC-MS*
Content, % peak area **HPLC-MS-UV analysis** **GC-MS analysis**	 99.7 99.7	 99.4 99.6	> 99 (GC-MS and LC-MS)*
Water content, % (w/w)	0.15	0.20	-
Ash, % (w/w)	<0.1	<0.1	-

* No raw data provided

3.1.5. Impurities / accompanying contaminants

2,4-Dinitroaniline: max. 0.60 % (w/w)

2-Nitro-p-phenylenediamine:	max. 0.06% (w/w) (listed in Directive 76/768/EEC, Annex III, part 2 n° 46; Classified by Germany MAK as carcinogen group 3B)

Unknown (tentatively identified as brominated 4-nitro-o-phenylenediamine): 0.17 % (GC-MS TIC peak area)

Fe:	≤ 44 ppm
Hg:	<0.5 ppm
AS:	<2 ppm
Cr, Cd, Co, Ni, Pb, Sb:	<1 ppm

3.1.6. Solubility

Water: 0.126 g/l, at 21.5±0.5ºC, pH 6.5

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.845 at 21.5±°C

3.1.8. Additional physical and chemical specifications

Melting point:	201 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	1.09×10^{-4} mm Hg
Density:	/
Viscosity:	/
pKa:	2.61
Refractive index:	/

3.1.9. Stability

4-Nitro-o-phenylenediamine is stable for minimal 3 months
4-Nitro-o-phenylenediamine solutions (1, 3 and 9 mg/ml in 1% CMC) were homogeneous and stable (within ±10%) up to 7 days
4-Nitro-o-phenylenediamine solution (200 mg/ml in 1% CMC) was homogeneous and stable (within ±15%) up to 7 days
Temperature for stability testing is not mentioned in the report

Comments on physicochemical characterisation

- No data is provided for the stability of 4-nitro-o-phenylenediamine in marketed products.

3.2. Function and uses

4-Nitro-o-phenylenediamine is used in oxidation hair dye formulations at a maximum concentration of 1.0 %, which after mixing typically in a 1:1 ratio with hydrogen peroxide just prior to use, corresponds to a concentration of 0.5% upon application.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: Rat, CFY
Test substance: 4-nitro-o-phenylenediamine
Batch: /
Purity: /
Vehicle: 36% suspension in aqueous gum tragacanth (0.5%) containing sodium sulphite (0.05%)
GLP: Not in compliance

Rats, 5 per sex/dose were administered the test substance by gavage (dose levels not reported). Shortly after dosing clinical signs observed were lethargy and piloerection. The acute median lethal oral dose of 4-nitro-o-phenylenediamine in rats was calculated to be 2100 (95% confidence limits: 1800-2500) mg/kg bw. Since, the results of acute oral toxicity tests of multiple substances were reported together, other substance specific results were lacking.

Ref.: 22

A LD_{50} of 681 mg/kg bw after oral administration is reported both for mouse and rat by ChemIDplus.

Ref.: A

The oral LD_{50} of 1,2-diamino-4-nitrobenzene in oil-in-water emulsion so in rats is 3720 mg/kg bw; the intraperitoneal LD_{50} of the compound in dimethylsulfoxide in rats is > 1600 mg/kg bw.

Ref.: B

Comment
The references are publications in open literature. Therefore no raw data are available. Despite the deficiencies of this study (purity and batch number unknown, not according to a guideline, no raw data available) the studies are useful for evaluation.

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: Conducted to US Code of Federal Regulations, Title 16, Sec. 1500.41
Species: New Zealand White rabbits
Group: 3 (sex not stated)
Substance: 4-nitro-o-phenylenediamine
Batch: not stated

Purity:	not stated
Dose:	2.5% w/v aqueous solution/suspension in 0.5% gum tragacanth
GLP:	not in compliance

Application Test solution applied to the intact and abraded skin of the rabbits. Observation methodology or intervals not specified (24 hour observation period mentioned) but observation of symptoms 72 hour observation period.

Results
None of the treated animals showed any response to treatment.

Conclusion
Under the conditions of the experiment, 2.5% w/v 4-nitro-o-phenylenediamine was not irritant to the rabbit skin.

Ref.: 22

3.3.2.2. Mucous membrane irritation

Guideline:	Conducted to US Code of Federal Regulations, Title 16, Sec. 1500.42
Species:	New Zealand White rabbits
Group:	3 (sex not stated)
Substance:	4-nitro-o-phenylenediamine
Batch:	not stated
Purity:	not stated
Dose:	2.5% w/v aqueous solution/suspension in 0.5% gum tragacanth
GLP:	not in compliance

Test preparation instilled into one eye of each rabbit; volume not specified. The eyes were irrigated with 50ml lukewarm (37°C) water 10 seconds after instillation of the test material. Observation methodology or periods not specified, but at least 3 days.

Results
Transient mild conjunctival inflammation observed, not persisting more than 24 hours.

Conclusion
Under the conditions of the experiment, 2.5% w/v 4-nitro-o-phenylenediamine was mildly irritant to the rabbit eye.

Ref.: 22

3.3.3. Skin sensitisation

Guideline:	/
Species:	Hartley albino guinea pigs
Group:	10 (sex not stated)
Substance:	4-nitro-o-phenylenediamine
Batch:	not stated
Purity:	not stated
Dose:	1% test substance dispersed in white petrolatum (50 mg of active)
GLP:	not in compliance

In a ranging study, concentrations of 0.1, 0.2, 0.5, 1, 2, 5 and 10% of the test material dispersed in white petrolatum did not produce any irritant reactions.

Induction phase
Test formulation 1% test material dispersed in white petrolatum (50mg in white petrolatum) applied under occlusion for 48 hours to the neck; repeated three times per week for two weeks.

Challenge phase
After resting for two weeks, 0.1% to 1% dispersions of the test substance in white petrolatum were applied to flanks occluded for 48 hours. Reactions were evaluated at 24 and 48 hours.

No Negative control.

Conclusion
Under the conditions of the experiment, 1% dispersion of 4-nitro-o-phenylenediamine was not shown to produce sensitisation to the guinea pig.

Ref.: 19

Comment
The study is inadequate because of the extremely low induction and challenge concentration used.

Local Lymph Node Assay (LLNA), study 1

Guideline:	OECD 429 (2002)
Species:	CBA/CaOlaHsd mice
Group:	4 female (8-12 weeks) per dose group
Substance:	4-NOPD
Batch:	I-31327
Purity:	99.7%
Dose:	Test material dissolved in Acetone:Olive Oil 4:1 (v/v). Dose levels and Negative Control Concentrations of 0.05, 0.1, 0.25, 0.5 and 1% tested. Vehicle control
GLP:	in compliance

25 µl of test material or vehicle control were applied to the dorsal surface of both ear lobes once daily for 3 consecutive days. 5 days after the first application animals were injected *iv* with 250µl ^{3}H-methyl thymidine in the tail vein. Mice were killed by Vetanarocol *ip* 5 hours later. Draining lymph nodes were excised and pooled to prepare a single cell suspension for each group. Thymidine incorporation was measured by β-scintillation counting. The disintegrations per minute per lymph node (DPM/node) was measured and expressed as the ratio of the control group (stimulation index, S.I.).

Results

Concentration % w/v	S.I.
0.05	3
0.1	5.6
0.25	5.3
0.5	8.5
1	14.4

Slight ear swelling was observed in the animals treated with 0.5 and 1% 4-NOPD. All doses had S.I. of at least 3. An EC3 was not calculated, as no dose gave a S.I. of less than 3.

Conclusion
The results indicate that 4-NOPD is an extremely potent skin sensitiser.

Ref.: 53

Local Lymph Node Assay (LLNA), study 2

Guideline:	OECD 429 (2002)
Species:	CBA/CaOlaHsd mice
Group:	4 female (8-12 weeks) per dose group
Substance:	4-NOPD
Batch:	A9366
Purity:	not stated
Dose:	Test material dissolved in Acetone:Olive Oil 4:1 (v/v). Dose levels and Negative Control Concentrations of 0.01, 0.025, 0.075, 0.125 and 0.2% tested. Vehicle control
GLP:	in compliance

25 µl of test material or vehicle control were applied to the dorsal surface of both ear lobes once daily for 3 consecutive days. 5 days after the first application animals were injected *iv* with 250µl 3H-methyl thymidine in the tail vein.
Mice were killed by CO_2 5 hours later. Draining lymph nodes were excised and pooled to prepare a single cell suspension for each group. Thymidine incorporation was measured by β-scintillation counting. The disintegrations per minute per lymph node (DPM/node) was measured and expressed as the ratio of the control group (stimulation index).

Results

Concentration % w/v	S.I.
0.01	1
0.025	1.5
0.075	2.2
0.125	2.0
0.2	1.8

No dose response relationship was observed. No test concentrations produced a stimulation index of 3 or higher.

Conclusion
Under the conditions of the experiment, 4-NOPD did not induce contact allergy when tested at concentrations below 0.2%. The purity of the test substance was not known.

Ref.: 54

3.3.4. Dermal / percutaneous absorption

***In vivo* percutaneous absorption (rat)**

Guideline:	/
Species:	Colworth Wistar rats
Group:	5 female
Substance:	^{3}H 4-NOPD
Batch:	/
Purity:	/
Dose levels:	0.6% 4-NOPD diluted with water to a 50% solution.
Test formulation:	Test compound was applied in shampoo/water base (1:1)

GLP: not in compliance

Hair from the back of each rat was removed using clippers 24 hours prior to treatment. 200 µl of hair dye solution was applied to 10cm^2 of skin for 20 minutes; then rinsed off with distilled water. The treated skin was covered with a non-occlusive patch. The animals were placed in individual metabolism cages to collect urine, faeces and expired air for 48 hours. Animals were then sacrificed. Levels of ^{3}H 4-NOPD was measured using a liquid scintillation counter.

Results

Dye	Application (µg/cm²)	Rinsings (% applied)	Amount in the skin (µg/cm²)	Penetration (µg/cm²)
[^{3}H] 4-NOPD	120	88 ± 18	2.1 ± 0.4	2.2 ± 0.7

88% of 4-NOPD was rinsed off after 20 minutes. 2.2 µg/cm^2 (1.8% of amount applied) of 4-NOPD penetrated the skin, which was recovered from the urine, faeces and carcass.

Ref.: 47

In vitro percutaneous absorption (pig)

Guideline: OECD 428 (draft, 2002)
Species: Landrace/Large White pigs
Group: Five (3 male, 2 female), from frozen pig skin library
Substance: Hair dye formulations containing:
a) 4-Nitro-o-[U-^{14}C]-phenylenediamine dihydrochloride, SEAC sample number S2600401 – purity 98.8%
b) Unlabelled 4-NOPD Lot A9366 (purity not stated)
Test formulation: Hoyu Complete Dye Base H and developer
Unilever Complete Dye Base U and developer
Dose: 4-NOPD was included at 0.5% in both dye bases.
Complete Dye Base H mixed with developer at 1:1 w/w giving an "on skin" concentration of 0.25% 4-NOPD.
Complete Dye Base U mixed with developer at 1:1.5 w/w giving an "on skin" concentration of 0.2% 4-NOPD
GLP: in compliance

Dye composition of each of the base formulations:

Dye	Percentage by weight in base formulation	
	Hoyu base	**Unilever base**
4-nitro-o-phenylenediamine	0.50	0.50
p-phenylenediamine	0.64	/
m-aminophenol	0.25	/
o-aminophenol	0.10	/

Split-thickness skin membranes were mounted into flow-through diffusion cells. Receptor fluid (5%, v/v new born calf serum in phosphate buffered saline) was pumped underneath the skin at a flow rate of ca. 1.5 ml/h with a skin surface temperature of *ca* 32°C. The skins were allowed to equilibrate under these conditions. A barrier integrity assessment was then performed using tritiated water. Skin samples that exhibited a tritiated water permeability coefficient > 3.5 x 10^{-3} cm/h were considered to be damaged and were rejected.

After the barrier integrity test, the remaining tritiated water was removed after which a *ca* 30 minutes pre-dose collection of receptor fluid was taken. [^{14}C]-4-NOPD was applied in the two hair dye formulations (Complete Dye Base H and Complete Dye Base U) after mixing with developer. The two formulations were applied at a formulation application rate of 10µl/cm^2. Absorption was assessed by collecting receptor fluid hourly from 0-24 hours post dose. At 30 minutes post dose, the skin was washed with 12 x 0.5ml water and dried. At 24 hours post dose the skin surface was washed with 2ml water and dried. The skin was removed from the cells and tape stripped with D-SquameR tape to remove the stratum corneum. The remaining skin was divided into exposed and unexposed skin. All liquid samples were analysed by liquid scintillation counting and the remaining samples were analysed by combustion/ liquid scintillation counting.

Results
Due to the reactive nature of hair dyes, the radio-labelled test item exists in different forms once the oxidative reactions are under way. The results in the table are therefore expressed as Dg equiv/cm^2 – mean (SD). Complete Dye Base H (n=10) Complete Dye Base U (n=12).

A summary of the mean results are provided in the table below.

Hair Dye Formulation	**Complete Dye Base H**	**Complete Dye Base U**
Application rate (µl/cm²)	10	10
Application rate (mg/cm²)	6.77	7.04
NOPD Concentration (mg/g)	2.90	2.29
NOPD (% Applied Dose)	**(Mean ± SD)**	
Extractable Dose	93.99 ± 7.29	92.96 ± 6.87
Unabsorbed Dose *	96.80 ± 5.53	94.41 ± 6.21
Absorbed Dose **	0.70 ± 0.46	1.04 ± 0.54
Dermal Delivery ***	4.21 ± 3.47	2.40 ± 1.44
Mass Balance	101.01 ± 2.63	96.81 ± 5.32
NOPD (µg equiv./cm²)	(**Mean ± SD)**	
Extractable Dose	18.44 ± 1.67	14.93 ± 0.83
Unabsorbed Dose *	18.99 ± 1.38	15.16 ± 0.72
Absorbed Dose **	0.14 ± 0.09	0.17± 0.09
Dermal Delivery ***	0.83 ± 0.69	0.39 ± 0.24
Mass Balance	19.82 ± 1.04	15.55 ± 0.61

* Unabsorbed dose = extractable dose + stratum corneum + unexposed dose
** Absorbed dose = receptor fluid + receptor rinse
*** Dermal delivery = exposed skin + absorbed dose

Ref.: 52

Comment
The concentration used in these experiments was approximately half that of the use concentration in hair dye products (not conform to the SCCP Notes of Guidance). The total absorbed of 4-NOPD was equivalent to 0.83 ± 0.69 µg eq/cm² and 0.39 µg equiv/cm² in the two hair dye formulations tested. The highest value (Complete Dye Base H) observed was 2.15 µg eq/cm² since the formulation contained 0.29% 4-NOPD a value of (2.15 x 0.50/0.29) 3.6 µg eq/cm² will be used for the calculation of the margin of safety.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

13-week study in rats

Guideline:	OECD 408 (1998)
Species/strain:	Rat, HanBrl:WIST (SPF)
Group size:	15 animals per sex and dose
Test substance:	4-nitro-o-phenylenediamine
Batch:	Lot: A9366
Purity:	Specified in other study to be >99%
Dose levels:	0, 5, 15 and 45 mg/kg bw/day
Vehicle:	Carboxymethylcellulose as a 1% aqueous solution
Route:	Oral, by gavage
Dosing schedule	13 weeks
GLP:	In compliance

In a thirteen-week oral gavage study 4-nitro-o-phenylenediamine was dosed in carboxymethylcellulose at levels of 0, 5, 15 and 45 mg/kg body weight/day for 13 weeks using 10 SPF-bred Wistar rats per sex per dose level, with a recovery group of 5 animals per sex per dose level. Following the 90-day treatment the recovery groups were maintained treatment free for a further 28 days. Clinical signs, ophthalmologic examination, food consumption and body weights were recorded throughout the study. Blood samples were taken at 4, 8, 13 week's treatment and from the recovery groups following the 4 weeks treatment free. Urinalysis was also conducted at the end of 13 week's treatment and the treatment free recovery period. Histopathological examination was conducted.

Results
Oral administration 5, 15 and 45 mg/kg bw/day, for 13 weeks resulted in one spontaneous death at the highest dose level, no clinical signs of adverse effects, no effects of for and hind limb grip strength, no effects on locomotor activity, no ophthalmologic changes, no effects on food consumption or body weights, no changes in the haematology or urinalysis parameters, no changes on thyroid hormone profiles, and no noteworthy macroscopical findings.

Test item-related changes in clinical biochemistry parameters, such as elevated levels of cholesterol, triglycerides and phospholipids (indicative of minor adaptive changes in liver function), as well as elevated albumin, globulins and proteins (supported by adaptive response of the liver and thyroid changes seen microscopically) were observed in males at of the highest dose group. Elevated sodium levels were seen in females at all dose levels (not dose-related) and males at 15 mg/kg bw/day and 45 mg/kg bw/day (dose-related), but remained within the ranges of the historical control data, and were reversible. Except for the changes in albumin, globulins and protein, the changes in clinical biochemistry were considered to be non-adverse.

Elevated absolute and relative liver weights were seen in both sexes at 5, 15 and 45 mg/kg bw/day. These differences correlated with microscopical changes (centrilobular hepatocellular hypertrophy) and were considered to be a consequence of metabolic adaptation. At the highest dose level, the increases of absolute (29% in males and 21% in females) and relative (20% in males and 19% in females) liver weights were considered adverse. At the other dose levels, increases of absolute and relative liver weights (<19%) were considered non-adverse. After recovery, all organ weights and ratios were considered to be unaffected.

Microscopic examination showed test item-related changes in the livers of males at all dose levels and in females of the highest dose group (centrilobular hepatocellular hypertrophy), and in the thyroid gland in males treated with 45 mg/kg bw/day (increased incidence of

follicular hypertrophy). Slight to moderate hepatocellular hypertrophy was observed in (8/10) males and (5/10) females of the highest dose group. At the other dose levels, only minimal to slight hepatocellular hypertrophy was observed. Hepatocellular hypertrophy a common adaptive response to xenobiotics, whereas follicular hypertrophy of the thyroid gland is commonly seen in male rats and may be exacerbated in rates with hepatocellular hypertrophy. Minimal hepatocellular hypertrophy persisted after recovery in treated males of all dose levels, whereas females reverted completely. After recovery, changes in the thyroid glands of treated male rats were reversible when compared with control males.

Marginal evidence of post-treatment changes in the bone marrow of males from the highest dose group (proportion of fatty to hematopoietic marrow was reduced in comparison to the control animals) was reversible after the recovery period.

Conclusion
Based on the results of this study, a NOEL could not be obtained for 4-nitro-o-phenylenediamine. However, the authors considered all findings to be adaptive or secondary responses to the treatment with the test item and, with the exception of minimal centrilobular hepatocellular hypertrophy of the liver in males previously treated with 45 mg/kg/day, reversible after the recovery period. The centrilobular hepacellular hypertrophy of the liver in males of the highest dose group (still observed after the recovery period) was considered non-adverse, because a clear trend for reversibility was established. The No Observed Adverse Effect Level (NOAEL) for 4-NOPD was considered to be 45mg/kg bw/day.
Ref.: 48

Comment
The authors considered the NOAEL to be equal to the highest dose level (45 mg/kg bw/day). They argued that the centrilobular hepacellular hypertrophy of the liver in males of the highest dose group (still observed after the recovery period) were non-adverse, because a clear trend for reversibility was established. However, after a 4-week recovery period minimal hepatocellular hypertrophy was still observed in males of all dose levels. Based on the partly reversible microscopic changes (centrilobular hepatocellular hypertrophy) and reversible increases in (absolute and relative) liver weights, albumin, globulins and proteins in males of the highest dose group, the SCCP considers the NOAEL to be 15 mg/kg bw/day.

3.3.5.3. Chronic (> 12 months) toxicity

Guideline:	/
Species/strain:	Beagle dogs
Group size:	6 per sex and dose group
Test substance:	A composite of hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine (and 13 other dyes)
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 19.5 and 97.5 mg/kg bw/day hair dye formulation
Route:	Oral, dietary
Vehicle:	None
Dosing schedule:	7 days/week, 2 years
GLP:	Not in compliance

A 2-year dietary feeding study was conducted in beagle dogs with a composite hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine at dose levels of 0, 19.5 and 97.5 mg/kg body weight/day (0.0312 and 0.156% 4-nitro-o-phenylenediamine respectively). Animals were observed for signs of toxic or pharmacologic effects, physical examinations were conducted, and body weight and food consumption records were kept. Haematological, blood chemistry and urinalysis parameters were determined. Necropsy was performed on one male and one female from each group at 6, 12 and 18 months and on all survivors at 24 months. Individual organ weights and organ to body weight ratios of the

major organs were recorded. Microscopic examination of 30 tissues/organs and electron microscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.

Results
No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5 mg/kg body weight/day. All dogs in the two test groups excreted urine of a blue-brown colour on a daily basis. However urine analysis showed no remarkable findings. Colour was normal in urine collected after overnight fasting.

Conclusion
Oral dietary exposure to a composite hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine up to 97.5 mg/kg body weight/day (0.156% 4-nitro-o-phenylenediamine respectively) did not result in any signs of toxicity.

Ref.: 38

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (batch number unknown, not according to a guideline, no raw data available) this study is useful for evaluation.

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA1535, TA1537, TA1538
Replicates:	Without S9-mix (plate incorporation test)
Test substance:	4-Nitro-o-phenylenediamine in DMSO
Batch:	/
Purity:	/
Concentrations:	0, 7, 20, 50 μg/plate without S9-mix (doses approximated from a figure)
GLP:	not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in strains TA1535, TA1537, and TA1538 of *Salmonella typhimurium* without S9-mix after a 48 h treatment (plate incorporation test).

Results
4-nitro-o-phenylenediamine was a direct mutagen in *Salmonella typhimurium* in the frame-shift strain TA1538, but not in TA1535 or TA1537. The mutagenic effect showed a clear dose-dependency at the dose range tested.
4-Nitro-o-phenylenediamine was suggested to be responsible for the direct mutagenic activity of a variety of hair dye formulations tested in the paper. The mutagenicity of the test substance could be exactly accounted by its 4-nitro-o-phenylenediamine content (0.25 mg/ml).

Conclusions
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced frame-shift mutations in *Salmonella typhimurium*.

Ref.: 2

Bacterial gene mutation assay

Guideline: /
Species/strain: *Salmonella typhimurium*, TA1538
Replicates: Without S9-mix (plate incorporation test)
Test substance: 4-Nitro-o-phenylenediamine in DMSO
Batch: No data
Purity: Not indicated.
Concentrations: 1, 5, 10 μg/plate without S9-mix
GLP: not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in strain TA1538 of *Salmonella typhimurium* without S9-mix (plate incorporation test).

Results
The test agent was a direct mutagen in *Salmonella typhimurium* in the frame-shift strain TA1538 starting from the lowest concentration tested (1 μg/plate). The effect was dose-dependent.

Ref.: 3

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced frame-shift mutations in *Salmonella typhimurium*. The purity of the test agent was not indicated. The assay was performed before OECD test guidelines were available.

Bacterial gene mutation assay

Guideline: /
Species/strain: *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537
Replicates: With and without S9-mix (plate incorporation test)
Test substance: 4-Nitro-o-phenylenediamine in water
Batch: No data
Purity: Not data
Concentrations: 0, 0.05, 0.5, 5, 50 and 500 μg/plate in water, with and without S9-mix
GLP: not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in strains TA98, TA100, TA1535, and TA1537 of *Salmonella typhimurium* with and without S9-mix (plate incorporation test).

Results
4-Nitro-o-phenylenediamine was a direct mutagen in *Salmonella typhimurium* in the frame-shift strain TA98.

Ref.: 5

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced frame-shift mutations in *Salmonella typhimurium*. The purity of the test agent was not indicated. The assay was performed before OECD test guidelines were available.

Bacterial gene mutation assay

Guideline: /
Species/strain: *Salmonella typhimurium*, TA1538 (preincubation test)

Replicates:	Not indicated
Test substance:	4-Nitro-o-phenylenediamine
Batch:	No data
Purity:	No data
Concentrations:	0, 63, 125, and 250 μg/plate, without S9-mix
GLP:	not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in strain TA1538 of *Salmonella typhimurium* without S9-mix (preincubation for 1 h).

Results
4-Nitro-o-phenylenediamine was mutagenic in *Salmonella typhimurium* TA1538 without S9-mix, with a dose-dependent effect.

Ref.: 36

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced frame-shift mutations in *Salmonella typhimurium*. The assay was performed before OECD test guidelines were available.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA1537 and TA1538 (plate incorporation test)
Replicates:	Two plates per point
Test substance:	4-Nitro-o-phenylenediamine in water
Batch:	No data
Purity:	>97%
Concentrations:	0, 5, and 50 μg/plate in water, with and without benzo[a]pyrene-induced S9-mix
GLP:	not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in strains TA1537, and TA1538 of *Salmonella typhimurium* with and without S9-mix (plate incorporation test, incubation for 3 days).

Results
4-nitro-o-phenylenediamine was mutagenic in *Salmonella typhimurium* TA1538 irrespective of the use of S9-mix. A weak positive effect was seen in strain TA1537.

Ref.: 33

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced frame-shift mutations in *Salmonella typhimurium*. The assay was performed before OECD test guidelines were available.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA100, TA104, TA4001, TA4006 (plate incorporation test)
Replicates:	Each test was triplicate and was performed at least twice
Test substance:	4-Nitro-o-phenylenediamine in DMSO
Batch:	Sigma Chemical Company
Purity:	Not indicated.

Concentrations: 0, 10, 30, 100, 300 μg/plate in water, without S9-mix
GLP: not in compliance

4-Nitro-o-phenylenediamine was assessed for the induction of revertant mutations in *Salmonella typhimurium* strains TA100, TA104, TA4001, TA4006 with and without S9-mix (plate incorporation test). Strains TA4001 and TA4006 exclusively detected TA to CG and CG to GC reversions, respectively. In addition, the nature of the mutants was studied by assessing sensitivity and resistance to DL-1,2,4-triazole-3-alanine.

Results
4-Nitro-o-phenylenediamine was a direct mutagen in *Salmonella typhimurium* TA100, TA104, with a dose-dependent effect. A weak effect was seen in TA4006, while TA4001 gave a negative result. In TA100, 88-95% of the mutations induced appeared to be point mutations and 5-12% suppressor mutations. In TA104, point mutations comprised 80-85% and suppressor mutations 15-20% of the mutations detected. The results indicated that base-pair mutations (GC to TA transitions, GC to AT transversions, and TA to AT transversions) were primarily induced.

Ref.: 9

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced base-pair mutations in *Salmonella typhimurium*. The purity of the test agent was not indicated.

Bacterial gene mutation assay

Guideline: /
Species/strain: *Salmonella typhimurium* TA98
Replicates: Not clear (text in Japanese) with and without S9-mix
Test substance: 4-Nitro-o-phenylenediamine alone and with hydrogen peroxide
Batch: Unclear (text in Japanese)
Purity: Unclear (text in Japanese)
Concentrations: 15, 50 and 150 μg/plate without and with S9-mix
GLP: Not in compliance

4-Nitro-o-phenylenediamine was tested for mutagenicity in strain TA98 of *Salmonella typhimurium* with and without S9-mix.

Results
4-Nitro-o-phenylenediamine induced a very clear dose-dependent increase in revertant colonies in the strain TA98 without and with metabolic activation. Treatment together with hydrogen peroxide appeared to slightly increase the mutagenicity of 4-nitro-o-phenylenediamine.

Conclusion
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium* and the effect seemed to be slightly enhanced by co-treatment with hydrogen peroxide.

Ref.: 42

Comment
Details remain unclear as the paper is in Japanese.

Bacterial gene mutation assay

Guideline: /

Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA1535, TA1537, TA1538
Replicates:	Triplicate, with and without S9-mix
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Provided by NCI
Purity:	Not indicated
Concentrations:	0.3, 1, 3.3, 10, 33, 100, 333 μg/plate in DMSO, with and without S9-mix
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was assessed for the induction of revertant mutations in strains TA98, TA100, TA1535, TA1537, and TA1538 of *Salmonella typhimurium* without and with S9-mix from Aroclor 1254-induced or not-induced liver from B6C3F1 mouse, Fisher 344 rats, or Syrian hamsters. Plate incorporation procedure was used.

Results
4-nitro-o-phenylenediamine induced a clear dose-dependent increase in revertant colonies in strains TA98, TA100, TA1537, and TA1538 without S9-mix. The clearest effect was seen in strain TA98 without S9-mix. The presence of mouse and rat liver S9-mix reduced the mutagenic response observed in TA98.

Ref.: 14

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium*.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA1538
Replicates:	Duplicate, two independent tests with and without S9-mix
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Gurr Products
Purity:	Purified by thin layer chromatography
Concentrations:	50, 100 μg/plate in DMSO, with and without S9-mix
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was assessed for the induction of revertant mutations in strain TA1538 of *Salmonella typhimurium* without and with S9-mix from phenobarbitone-induced male Wistar rat liver. Plate incorporation procedure was used.

Results
4-nitro-*o*-phenylenediamine induced a very clear dose-dependent increase in revertant colonies in strain TA1538 without metabolic activation. The presence of S9-mix slightly reduced the mutagenic response observed.

Ref.: 15

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium*.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA98NR, TA100NR
Replicates:	With and without S9-mix (pre-incubation test)

Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Sigma Chemical Company
Purity:	Not indicated
Concentrations:	1, 10, 30, 100, 300, 1000 μg/plate in DMSO, with and without S9-mix
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was assessed for the induction of revertant mutations in strains TA98, TA100, TA98NR, and TA100NR of *Salmonella typhimurium* without and with S9-mix from Sprague-Dawley rat liver. Pre-incubation procedure was used.

Results
4-nitro-*o*-phenylenediamine induced a very strong dose-dependent increase in revertant colonies in all tester strains without and with S9-mix. The clearest effect was seen in strain TA98 without S9-mix. The presence of S9-mix reduced the mutagenic response observed.
Ref.: 10

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium*.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA7001, TA7002, TA7003, TA7004, TA7005, TA7006
Replicates:	With and without S9-mix (plate incorporation test)
Test substance:	4-Nitro-o-phenylenediamine
Batch:	No data. Sigma
Purity:	Not indicated
Concentrations:	600, 900, 1200, 1500, 1800 μg/plate in DMSO, with and without S9-mix
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was assessed for the induction of revertant mutations in the Xenometrix strains TA7001, TA7002, TA7003, TA7004, TA7005, and TA7006 of *Salmonella typhimurium* without and with Aroclor 1254 -induced S9-mix. Plate incorporation assay was used.

Results
4-nitro-*o*-phenylenediamine induced a dose-dependent increase in revertants in strains TA7002, TA7004, and TA7005 without and with S9-mix. The highest response was observed in strain TA7005 without metabolic activation. The presence of S9-mix reduced the mutagenic response observed. The results indicated that the test agent induced GC to AT transitions as well as TA to AT and CG to AT transversions.
Ref.: 11

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium*.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA1538
Replicates:	Triplicate, with and without S9-mix

Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Eastman Kodak
Purity:	At least 95%
Concentrations:	10, 20, 40, 60 μg/plate in DMSO, without S9-mix
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was assessed for the induction of revertant mutations in strains TA98, TA100, and TA1538 of *Salmonella typhimurium* without S9-mix. Plate incorporation procedure and proper positive controls were used.

Results
4-nitro-*o*-phenylenediamine induced a clear dose-dependent increase in revertant colonies in strains TA98 and TA1538 without metabolic activation. The clearest effect was seen in strain TA1538.

Ref.: 16

Conclusions
Under the experimental conditions reported, 4-nitro-*o*-phenylenediamine induced mutations in *Salmonella typhimurium*.

Bacterial gene mutation assay

Guideline:	/
Species/strain:	*Salmonella typhimurium,* TA98
Replicates:	Three experiments with and without S9-mix, each triplicate
Test substance:	4-Nitro-o-phenylenediamine oxidized by hydrogen peroxide
Batch:	No data. Tokyo Kasei Co. Ltd
Purity:	No data
Concentrations:	1 and 3 µg/plate in DMSO, without and with S9-mix from livers of Sprague-Dawley rats
GLP:	Not in compliance

Two moles of 4-nitro-o-phenylenediamine in 91 ml of ethanol was oxidized in alkaline conditions by the addition of 1.4:1 moles of 30% hydrogen peroxide. The reaction mixture was allowed to stand at 30 °C for 2 days, after which ethanol was evaporated, and the residue was mixed with same volumes of ethyl acetate and water. Ethyl acetate was evaporated to dryness, and the extract was dissolved in DMSO and tested for mutagenicity in strain TA98 of *Salmonella typhimurium* with and without S9-mix. Plate incorporation procedure and proper negative and positive controls were used. The results were compared to those obtained with 4-nitro-o-phenylenediamine alone.

Results
4-Nitro-o-phenylenediamine induced a clear increase in revertant colonies in the strain TA98 with and without metabolic activation. Treatment with hydrogen peroxide did not increase the mutagenicity of 4-nitro-o-phenylnediamine alone.

Ref.: 37

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium,* but the effect was not increased by oxidation with hydrogen peroxide.

Bacterial gene mutation assay (photo-mutagenicity)

Guideline:	/
Species/strain:	*Salmonella typhimurium.* TA98
Replicates:	Duplicate assays without S9-mix
Test substance:	4-Nitro-o-phenylenediamine irradiated with mercury vapour lamp for 5, 10 or 15 min
Batch:	No data. Wako Pure Chemicals
Purity:	No data
Concentrations:	5 and 10 µg/plate in DMSO, without S9-mix
GLP:	Not in compliance

4-Nitro-o-phenylenediamine was irradiated by a mercury vapour lamp (cooled by an electric fan) at 43 °C from a distance of 30 cm. The irradiated test substance was tested for mutagenicity in strain TA98 of *Salmonella typhimurium* without S9-mix. Pre-incubation procedure and proper negative and positive controls were used. The results were compared to those obtained with 4-nitro-o-phenylenediamine irradiated in a brown tube.

Results
4-Nitro-o-phenylenediamine (10 µg/plate) induced a very clear increase in revertant colonies in the strain TA98 without metabolic activation. Treatment of the compound with mercury lamp resulted in a more than 5-fold increase in mutagenicity. A linear increase in mutagenicity of 4-nitro-o-phenylenediamine was observed when the time of irradiation was increased from 5 to 15 min.

Ref.: 41

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in *Salmonella typhimurium,* and the effect was greatly increased by treatment of the test agent with light from a mercury vapour lamp. The authors considered that the enhancement of mutagenicity "must be the result of photo-oxidation of amine and nitro".

Urine mutagenicity test by bacterial gene mutation assay

Guideline:	/
Species/strain:	Male Texas: Sprague-Dawley rats treated; urine mutagenicity tested with *Salmonella typhimurium*, TA1538 without S9-mix (plate incorporation test)
Replicates:	3 plates for each point.
Test substance:	4-Nitro-o-phenylenediamine
Batch:	No data
Purity:	Not indicated.
Doses:	5 mg 4-nitro-o-phenylenediamine, in 0.9% NaCl and 1.75% gum acacia solution, was injected *i.p.* into rats. Alternatively, 120 mg of 4-nitro-o-phenylenediamine in acetone or isopropanol was applied topically to shortened hair on the back of the rats for 20 min and removed by shampooing and thorough rinsing. Urine was collected before treatment and 24 after the treatment.
GLP:	not in compliance

5 mg 4-nitro-o-phenylenediamine, in 0.9% NaCl and 1.75% gum acacia solution, was injected *i.p.* into rats. Alternatively, 120 mg of 4-nitro-o-phenylenediamine in 4 ml acetone or 8 ml isopropanol was applied topically to shortened hair on the back of the rats for 20 min and removed by shampooing and thorough rinsing. Urine was collected before treatment and every 24 thereafter.

Results
The urine, collected 24 h after the treatments was mutagenic in *Salmonella typhimurium* in the frame-shift strain TA1538 without metabolic activation. 1-1.5% of the 4-nitro-o-phenylenediamine injected *i.p.* appeared in the urine in a form that was directly mutagenic to strain TA1538 of *Salmonella typhimurium* (plate incorporation test). Topically applied 4-nitro-o-phenylenediamine resulted in four times more mutagenic urine if applied in acetone rather than isopropanol. 4-nitro-o-phenylenediamine was a direct mutagen.

Ref.: 3

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine given to rats resulted in urine that was directly mutagenic to *Salmonella typhimurium*. The purity of the test agent was not indicated.

Mammalian cell gene mutation test *(tk* locus)

Guideline:	/(OECD 476)
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Not indicated
Metabolic act.:	S9-mix from Aroclor 1254-induced Fischer 344 rat liver
Test substance:	4-Nitro-o-phenylenediamine
Batch:	Not given; Clairol. Inc.
Purity:	At least 98% pure
Concentrations:	50. 100. and 200 µg/ml without S9-mix
Treatment:	24 h, in the absence of metabolic activation
Solvent:	DMSO
GLP:	Not in compliance

The induction of mutations at the thymidine kinase locus by 4-nitro-o-phenylenediamine was studied in the mouse lymphoma L5178Y cell line. Dose levels were chosen on the basis of a range-finding study. The used concentrations, 50, 100, and 200 µg/ml, produced about 80%, 50%, and 20% survival rates, respectively. Proper negative and positive controls were included.

Results
4-Nitro-o-phenylenediamine was observed to increase mutants in the assay without metabolic activation. The highest effect (6.9-fold increase) was obtained at 200 µg/ml.

Ref.: 29

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in the mouse lymphoma thymidine kinase locus in L5178Y cells in the absence of metabolic activation. No statistical tests were used for the evaluation of the results.

Mammalian cell gene mutation test *(tk* locus)

Guideline:	/ (OECD 476)
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Not indicated
Metabolic act.:	S9-mix from Aroclor 1254-induced Fischer 344 rat liver
Test substance:	4-Nitro-o-phenylenediamine
Batch:	Not given: Aldrich Chemical Company
Purity:	Not given: "Highest technical grade available"

Concentrations:	Data shown for 25, 50, 75,100, 125, 150, 175, and 200 µg/ml without S9-mix
Treatment:	4 h, in the absence and presence of S9-mix
Solvent:	DMSO
GLP:	Not in compliance

The induction of mutations at the thymidine kinase locus by 4-nitro-o-phenylenediamine was studied in the mouse lymphoma L5178Y cell line. 4-Nitro-o-phenylenediamine was one of 42 test agents chosen to evaluate the performance of the mutation assay. The assay was performed in three independent trials without and with S9-mix. Proper negative and positive controls were included.

Results
4-Nitro-o-phenylenediamine induced a 2.5-fold increase in mutant frequency at 50 µg/ml and above without S9-mix. The highest effect (5-fold increase) was obtained at 150 µg/ml without metabolic activation.

Ref.: 28

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in the mouse lymphoma thymidine kinase locus in L5178Y cells in the absence of metabolic activation. No statistical tests were used for the evaluation of the results.

Mammalian cell gene mutation test (*tk* locus)

Guideline:	OECD 476
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Two experiments
Metabolic act.:	S9-mix from Aroclor 1254-induced Fischer 344 rat liver
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	Not given (NTP study)
Purity:	Not given (NTP study)
Concentrations:	Without S9-mix: 44, 128, 256, 512, 640, 800, and 1000 µg/ml (1st experiment); 20, 45, 101, 226, and 312 µg/ml (2nd experiment). With S9-mix (1st and 2nd experiment): 13.1, 16.4, 20.5, 25.6, 32, 40 and 50 µg/ml
Treatment:	4 h in the absence and presence of S9-mix; 2 days were allowed for mutant expression
Solvent:	DMSO
GLP:	Not in compliance

The study was performed to investigate the potential of 4-nitro-*o*-phenylenediamine to induce mutations at the thymidine kinase locus of the mouse lymphoma L5178Y cell line. The assay was performed in two independent experiments, using two parallel cultures without S9-mix and three parallel cultures with S9-mix. Proper negative and positive controls were included.

Results
4-Nitro-*o*-phenylenediamine induced mutations in both experiments without and with S9-mix. In the first experiment, there was a 2.3-fold increase in mutants at 256 µg/ml without metabolic activation. In the second experiment, an approximately 3-fold increase was seen at 228 µg/ml. The mutagenic effect was increased with S9-mix, with approximately a 3-fold increase at 50 µg/ml in the first experiment and a 5-fold increase at 40 µg/ml in the second experiment where 50 µg/ml was toxic.

Ref.: 25

Comment
Under the experimental conditions reported, 4-nitro-*o*-phenylenediamine induced mutations in the mouse lymphoma thymidine kinase locus assay using cell line L5178Y in the absence and presence of metabolic activation.

Mammalian cell gene mutation test *(tk* locus)

Guideline:	/ (OECD 476)
Species/strain:	Mouse lymphoma cell line L5178Y
Replicates:	Three trials: triplicate in the 1st trial, duplicate in the 2nd and 3rd trials
Metabolic act.:	S9-mix from Aroclor 1254-induced Fischer 344 rat liver
Test substance:	4-Nitro-o-phenylenediamine
Batch:	Not given (NTP study)
Purity:	Not given (NTP study)
Concentrations:	Without S9-mix: 75, 150, 225, 300, 450, and 600 µg/ml (1st trial); 75, 150, 250, 300, 319, 425, and 500 µg/ml (2nd trial); 150, 250, 300, 375, 425, and 500 µg/ml (3rd trial) With S9-mix: 12.5, 25, 50, 100, and 150 µg/ml (1st trial); 15, 30, 60, 100, and 120 µg/ml (2nd trial); 20, 40, 80, 100, 190, and 200 µg/ml (3rd trial)
Treatment:	4 h in the absence and presence of S9-mix
Solvent:	DMSO
GLP:	Not in compliance

The induction of mutations at the thymidine kinase locus by 4-nitro-o-phenylenediamine was studied in the mouse lymphoma L5178Y cell line. The assay was performed in three independent trials without and with S9-mix. Proper negative and positive controls were included.

Results
4-Nitro-o-phenylenediamine induced dose-related increases in mutant frequency in three trials without metabolic activation. The lowest effective concentration was 75-100 µg/ml (at 38-52% relative total growth) and maximum values of approximately 3- to 6-fold -fold increases in mutant frequency were reached near the solubility limit (375-450 µg/ml). When S9-mix was present (3 different batches of S9 fraction), the compound became more toxic and induced significant increases in mutant frequency at 20-30 µg/ml. The first trial with S9-mix showed a 2.3-fold increase in mutants at a toxic dose of 100 µg/ml. Trial 3 showed the highest (4-fold) response with S9-mix at 150 µg/ml.

Ref.: 26

Comment
Under the experimental conditions reported, 4-nitro-o-phenylenediamine induced mutations in the mouse lymphoma thymidine kinase locus assay using cell line L5178Y in the absence and presence of metabolic activation.

***In vitro* chromosome aberration test**

Guideline:	/
Cells:	A(T_1)C1-3 hamster cells
Replicates:	None
Test substance:	4-Nitro-o-phenylenediamine
Batch:	No data
Purity:	No data
Concentrations:	0, 0.01, 0.1, and 0.3 mM for 24 h without metabolic activation

GLP: not in compliance

4-Nitro-o-phenylenediamine was investigated for the induction of chromosomal aberrations in hamster A(T_1)C1-3 cells. Concentrations were chosen for scoring based upon reduction in mitotic index. Negative and positive controls were in accordance with the OECD guidelines.

Results
4-Nitro-o-phenylenediamine was reported to induce chromatid breaks, but the data were based on only 50 cells per concentration. The data suggested a dose-dependent effect.
Ref.: 4

Comment
4-Nitro-o-phenylenediamine induced chromosomal aberrations in hamster cells in vitro when tested in the absence of a metabolic activation system. However, the purity of the test agent was not indicated, and the cytogenetic analysis was based on only 50 cells per point without replications.

***In vitro* chromosome aberration test**

Guideline:	/
Cells:	Chinese hamster ovary cells (CHO-K1 cells)
Replicates:	Three experiments
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Sigma Chemical Company
Purity:	Not indicated
Concentrations:	138, 276, 552, 828 µg/ml for 2 h without S9-mix
Solvent:	DMSO
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was investigated for the induction of chromosomal aberrations in Chinese hamster ovary cells (CHO-K1 cells) *in vitro*, using a 2-h treatment without metabolic activation. The concentrations were chosen on the basis of a preliminary toxicity test, so that they were 0.5, 1, 2 and 3 times the TC_{50} obtained in the toxicity tests. Control cultures received the solvent used (DMSO). TEM was used as a positive control.

Results
4-Nitro-*o*-phenylenediamine induced chromosomal aberrations in a dose-dependent manner starting from the lowest concentration tested. Breaks, deletions, and dicentric chromosomes were mostly induced.
Ref.: 10

Comment
4-Nitro-*o*-phenylenediamine induced chromosomal aberrations in Chinese hamster ovary cells *in vitro* when tested in the absence of a metabolic activation system.

***In vitro* chromosome aberration test**

Guideline:	/
Cells:	Chinese hamster lung cells(CHL cells)
Replicates:	Not indicated
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Provided by the project team
Purity:	Not indicated
Concentrations:	Three doses, maximum effective dose was 60 µg/ml (other doses not shown). The tests were performed with 24-h and 48-h treatments

Solvent:	DMSO
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was investigated for the induction of chromosomal aberrations in Chinese hamster lung cells (CHL cells) *in vitro*. A preliminary growth inhibition test was used to determine 50% inhibition dose to be used as the highest dose in the cyto-genetic test.

Results
4-Nitro-*o*-phenylenediamine induced chromosomal aberrations. The maximum effective dose (60 µg/ml, 0.39 mM) produced 30% of aberrant cells after a 48-h treatment. Gaps, breaks and translocations were induced.

Ref.: 18

Comment
4-Nitro-*o*-phenylenediamine induced chromosomal aberrations in Chinese hamster lung cells *in vitro* when tested in the absence of a metabolic activation system. The report contained data on very many chemicals and lacked details. The study was performed before OECD guidelines were available.

In vitro chromosome aberration test

Guideline:	/
Cells:	Chinese hamster prostate cells (CHMP/E cells)
Replicates:	None
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Aldrich Chemical Company.
Purity:	>98%
Concentrations:	25 µg/ml for 1, 2, 3, 4, 5 and 7 days without metabolic activation
Solvent:	DMSO
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was investigated for the induction of chromosomal aberrations in Chinese hamster prostate cells (CHMP/E cells) *in vitro*. In a colony forming assay, 4-nitro-*o*-phenylenediamine showed a dose-dependent toxic effect at the concentration range of 5-100 µg/ml in a continuous exposure for 5 days.

Results
4-Nitro-*o*-phenylenediamine was reported to induce chromosomal aberrations at the only dose tested particularly after 4 days (11.5-fold in comparison with controls) and 7 days (6.4-fold) of exposure. After 24-h exposure, a 4-fold increase in cells with chromosomal aberrations (excluding gaps) was seen in comparison with a control culture. At other time points, the difference to the control culture was 4-fold (24-h exposure), 5-fold (48-h exposure), and 2.7-fold (72-h exposure). Chromatid breaks were mostly induced.

Ref.: 21

Comment
4-Nitro-*o*-phenylenediamine induced chromosomal aberrations in Chinese hamster prostate cells *in vitro* when tested in the absence of a metabolic activation system. Although several time points after the exposure were assayed, only one concentration was studied. The cytogenetic analysis was based on only 50 cells per point without replications, and no statistical tests were applied. The study was performed before OECD guidelines were available.

***In vitro* micronucleus test**

Guideline:	/
Cells:	Human lymphocytes
Replicates:	Two independent experiments, duplicate cultures
Test substance:	4-Nitro-o-phenylenediamine
Batch:	A9366
Purity:	>99%
Concentrations:	Experiment 1 (treatment 24 h after culture start): 400, 550, 700 µg/ml and 1531 µg/ml for 20 h without S9-mix (+ 28 h recovery period) 501.7, 627.1 and 783.9 µg/ml for 3 h (+ 45-h recovery period) with S9-mix (from Aroclor 1254 -induced rat liver) Experiment 2 (treatment 48 h after culture start): 500, 600 and 700 µg/ml for 20 h without S9-mix (+ 28 h recovery period) 600, 750 and 900 µg/ml for 3 h (+ 45-h recovery period) with S9-mix
Solvent:	DM50
GLP:	in compliance

4-Nitro-o-phenylenediamine was tested in the *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in two independent experiments. To find the dose range, treatments covering a broad range of doses, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9-mix). The test article was dissolved in DMSO. In Experiment 1, treatment of cells commenced approximately 24 h following mitogen stimulation by phytohaemagglutinin (PHA). In the absence of S9-mix, this was for 20 h followed by a 28-h recovery period prior to harvest (20+28). Treatment in the presence of S9-mix was for 3 h followed by a 45-h recovery period prior to harvest (3+45). The S9-mix used was prepared from a rat liver postmitochondrial fraction (S9-mix) from Aroclor 1254 -induced animals. The test article dose levels for micronucleus analysis were selected by evaluating the effect of 4-nitro-o-phenylenediamine on the replication index (RI). Micronuclei were analysed at three dose levels. The highest concentrations chosen for analysis, 700.0 µg/ml in the absence of S9-mix and 783.9 µg/ml in the presence of S9-mix, induced approximately 62% and 52% reduction in RI respectively. In Experiment 2, treatment of cells commenced approximately 48 h following mitogen stimulation. In the absence of S9-mix, this was for 20 h followed by a 28-h recovery period prior to harvest (20+28). Treatment in the presence of S9-mix was for 3 h followed by a 45- h recovery period prior to harvest (3+45). Micronuclei were analysed at three dose levels (see below). The highest concentrations chosen for analysis, 700.0 µg/ml in the absence of S9-mix and 900.0 µg/ml in the presence of S9-mix, induced approximately 64% and 53% reduction in RI respectively. Appropriate negative (solvent) control cultures were included in the test system under each treatment condition. The proportion of binucleate cells with micronuclei in these cultures fell within historical solvent control ranges. 4-Nitroquinoline 1-oxide (NQO) and vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals respectively in the absence of liver S9-mix. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of liver S9-mix. Cells receiving these agents were sampled at 48 h after the start of treatment. The positive control compounds induced statistically significant increases in the proportion of cells with micronuclei.

Results
Treatment of cells with 4-nitro-o-phenylenediamine resulted in frequencies of micronucleated binucleate cells (MNBN) that were similar to those observed in concurrent vehicle control cultures at all concentrations analysed in the absence and presence of S9-mix in Experiments 1 and 2. The MNBN cell frequency exceeded the historical negative

control range in only one culture (at 400.0 µg/ml, the lowest concentration analysed in the absence of S9-mix in Experiment 1) in either experiment. This was not observed in the replicate culture and was therefore not considered biologically relevant.

Conclusion
It is concluded that 4-nitro-o-phenylenediamine did not induce micronuclei in cultured human peripheral blood lymphocytes when tested under two different experimental conditions at concentrations up to its limit of cytotoxicity, in both the absence and presence of a rat liver metabolic activation system (S9-mix).

Ref.: 49

In vitro UDS (DNA repair) test in mammalian cells

Guideline:	/
Cells:	HeLa S3 cells
Replicates:	Duplicate flasks, triplicate flasks with S9-mix
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Aldrich Chemical Company Ltd
Purity:	No data
Concentrations:	"Tenfold dilutions" tested, data shown only for 1 mM without metabolic activation
Solvent:	DMSO
GLP:	Not in compliance

Human HeLa S3 breast cancer cells were exposed to 4-nitro-*o*-phenylenediamine in the presence of tritiated thymidine. Untreated controls, solvent controls, and positive controls were also included in the series. After the treatment, DNA was extracted and radioactivity was measured by a liquid scintillation counter.

Results
4-Nitro-*o*-phenylenediamine induced UDS in HeLa S3 cells at 1 mM without metabolic activation.

Ref.: 24

Comment
Data were shown only for "active dose range" which in the case of 4-nitro-*o*-phenylene-diamine was 1 mM.

In vitro UDS (DNA repair) test in mammalian cells

Guideline:	/
Cells:	Primary hepatocytes from F344 rats
Replicates:	Two experiments
Test substance:	4-Nitro-o-phenylenediamine
Batch:	No data, Aldrich Chemical Co
Purity:	No data
Concentrations:	8 concentrations, covering the range of 0.5-1000 µM; Results shown only for 100 µM without exogenous metabolic activation: treatment times were 5 h and 20 h
Solvent:	DMSO
GLP:	Not in compliance

Rat hepatocytes were exposed to 4-nitro-o-phenylenediamine for 5 h in the presence *of* tritiated thymidine. After auto-radiography, the cells were analysed for nuclear grain number using an automated colony counter, to assess the induction of unscheduled DNA

synthesis (UDS). As the result of the initial test was negative, a retest was performed with a 20-h treatment. Proper negative and positive controls were applied.

Results
4-Nitro-o-phenylenediamine did not induce UDS in the assay.

Ref.: 31

Comment
4-Nitro-o-phenylenediamine did not induce UDS in rat primary hepatocytes *in vitro.* The paper included results on a number of compounds. Detailed results were not shown for 4-nitro-o-phenylenediamine.

***In vitro* UDS (DNA repair) test in mammalian cells**

Guideline:	/
Cells:	Primary hepatocytes from F344 rats
Replicates:	Two experiments
Test substance:	4-Nitro-*o*-phenylenediamine
Batch:	No data. Provided by NCI
Purity:	No data
Concentrations:	10, 50, 100 µg/ml for 18-20 h without exogenous metabolic activation
Solvent:	DMSO
GLP:	Not in compliance

Viable rat hepatocytes were exposed to 4-nitro-*o*-phenylenediamine in serum-free WME medium in the presence of tritiated thymidine. An untreated control, a solvent control, and a positive control were also included in the series. After autoradiography, viable (swollen) cells were analyzed for nuclear grain number in the microscope, to assess induction of unscheduled DNA synthesis (UDS). Cytotoxicity was assessed by the absence of S-phase cells and general morphology. The highest tested dose (100 µg/ml) was toxic in both of the experiments.

Results
4-Nitro-*o*-phenylenediamine did not induce UDS in the assay.

Ref.: 40

Comment
4-Nitro-*o*-phenylenediamine did not induce UDS in rat primary hepatocytes *in vitro*.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mouse bone marrow micronucleus test

Guideline:	/
Species/strain:	Mouse, C57BL76 x C3H/He
Group size:	4 females
Test substance:	4-Nitro-o-phenylenediamine in water
Batch:	No data
Purity:	Not given
Dose levels:	*i.p.* injection on 5 consecutive days, 312, 625, 1225, 2500 mg/kg
Sacrifice time:	About 4 h after the last dosing
GLP:	Not in compliance

4-Nitro-o-phenylenediaminewas tested for the induction of micronuclei in the bone marrow of female C57BL76 x C3H/He mice after daily *i.p.* injections (in water) on 5 consecutive days. Control animals received only water. The mice were killed about 4 h after the last

treatment. The highest dose tested was chosen to be near LD50, and the lower doses were 1/2, 1/4 and 1/8 of the highest dose. The frequency of micronucleated polychromatic erythrocytes was investigated in bone marrow. Various other chemicals were tested in the same series.

Results
No increase in micronucleated polychromatic erythrocytes by 4-nitro-o-phenylenediamine was noted in the experiments. Various other test agents gave a positive response.

Ref.: 5

Comment
Under the test conditions reported, 4-nitro-o-phenylenediamine did not induce micronuclei in bone marrow polychromatic erythrocytes of mouse. The study was performed before OECD guidelines were available.

Rat bone marrow micronucleus test

Guideline:	OECD 474
Species/strain:	Rat, Sprague-Dawley
Group size:	7 males
Test substance:	4-Nitro-o-phenylenediamine in 1% carboxymethylcellulose
Batch:	A9366 (52575501)
Purity:	Not given
Dose levels:	Single 500, 1000, 2000 mg/kg bw oral gavage on 5 consecutive days, 312, 625, 1225, 2500 mg/kg bw
Sacrifice time:	24 h or 48 h after treatment
GLP:	In compliance

4-Nitro-o-phenylenediamine was tested in the rat micronucleus assay. The vehicle used, 1% carboxymethylcellulose, was used as the negative (vehicle) control and Cyclophosphamide monohydrate (CP: 40 mg/kg bw) as the positive control article. The micronucleus assay was carried out using 3 dose levels of the test article with two bone marrow harvest times. In addition, two groups of 3 animals were dosed with either the vehicle or 2000 mg/kg bw of the test agent for blood collection at 1, 2, 8, and 24 h post-dose. No mortality was observed in the experiment, but lethargy, piloerection, orange skin tone, and orange urine on bedding were seen at 1000 and 2000 mg/kg bw.

Results
No appreciable reduction in the ratio of polychromatic to total erythrocytes was observed in the test article treated animals, suggesting that the test article did not inhibit erythropoiesis. No statistically significant increase in the number of polychromatic erythrocytes in the test article treated animals in comparison with the respective vehicle control animals was observed at either sampling time. In the blood samples, the concentrations of 4-nitro-o-phenylenediamine peaked over the first two sampling times (1 and 2 h) with mean values of 12.2 and 12.3 µg/ml, respectively, and decreased at the later sampling times of 8 and 24 h. This provided evidence of systemic exposure to the test agent in addition to the clinical signs of toxicity observed following treatment with the test article.

Conclusion
The results of the assay indicate that under the conditions described in this report, a single oral administration of 4-nitro-o-phenylenediamine, at doses up to 2000 mg/kg bw, did not induce a statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in male rats. Therefore, 4-nitro-o-phenylenediamine was concluded to be negative in the rat micronucleus test.

Ref.: 50

Rat bone marrow micronucleus test

Guideline:	/
Species/strain:	Rat, CFY
Group size:	5 males and 5 females
Test substance:	4-Nitro-*o*-phenylenediamine
Batch No.:	No data. Merck-Schuchardt
Purity:	Not given
Dose levels:	A total of 5000 mg/kg bw by two gastric intubations, 24 h apart, in 0.5% (w/v) gum tragacanth containing 0.05% (w/v) sodium sulphate
Sacrifice time:	6 h after the last dosing
GLP:	Not in compliance

4-Nitro-*o*-phenylenediaminewas tested for the induction of micronuclei in the bone marrow of CFY rats after two gastric intubations *i.p.* injections at a total dose of 5000 mg/kg bw. The dose was chosen on the basis of preliminary toxicity tests. The rats were killed 6 h after the last treatment. The frequency of micronucleated polychromatic erythrocytes was investigated in bone marrow. Control animals received only the vehicle. Historical control values were reported for 175 rats.

Results
One animal in the group treated with the test agent died. Other animals showed signs of agitation and convulsion. No increase in micronucleated polychromatic erythrocytes by 4-nitro-*o*-phenylenediamine was noted in the experiments. Diaminobenzidine (subcutaneously) and 2-aminofluorene (orally) were reported to give clear positive results (data were not shown).

Ref.: 17

Comment
Under the test conditions reported, 4-nitro-*o*-phenylenediamine did not induce micronuclei in bone marrow polychromatic erythrocytes of mouse. However, only one dose used. No information was given on the bone marrow toxicity of the treatment. Therefore, it is not known if bone marrow toxicity could have affected the negative result. The study was performed before OECD guidelines were available.

Mouse bone marrow micronucleus test

Guideline:	/
Species/strain:	Mouse, NMRI
Group size:	2 males and 2 females
Test substance:	4-Nitro-*o*-phenylenediamine in 0.9% NaCl
Batch:	No data. Merck-Schuchardt
Purity:	>97%
Dose levels:	two *i.p*. injections 24 h apart, 2 x 75, 2 x 150, 2 x 225, 2 x 300 mg/kg bw
Sacrifice time:	6 h after the last dosing
GLP:	Not in compliance

4-Nitro-*o*-phenylenediamine was tested for the induction of micronuclei in the bone marrow of NMRI mice after two i.p. injections (in water) at 4 dose levels. The mice were killed about 6 h after the last treatment. The frequency of micronucleated polychromatic erythrocytes was investigated in bone marrow. Control animals received only the vehicle (saline). A pooled control value was available from previous tests on 313 mice.

Results
All doses were non-lethal. No increase in micronucleated polychromatic erythrocytes by 4-nitro-*o*-phenylenediamine was noted in the experiments. However, the ratio of polychromatic erythrocytes to total erythrocytes was not affected by treatment with 4-nitro-*o*-phenylenediamine, suggesting that the dose range did not include toxic doses.

Ref.: 39

Comment
Under the test conditions reported, 4-nitro-*o*-phenylenediamine did not induce micronuclei in bone marrow polychromatic erythrocytes of mouse. However, as there were neither signs of toxicity nor a decrease in the ratio of polychromatic erythrocytes, the doses tested may have been too low. The study was performed before OECD guidelines were available.

UDS test with rat liver cells *in vivo*

Guidelines:	OECD 486 (1998)
Species/Strain:	Male Sprague-Dawley rats.
Replicates:	Two exposure times (2-4 and 15-16 h)
Animals per dose:	3 per exposure time and dose
Assay conditions:	Single oral gavage at 10 ml/kg bw dosing volume
Test Substance:	4-Nitro-o-phenylenediamine in 1% carboxymethylcellulose
Batch:	A9366 (52575501)
Purity:	As in SEAC study AC040096
Doses:	500, 1000, and 2000 mg/kg bw
GLP:	In compliance

4-Nitro-o-phenylediamine was tested in the unscheduled DNA synthesis (UDS) test with male rat hepatocytes *in vivo* to evaluate the potential of the test article to induce unscheduled DNA synthesis in hepatocytes. The test agent was administered by single oral gavage injection. In a study of the maximum tolerable dose, no mortality was seen among rats (3 animals per group) treated with 500, 1000, or 2000 mg/kg bw. At the highest dose, clinical signs included lethargy, piloerection, cowering, orange coloured urine, yellow fur around tail and anal area, yellow bedding and weight loss. At 500 mg/kg bw piloerection, yellow fur around anal area and on tail, and weight loss were observed. Based on these results, the UDS test was set at the maximum tolerated dose estimated to be 2000 mg/kg bw.

Male rats were treated for either 2 to 4 h or 12 to 16 h. Control animals received the vehicle (1 % carboxymethylcellulose) and positive controls (for both exposure times) dimethylnitrosamine (35 mg/kg bw). No mortality was seen. Prior to harvest, clinical signs included piloerection and orange yellow urine in all animals treated with the test agent, cowering in animals in the 1000 and 2000 mg/kg bw groups, puffy eyes for two animals in the 1000 mg/kg bw group (2-4 h exposure), lethargy and orange-yellow colour around the anal and genital area in the 2000 mg/kg bw group (12-16 h exposure), and excessive strong ammonia odour and tremor in the 2000 mg/kg bw group (2-4 h exposure).

Results
The group mean net nuclear grain (NG) counts for animals treated with 4-nitro-o-phenylenediamine were not increased when compared with vehicle control. As the vehicle control group of the 2-4 h treatment group showed a mean net NG count of 1.4 - when less than 1 is specified in the protocol - the usual analysis of 3 slides per animal was expanded by rescoring two slides in the vehicle control and test agent treated rats. Based on re-scoring of the slides, the group mean NG counts for the test article -treated animals ranged from -1.8 to -2.7 with ≤5% of cells in repair (≥5 NG). The group mean NG count for the vehicle control group was -1.5 with 5% of cells in repair. For the 12 to 16 -h time point, the group mean NG counts for the test article -treated animals ranged from-1.0 to 1.2 with

≤16% of cells in repair. The group mean NG count for the vehicle control group was -1.1 with 5% cells in repair. The positive control group mean NG counts were 23.2 and 21.0 for the 2-4 -h and 12-16 -h exposure, respectively.
Plasma concentrations of 4-nitro-o-phenylenediamine peaked at the first sampling time (1 h) with a mean of 8.8 µg/ml and decreased at 2 and 12 h post-dose. This provided evidence of systemic exposure to the test agent, in addition to the clinical signs of toxicity.

Conclusion
4-Nitro-o-phenylenediamine did not induce a biologically significant increase in the mean number of net nuclear grain counts not an increase of nuclei with at least 5 counts over the negative control group) in hepatocytes isolated either 2 to 4 h or 12 to 16 h after dose administration. Therefore, 4-nitro-o-phenylenediamine was concluded to be negative in the UDS test in mammalian cells *in vivo.*

Ref.: 56

Comment
The reason for the high NG count (outside historical control range) in the 2-4 -h vehicle control group in the 1st scoring was left unclear which casts doubts on the relevance of the assay.

Mouse sperm abnormality assay

Guideline:	OECD 474
Species/strain:	Mouse, C57BL76 x C3H/He
Group size:	4 males
Test substance:	4-Nitro-o-phenylenediamine in water
Batch:	No data
Purity:	Not given
Dose levels:	*i.p.* injection on 5 consecutive days, 312, 625, 1225, 2500 mg/kg
Sacrifice time:	35 days after the last dosing
GLP:	Not in compliance

4-Nitro-o-phenylenediamine was tested for the induction of sperm abnormalities in male C57BL76 x C3H/He mice after daily *i.p.* injections (in water) for 5 consecutive days. Control animals received only water. The mice were killed 35 days after the last treatment and sperm samples were prepared for investigation of sperm abnormalities. The highest dose tested was chosen to be near LD50, and the lower doses were 1/2, 1/4 and 1/8 of the highest dose. Various other chemicals were tested in the same series.

Results
No increase in sperm abnormalities by 4-nitro-o-phenylenediamine was noted in the experiments. Various other test agents gave a positive response.

Conclusion
Under the test conditions reported, 4-nitro-o-phenylenediamine did not induce sperm abnormalities in mice.

Ref.: 5

3.3.7. Carcinogenicity

4-Nitro-o-Phenylenediamine (4-NOPD) alone

In vitro Syrian Hamster Embryo (SHE) transformation assay

Guideline:	/
Species/strain:	Syrian golden hamster embryo cells

Group size:	12 – 15 plates per dose
Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Concentration:	0.05 – 50 µg/ml
Route:	/
Exposure:	8 days
GLP:	not in compliance

SHE cells were isolated from 13th day gestation embryos. These are then cultured for up to 3 days in DMEM with glucose and sodium pyruvate supplemented with L-glutamine and 20% heat inactivated FBS was used. Medium was at neutral pH. SHE cells are cultured in DMEM with 20% FBS on feeder cells (irradiated SHE cells). After 1 day incubation 4ml of test chemical was added to each plate to obtain the desired test concentration (12 – 15 plates per dose). Benzo(a)pyrene or 3-methylcholanthrene were used as positive controls. After 8 days medium was removed and plates were washed and fixed with methanol. Cells were stained with 10% Giemsa and air-dried. Plates were then examined for transformed colonies. A preliminary toxicity test was conducted to determine a dose level that yielded at least 50% cytotoxicity as measured by cloning efficiency. Chemicals were then tested at 2-fold dilutions of this level.

4-NOPD was positive for morphological transformation under the conditions of this assay.

Ref.: 30

Guideline:	/
Species/strain:	Syrian golden hamster embryo cells
Group size:	At least 20 dishes
Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Concentration:	24 h: 50 – 250 µg/ml, 7 days: 5 – 35 µg/ml
Route:	/
Exposure:	24 h or 7 days
GLP:	not in compliance

The standard SHE cell transformation assay has been used but using an alternative culture medium (DMEM with reduced sodium bicarbonate, glucose, phenol levels and with magnesium chloride rather than magnesium sulphate) with reduced pH (6.7 versus 7.2). Benzo(a)pyrene was used as positive control.

SHE cells were isolated from thirteen day gestation embryos and used to determine a dose level that yields at least 50% cytotoxicity. This level is then used as the top dose with three or more additional doses tested. Each test consisted of at least 20 culture dishes per test dose group with between 25 and 45 SHE cell colonies/culture dish. After 24 hour (with 7 days additional culture) or 7 day exposure to the test chemical the cell colonies are fixed with methanol, stained with Giemsa and scored for morphological transformation (MT). At least two tests are then pooled and analysed at each test exposure time.

4-NOPD was negative in the SHE cell transformation assay under the conditions of this assay.

Ref.: 20

Guideline:	/
Species/strain:	Syrian golden hamster embryo cells
Group size:	/

Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Concentration:	20 – 35 µg/ml
Route:	/
Exposure:	7 days
GLP:	not in compliance

The SHE assay was conducted at pH6.7. SHE cells were isolated from 13th day gestation embryos and frozen. SHE cells were seeded into dishes onto feeder cells (x-ray irradiated SHE cells) at a density to produce 25 - 45 colonies per dish. Twenty-four hours later the cells were exposed to the test compound continuously for 7 days. Benzo(a)pyrene was used as positive control. The SHE cell colonies were then fixed, stained and scored for morphological transformation. Plating efficiency was used to assess cytotoxicity.

35µg/ml resulted in relative cytotoxicity of 65% of control as measured by relative plating efficiency (RPE). Concentrations of 4-NOPD up to 35µg/ml did not induce morphological transformations.

Ref.: 43

Guideline:	OECD 495 (under preparation)
Species/strain:	Syrian golden hamster embryo cells
Group size:	/
Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	Lot A9366 Katayama Chemical Ind. Co, Ltd.
Purity:	not stated
Concentration:	24 h: 100 – 300 µg/ml 7 days: 1.0 - 40 µg/ml
Route:	/
Exposure:	24 h or 7 days
GLP:	in compliance

The SHE assay was conducted at pH 6.7. SHE cells were isolated from 13th day gestation embryos and frozen. SHE cells were seeded into dishes onto feeder cells (x-ray irradiated SHE cells) at a density to produce 25 - 40 colonies per dish. Twenty-four hours later the cells were exposed to the test compound continuously for either 24 hours (with 7 days additional culture) or 7 days. The SHE cell colonies were then fixed, stained and scored for morphological transformation. Relative plating efficiency (RPE) was used to assess cytotoxicity.

In the 7 day study, statistically significant increases in morphological transformation frequency were seen at concentrations of 1.0, 2.5, 5.0, 10 and 40µg/ml 4-NOPD. At 24 hours, statistically significant increases in morphological transformation frequency were seen at concentrations of 100, 150, 200, 225 and 250µg/ml 4-NOPD.

4-NOPD was positive for morphological transformation under the conditions of this assay.

Ref.: 55

Comment
In the *in vitro* Syrian Hamster Embryo (SHE) cell transformation assay there was conflicting evidence. One early study from 1980 and a recent study conducted to GLP and draft OECD guidelines showed that 4-NOPD induced SHE cell transformations. On the other hand, two apparently well conducted studies were negative. Thus, it is difficult to draw any conclusions from the SHE transformation studies.

Oral administration, mice

Guideline:	/
Species/strain:	B6C3F1 mice
Group size:	50 Animals per sex and dose, control 20 male and 20 female
Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	Aldrich Chemical Company
Purity:	98%
Dose:	3750 and 7500 ppm in the diet
Route:	Oral
Exposure:	102 weeks
GLP:	in compliance

US National Cancer Institute carried out the study.

A 2-year oral dietary feeding carcinogenicity bioassay was conducted on 4-NOPD using treatment groups of 50 male and 50 female B6C3F1 mice (6 weeks old) and control groups of 20 animals of each sex. The low and high dietary concentrations of 4-NOPD were 3750 and 7500 ppm (equivalent to approximately 450 and 900 mg/kg/day) and were administered for 102 weeks. The treatment period was followed by a treatment free observation period of 2 weeks. Animals were observed for signs of toxicity and tissue masses or lesions throughout the study. Necropsy and histopathology were conducted on all animals.

There were no significant positive associations between exposure to 4-NOPD and mortality in either sex. Animal survival in all groups was sufficient to be able to detect late-developing tumours. Dose-related mean body weight depression was observed in mice, indicating that the concentrations of 4-NOPD administered to these animals may have approximated the maximum tolerated concentrations. There was an increase in the incidence of hepatocellular adenomas in low dose females, compared to the controls (19% compared to 6%), but this was not statistically significant. There was no statistically significant increase in tumour incidence at any site in either sex.

It is concluded that 4-NOPD at the dosage levels used did not induce neoplastic or nonneoplastic lesions in the male or female mice under the conditions of this study.

Ref.: 1

Oral administration, rats

Guideline:	/
Species/strain:	F344 rats
Group size:	50 Animals per sex and dose, control 20 male and 20 female
Test substance:	4-Nitro-o-phenylenediamine (4-NOPD)
Batch:	Aldrich Chemical Company
Purity:	98%
Dose:	375 and 750 ppm in the diet
Route:	Oral
Exposure:	103 weeks
GLP:	in compliance

US National Cancer Institute carried out the study.

A 2-year oral dietary feeding carcinogenicity bioassay was conducted on 4-NOPD using treatment groups of 50 male and 50 female Fischer 344 rats (6 weeks old) and control groups of 20 animals of each sex. The low and high dietary concentrations of 4-NOPD were 375 and 750 ppm (equivalent to approximately 15 and 30 mg/kg/day) and were

administered for 103 weeks. The treatment period was followed by a treatment free observation period of 2 weeks. Animals were observed for signs of toxicity and tissue masses or lesions throughout the study. Necropsy and histopathology were conducted on all animals.

Slight dose-related mean body weight depression was seen in the male rats. A slight depression was seen in the female rats but not dose-related. There were no other indications of chronic toxicity associated with administration of 4-NOPD to either sex. There was no significant association between dose and mortality for male or female rats. Animal survival in all groups was sufficient to be able to detect late-developing tumours.

There was a greater incidence of neoplasms of the adrenal gland in dosed male rats and the hematopoetic system of both male and female rats compared with control rats. These neoplasms are frequently seen in aged F344 rats and the incidence observed is similar to the incidence of spontaneous occurrence of these neoplasms in this strain of rats.

It is concluded that 4-NOPD at the dosage levels used did not induce neoplastic or non-neoplastic lesions in the male or female rats under the conditions of this study.

Ref.: 1

Comment
4-NOPD did not induce tumours in mice or rats after oral administration under the conditions of an US National Cancer Institute study.

4-Nitro-o-Phenylenediamine (4-NOPD) in semipermanent hair dye formulations

Oral administration, dogs

Guideline:	/
Species/strain:	Beagles
Group size:	6 Animals per sex and dose
Test substance:	Hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Dose:	0, 19.5 and 97.5 mg/kg/d (0.0312 and 0.156% 4-NOPD, respectively)
Route:	Oral - diet
Exposure:	24 months
GLP:	not in compliance

Diets were prepared daily with the incorporation of the hair dye formulation to give doses of 0, 19.5 and 97.5mg/kg body weight/day to beagles dogs (7 – 9 month of age when the study was started). Adjustments of concentrations in the diet were made weekly according to body weight changes. Each animal was observed daily 7 days/week for signs of toxic or pharmacologic effects. Individual records of body weight and food consumption were kept on a weekly and daily basis.

Physical examinations including funduscopic, EKG, blood pressure, pulse rate and body temperature were conducted initially and at 3, 6, 12, 18 and 24 months. Haematological, blood chemical and urinalysis parameters were determined on all high dose and control dogs and on 3 males and 3 females from the low dose group. Haematologic studies included determination of total and differential leucocyte counts, haematocrit, haemoglobin concentration, erythrocyte sedimentation rate and prothrombin time. Clinical chemistry determinations were conducted on animals that had been fasted for 18 hours. These included serum glucose, blood urea nitrogen, creatinine and uric acid concentrations and alkaline phosphatase and serum glutamic pyruvic transaminase activities. Urinalysis

included detection of occult blood, albumin, glucose, pH and microscopic examination of urinary sediment.

Necropsy was performed on one male and one female from each group at 6, 12 and 18 months. Individual organ weights and organ to body weight ratios of the major organs were recorded. Sections from 30 tissues or organs were prepared and examined microscopically. Electronmicroscopic evaluation of the livers and urinary bladder from all 18 dogs at 24 months was performed.

No noteworthy differences were seen in any of the parameters studied between the controls and the animals receiving 19.5 or 97.5mg/kg body weight/day. All dogs gained weight normally and survived to end of the 104 weeks. All dogs in the two test groups excreted urine of a blue-brown colour on a daily basis. However urine analysis showed no remarkable findings. Colour was normal in urine collected after overnight fasting.

Oral dosing exposure of a hair dye formulation containing 0.16% 4-NOPD up to 97.5 mg/kg/day did not result in any signs of toxicity.

Ref.: 38

Topical application, mice

Guideline:	/
Species/strain:	A and DBAf mice
Group size:	24 - 26 Animals per sex and dose, control 16 animals per sex
Test substance:	Semi-permanent hair dye formulation containing approximately 0.06% 4-nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Dose:	Approximately 0.06 % 4-NOPD
Route:	Topical application twice weekly
Exposure:	80 weeks
GLP:	not in compliance

Groups of 48 male and female DBAf or 52 male and female A young adult mice were given twice weekly skin applications on the clipped dorsal skin (DBAf mice: 0.4 ml reduced to 0.2 ml at 24 weeks. The toxic effects may have been due to obstruction of the uro-genital tract by crystals which were sometimes seen in the bladder and skin round the penis. A mice: 0.4 ml) of a 10% solution of a commercially available hair dye, colorant 'GS', containing, among other constituents, 4-NOPD in 50% aqueous acetone; 32 control mice of each strain received acetone only.

When the experiment was terminated at 80 weeks, five lymphomas and six tumours of the female reproductive tract (four ovarian cystadenomas and two uterine fibrosarcomas) and three small squamous papillomas developed had developed in the treated DBAf mice within 26–80 weeks; in the DBAf control mice, one lymphoma, one liver tumour and one lung tumour were found. No differences occurred in the incidence of lymphomas, liver or lung tumours between treated and control A mice. Of the treated animals, 19 DBAf mice and 27 A mice survived 60 or more weeks and died without tumours.

It is concluded that twice weekly topical application of a semi-permanent hair dye containing 4-NOPD for 80 weeks to strain A-mice did not cause statistically significant increase in tumour incidence. However, the DBAf strain mice did show a statistically significant increase in tumour incidence. The topical applications were made using a relatively complex mixture including other dye components. It is therefore difficult to attribute any of these findings to 4-NOPD alone.

Ref.: 34

Comment
No effect was found with a hair dye formulation containing 0.16% 4-NOPD in a two-year oral study with beagle dogs. Topical application of a semi-permanent formulation containing 0.06% 4-NOPD caused increase tumour incidence in DBAf mice but not in A mice. It is not possible to assess the role of 4-NOPD in the tumour induction.

4-Nitro-o-Phenylenediamine (4-NOPD) and hydrogen peroxide in permanent hair dye formulations

Mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex and dose
Test substance:	One hair dye formulations (7403) containing 0.25% 4-nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated
Dose level:	0.05 ml of a solution containing 0.25% 4-NOPD (dye formulation 7403) prior to mixing with an equal volume of 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Route:	Topical, 1 application weekly
Exposure:	21 months
GLP:	not in compliance

The experiment involved altogether 12 different dye formulations and 3 negative control groups.

Dye applied topically to a 1 cm^2 area on a clipped (24 hours prior to application) site in the interscapular region. Mice received a dose of 0.05 ml topically without occlusion once weekly from 8 – 10 weeks of age for 21 months. The animals were observed daily for mortality and signs of toxicity, and were weighed monthly. A continuous weekly record was maintained for any skin lesions noted. After 7 months of treatment, 10 males and 10 females per group were necropsied and the study was terminated after 21 months. Skin and internal organs were evaluated histologically.

Six males and 12 females survived to 21 months in the group receiving the oxidative formulation containing 4-NOPD. At 21 months, there were 8 – 12 males and 11 – 14 females surviving in the control groups. There were no significant differences in absolute or relative liver or kidney weights in groups of 10 male and 10 female mice necropsied after 7 months. There were no statistically significant differences in the distribution of tumours among treated and control groups.

Ref.: 7

Rat

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats
Group size:	60 animals per sex and dose
Test substance:	One hair dye formulation (7403) containing 0.25% 4-nitro-o-phenylenediamine (4-NOPD)
Batch:	/
Purity:	not stated

Dose level:	0.5 ml of a solution containing 0.25% 4-NOPD (dye formulation 7403) prior to mixing with an equal volume of 6% hydrogen peroxide. The mixture was used within 15 minutes after mixing
Route:	Topical. 1 application twice weekly
Exposure:	114 weeks
GLP:	not in compliance

The experiment involved altogether 10 different dye formulations and 3 negative control groups.

Groups of 60 male and 60 female were obtained from the first mating (F_{1a}) of a multigeneration reproduction study in rats treated with a hair dye formulation containing 1% N-phenyl-p-phenylenediamine. The F_0 parents had received topical application of the hair dye formulation from the time of their weaning to the weaning of their offspring. The dye formulation was administered topically to the shaved (24 hours prior to application) neck and back area twice weekly. An initial dosage level of 0.2 ml/rat was increased incrementally by 0.1 ml per week until 0.5 ml was achieved. There were three independent control groups each containing 60 males and 60 females, which received no treatment.

The rats were observed daily for overt signs of toxicity and for mortality. Detailed observations were recorded weekly. Individual body weights were recorded weekly for the first 14 weeks and monthly thereafter. Group food consumption was recorded weekly. Haematological, biochemical and urinalysis studies were done on 5 males and 5 females per group at 3, 12, 18, and 24 months of study. After 12 months of treatment, 5 males and 5 females from each group were sacrificed and necropsied and all rats of a sex group were sacrificed and necropsied when survival reached 20%. Histopathological evaluations were performed on 18 tissues (plus tumour masses) including treated skin.

Survival just prior to terminal sacrifice at week 114 was 22 males and 14 females for the formulation group. Survival in the control groups was 17 – 20 males and 22 – 26 females for the control groups. The mean body weights at week 114 in the treated group were 656 g in males and 527 g in females. Control group values ranged from 682 to 759 gm in males and 477 to 513 g in females.

There were no significant changes in haematological values in the treated groups at 18 and 24 months. No significant differences considered to be treatment related were observed in the biochemical studies or in the urinalysis. Non-neoplastic lesions were those commonly found in ageing rats and were considered to be spontaneous.

The incidence of adenomas in the mammary gland was increased in animals treated with the dye formulation when compared with the incidence in control group 3 (where there were no adenomas), but not when compared with control groups 1 and 2. The incidence of all other tumours observed was comparable between the treated and control groups, with the exception of pituitary adenomas which were reduced in treated animals compared with control group 2.

It is concluded that twice-weekly topical application of an oxidative hair dye formulation containing 0.25% 4-NOPD did not result in increased tumour incidence in any of the tissues examined.

Ref.: 8

Comment
One study on 4-NOPD together with hydrogen peroxide involving topical application of mice and one involving topical application on rats have been identified. The concentration of 4-NOPD after mixing with hydrogen peroxide was in both studies 0.125% (the maximum concentration on the human scalp is 0.5%). A number of different hair dye formulations were tested in the same study. Although some of the formulations contained 2,4-

diaminoanisol (classified as carcinogen category 2 in EU), none of the formulations induced tumours in the mice or rats. Thus, no conclusion with regard to carcinogenicity can be made from the study.

Conclusion
Conflicting results have been found in *in vitro* studies using the Syrian hamster embryo (SHE) cell transformation assay. 4-NOPD did not induce tumours in mice or rats after oral administration under the conditions of an US National Cancer Institute study. No conclusions with regard to carcinogenicity can be drawn from animal studies were hair dye formulations were tested. IARC has classified 4-NOPD in Group 3 (Not classifiable as to carcinogenicity to humans).

3.3.8.	Reproductive toxicity

Guideline:	/
Species/strain:	Rat, Sprague-Dawley CD
Group size:	10 males and 20 females per dose group
Test substance:	A composite of hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine (and 13 other dyes)
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 1950 and 7800 ppm hair dye formulation in diet
Route:	Oral, dietary
Vehicle:	None
Dosing schedule:	Females: from 8 weeks prior to mating until 21st day of lactation Males: from 8 weeks prior to mating until after mating
GLP:	Not in compliance

A fertility and reproduction study in rats was carried out in two parts in which groups of 10 males and 20 females were treated separately with a diet containing 1950 or 7800 ppm of a hair dye formulation with 0.16% 4-nitro-o-phenylenediamine (corresponding to about 0.125 and 0.5 mg/kg bw/d). In the first part, females received basal diet 8 weeks prior to mating and during gestation to 21 days of lactation, while males were fed test diets for 8 weeks prior to and during mating period. In the second part, males received basal diet for 8 weeks prior to and during mating, while the females received test diet 8 weeks prior to mating and during gestation to 21 days of lactation. In both parts of the study, one male and two females were paired from until the presence of sperm during daily vaginal smears was confirmed (day 0 of pregnancy). One female pregnant by each male was sacrificed at day 13 of gestation to obtain information regarding the early states of gestation. The uterus was examined for the number and distribution of embryos, the presence of empty implantation sites and the number of embryos undergoing resorption. Each embryo was examined under a microscope. The remaining females were allowed to deliver normally. Females that did not deliver a litter were examined. The duration of gestation was noted and the litters were examined for numbers of live and stillborn pups and gross abnormalities. Pups were weighed at birth, 4, and 21 days. At 21 days all surviving pups were sacrificed and examined for gross abnormalities.

Results
Other than blue-brown staining of the urine, no treatment related adverse effects were observed in male or female parents fed either dose level, or in the pups of these parents.

Conclusion
No evidence was found to indicate that dietary exposure up to 7800 ppm hair dye formulation in the diet from 6 to 15 days of pregnancy had any adverse effect on the males, females or the pups.

Ref.: 38

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (batch number unknown, not according to a guideline, no raw data available) this study is of limited use for evaluation.

3.3.8.1. Multigeneration reproduction toxicity

Guideline:	/
Species/strain:	Rat, Sprague-Dawley
Group size:	40 per sex and dose group
Test substance:	Hair dye formulation (7403) containing 0.25% 4-nitro-o-phenylenediamine (and 3 other dyes)
Batch:	Not specified
Purity:	Not specified
Dose levels:	0.5 ml/kg hair dye formulation twice a week
Route:	Dermal
Vehicle:	None
GLP:	Not in compliance

Treatment was continuous through growth, mating, gestation, and lactation to weaning at the F_{1b}, and F_{2b} litters of the respective generations. The hair dye formulation was mixed with an equal volume of 6% H_2O_2 just prior to use. It was applied topically to the shaved (24 hours prior to applications) backs of the rats twice weekly at a final dosage level of 0.5 ml/rat. Application was made to the parental generation (F_0) from 6-8 weeks of age. They were mated at 100 days of age. The females were permitted to deliver their young (F_{1a}) naturally and litter size was reduced to 10 pups on Day 4 of lactation. F1a pups were assigned to a lifetime chronic toxicity study. F_0 parents were reduced to 20 males and 20 females per group and were mated again to produce F_{1b} litters. Twenty males and 20 females were selected from the F_{1b} litters to become the F_1 parents of the next generation. Animals were treated from weaning as for the F_0 rats, and were mated twice to produce the F_{2a} and F_{2b} litters. Twenty males and 20 females from the F_{2b} litters were mated to produce a third generation, but results of this were not reported as they were confounded by the presence of a viral infection. Rats were observed daily for behaviour and appearance. Body weight and food consumption were recorded weekly. Reproductive parameters were evaluated to determine fertility index, gestation anomalies, and affects on parturition and lactation. Pups were counted and weighed (as a litter) on Days 0, 5, and 14 and were weighed individually, usually on Day 21 of lactation. Live birth and survival indices were calculated. Gross necropsies and microscopic examinations were performed on 5 males and 5 females from the F_1 generation.

Results
Parental generations: Body weight gains, food consumption, and survival were similar among treated and control parents (F_1 and F_2 generations). Changes in appearance and behaviour attributable to treatment were restricted to local skin reactions.
Reproductive performance: Performance of F_0, and F_1 parental rats showed no difference for treated and control rats with respect to fertility, gestation, and live birth indices.
Offspring: Litter size, body weights, and survival of young were similar for test and control groups. There were no gross or microscopic lesions considered to be related to treatment in F_1 parents that were sacrificed, necropsied, and evaluated microscopically.

Conclusion
Topical application of a hair dye formulation containing 0.25% 4-nitro-o-phenylenediamine twice weekly throughout growth, mating, gestation and lactation phases of the F_0 parents to the F_{1a} and F_{2b} litters was not associated with any adverse developmental effects.

Ref. 8

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (purity and batch number unknown, not according to a guideline, no raw data available) this study is of limited use for evaluation.

3.3.8.2. Teratogenicity

Guideline:	/
Species/strain:	Mouse, albino CD-1
Group size:	20 mated females per dose group
Test substance:	4-nitro-o-phenylenediamine
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 16, 32, 64, 128, 256, 512, 768, and 1024 mg/kg bw/day
Exposure:	Gestation Days (GD) 1 through 15, once a day
Route:	Dermal, subcutaneous injection
Vehicle:	Sterile, distilled water
GLP:	Not in compliance

Two females per male were housed together; day 1 of gestation was defined as the morning a vaginal plug was found. Females were then housed 10 per cage. By day 6 females were divided into experimental and control groups, minimising body weight differences. Dosing by subcutaneous injection once a day on days 6 - 15 of gestation. On day 18 of gestation the mice were sacrificed by cervical dislocation. Implantation sites on each uterine horn were recorded. The uterine contents were removed and examined. Visceral examination on at least one third of the foetuses from each litter, stunted foetuses and those with external malformations was conducted. All foetuses were then processed for skeletal examination. The heads of those used in the visceral examination (except those with external head malformations) were cut off and prepared for free-hand sectioning. Histopathological examination of some of the foetal hearts with white deposits in the left ventricle was conducted.

Results
Maternal deaths occurred at 768 mg/kg/day (6/36) and 1024 mg/kg/day (7/37). Doses of 256 mg/kg/day and above showed significant reductions in average weight gain of the dams during pregnancy and average foetal weight, while the 768 mg/kg/day dose had a significant effect on the average number of live foetuses per dam. At days 6 – 17 the reduction in average weight gain during pregnancy seen with at 256 mg/kg/day appeared to be accounted for by the reduction in average foetal weight. However, the gravid uteri were not weighed at necropsy so this could not be definitively proven. At this dose 4-nitro-o-phenylenediamine shows indications of embryotoxicity and an equivocal effect on maternal weight gain. There was no assessment of maternal toxicity other than body weight.
At 256 mg/kg/day and above there was a significant increase in the number of foetuses with cleft palate, resulting in a significant increase in the average percentage of malformed foetuses. Major blood vessels were also affected at doses of 512 and 1024 mg/kg/day. Dose levels of 512 mg/kg/day and above were significant for white foci in the left ventricle of the foetal heart, these lesions were positive for calcium with appropriate stains. 4-Nitro-o-phenylenediamine was teratogenic at 256 mg/kg/day, although the major defect was cleft palate, the cardiovascular effects were more remarkable.

Conclusion
Teratogenic and embryotoxic effects of 4-nitro-o-phenylenediamine were only observed at maternally toxic doses. The lowest observed effect level (265mg/kg/day) was associated with an equivocal effect on maternal weight gain (no other measurements of maternal toxicity were conducted), reduced foetal weight and increase in the incidence of cleft palate (16 compared to 6 in control). The no observed effect level for maternal and embryo-foetal effects was 128 mg/kg/day.

Ref.: 23

Comment
This reference is a publication in open literature. Therefore no raw data are available. In the submission it is argued that teratogenic and embryotoxic effects were only observed at maternally toxic doses. The effects on maternal weight gain observed at the lowest observed effect level for teratogenic and embryotoxic effects (265 mg/kg/day) were judged to be equivocal by the applicant. The authors of the publication, however, argued that the effects could be accounted for by the reduction in average foetal weight and concluded that this dose was not toxic to pregnant dams. Since no other measurements of maternal toxicity were conducted, no definite conclusion with respect to the maternal toxic dose could be drawn. Therefore, it can not be ruled out that teratogenic (increased incidence of cleft palate) and embryotoxic (reduced foetal weight) effects occurred at dose levels which were not toxic to the pregnant dams.
Despite the deficiencies of this study (purity and batch number unknown, not according to a guideline, no raw data available, no assessment of maternal toxicity other than body weight), this study is useful for evaluation.

Guideline:	/
Species/strain:	Rat, Charles River CD
Group size:	20 mated females per dose group
Test substance:	Hair dye formulation (7403) containing 0.25% 4-nitro-o-phenylenediamine
Batch:	Not specified
Purity:	Not specified
Dose levels:	2 ml/kg
Exposure:	Gestation Days (GD) 1, 4, 7, 10, 13, 16, and 19
Route:	Dermal, topical application, non-occluded
Vehicle:	None
GLP:	Not in compliance

Groups of 20 pregnant Sprague Dawley rats were exposed topically to a hair dye formulation (7403). The hair colorant formulation containing 0.25% 4-nitro-o-phenylenediamine (mixed with an equal volume of 6% H_2O_2 just prior to use) was applied at a dose of 2 ml/kg on gestation days 1, 4, 7, 10, 13, 16, and 19 to a site on the dorso-scapular area. Three separate negative control groups (untreated but shaved) were employed and a positive control group received acetylsalicylic acid by gavage at a dose of 250 mg/kg on gestation Days 6 through 16. Caesarean sections were performed on Day 20. Uterine contents and foetuses were examined and preserved for evaluation. Approximately one third of each litter underwent visceral examination and the remaining foetuses underwent examination for skeletal abnormalities.

Results
No signs of toxicity were seen throughout the study. Maternal food consumption and body weight gain was similar between treated and control groups. There were no significant differences between treated and control rats with respect to maternal parameters (numbers of corpora lutea, implantation sites, resorption sites) or with respect to foetal parameters (numbers of live foetuses, dead or resorbed foetuses). There were no statistically significant variations in skeletal or soft tissue findings. Embryotoxic and teratogenic effects consistent with the reported effects were seen in the acetylsalicylic acid positive control group.

Conclusion
Topical application of a hair dye formulation containing 0.25% 4-nitro-o-phenylenediamine to rabbits was not associated with teratogenic or other adverse developmental effects.

Ref.: 6

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (batch number unknown, not according to a guideline, no raw data available) this study is of limited use for evaluation.

Guideline:	/
Species/strain:	Rat, CRE-S
Group size:	20 mated females per dose group
Test substance:	A composite of hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine (and 13 other dyes)
Batch:	Not specified
Purity:	Not specified
Dose levels:	0, 1950 and 7800 ppm in diet
Exposure:	Gestation Days (GD) 6 -15
Recovery:	GD 16 -19
Route:	Oral, dietary
Vehicle:	None
GLP:	Not in compliance

Sixty male and sixty female virgin CFE-S rats were divided into three groups of 20 males and 20 females each. One male and one female were mated until the presence of sperm during daily vaginal inspections was confirmed (day 0 of pregnancy). Females were then fed diet containing the dye formulation from day 6 to day 15 of gestation at levels of 0, 1950 and 7800 ppm (corresponding to about 0.125 and 0.5 mg/kg bw/d). Caesarean section was performed on the 19th day of pregnancy. The number and distribution of foetuses and number of corpora lutea, live and stillborn foetuses, and early and late resorptions were recorded. Each foetus was weighed, measured and examined for gross abnormalities. One third of each litter was examined for visceral abnormalities by Wilson serial sectioning and the other two thirds were examined for skeletal abnormalities.

Results
No evidence of adverse effects on the pregnant rat or pups was found. No dose-related significant differences were observed in the average number of implantation sites, live pups, or early and late resorptions per litter or in the number of females with one or more resorption sites. No grossly abnormal pups were noted in the low dose group; there was one in the control group. One grossly abnormal pup was noted out of the 262 examined in the high dosage group. In the litter with the abnormal pup, there were 13 other pups, all of which were normal. The rats in the test groups excreted urine of a blue-brown colour.

Conclusion
No evidence was found to indicate that administration of up to7800 ppm (616mg/kg/day) the hair dye formulation in the diet from 6 to 15 days of pregnancy had any adverse effect on the pregnant rat or the pups.

Ref.: 38

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (batch number unknown, not according to a guideline, no raw data available) this study is of limited use for evaluation.

Guideline:	/
Species/strain:	Rabbit, New Zealand White
Group size:	12 artificially inseminated females per dose group
Test substance:	A composite of hair dye formulation containing 0.16% 4-nitro-o-phenylenediamine (and 13 other dyes)

Batch:	Not specified
Purity:	Not specified
Dose levels:	19.5 or 97.5 mg/kg bw/day composite with hair dyes (corresponding to 0.03 or 0.15 mg 4-NOPD) or 97.5 mg/kg bw/day composite without hair dyes
Exposure:	Gestation Days (GD) 6 -18
Recovery:	GD 18 -30
Route:	Oral, by gavage
Vehicle:	0.5 % aqueous methylcellulose
GLP:	Not in compliance

The rabbits were artificially inseminated by the method of Gibson et al. (1966), day 0 of pregnancy. All animals were dosed daily by gavage on days 6 to 18 of gestation with test material or vehicle at 1 ml/kg. On the 30th day of gestation rabbits were sacrificed and their uterine contents examined. The number of pregnancies, average foetal weight, and maternal weight gains per group and mean values per female for number of corpora lutea, implantations, resorptions and live and stillborn foetuses were assessed. Half of the foetuses' viscera were examined for gross abnormalities and then the carcasses cleared and the skeletons stained and examined for defects. The remaining foetuses were incubated for 24 hours for an evaluation of foetal viability. The foetuses then had viscera examination and the skeletons stained and examined for defects.

Results
No evidence of adverse effects on the pregnant rabbit or offspring was found. Foetal survival was not adversely affected. No grossly abnormal foetuses or soft tissue defects were seen. Skeletal examination showed variations in the degree of ossification and in the number of ribs; the distributions of these changes showed no relationship to treatment. Animals in the high dose group excreted urine of a blue-brown colour. Urine colour was normal the following day prior to dosing.

Conclusion
No evidence was found to indicate that dosing with up to 97.5 mg/kg/day of the hair dye formulation from 6 to 18 days of pregnancy had any adverse effect on the pregnant rabbit or offspring.

Ref.: 38

Comment
This reference is a publication in open literature. Therefore no raw data are available. Despite the deficiencies of this study (batch number unknown, not according to a guideline, no raw data available) this study is of limited use for evaluation.

3.3.9. Toxicokinetics

Absorption, distribution, metabolism and excretion of 4-nitro-o-[U-^{14}C]-phenylenediamine after oral (gavage) administration to female rats

Guideline:	OECD 417 (1984)
Species/strain:	Rat, albino, Han Brl: Wist (SPF) Wistar
Group size:	8 Females
Test substance:	4-nitro-o-phenylenediamine
Vehicle:	1% carboxymethylcellulose solution
Batch:	4-nitro-o-[U-^{14}C]-phenylenediamine dihydrochloride: Batch number: 1, SEAC sample number: S2600401, RCC sample number: 152064/A Unlabelled 4-nitro-o-phenylenediamine: Lot number: A9366, Batch no./SEAC sample number: S2575501, RCC sample number: 152019/A

Purity:	Radiochemical purity: >97 % by HPLC and TLC on June 14, 2004 and 98.8% by HPLC on June 30, 2004 Non-labelled: not specified
Dose levels:	45 mg/kg bw (containing approximately 63 kBq/mg or 1.7 µCi/mg radioactivity)
Dose volume:	5 ml/kg bw
Route:	Oral, by gavage
Dosing schedule:	Single administration
GLP:	In compliance

Eight female Wistar rats were dosed orally by gavage with 45 mg/kg bw ^{14}C 4-nitro-o-phenylenediamine. The levels of radioactivity appearing in the urine and faeces were followed up to 96 hours after dosing. From one rat per time point, blood was taken from the sublingual vein at 30 minutes, 1, 1.5, 2, 3, 4, 5 and 7 hours after dosing. At sacrifice 96 hours after dosing, residual radioactivity was determined in the blood/plasma and residual carcass and a balance was given. The metabolite pattern in selected urine samples and faecal extracts was also determined.

Results
Maximal blood and plasma levels of radioactivity were observed at 30 minutes after dosing. The majority of the radioactivity was rapidly excreted via urine. 57.4 ± 8.7% was found in urine after 24 hours, and a total of 64.6 ± 7.1% was found by 96 hours. In the faeces, 16.8 ± 5.7% was found at 24 hours and a total of 27.0 ± 5.1% was found by 96 hours. Including the cage wash (3.9 ± 2.6%) the total excreted radioactivity by 96 hours amounted to 95.5 ± 1.9%. Low residual radioactivity was found in blood (<0.05%) and the carcass (0.9 ± 0.2%). The total recovery of radioactivity on average amounted to 96.4 ± 1.8% of the total administered dose (Table 1).

Table 1: Balance of radioactivity and excretion pattern

	Mean	**± S.D.**
Urine 0-24 hours	57.4	8.7
Total urine 0-96 hours	**64.6**	**7.1**
Faeces 0-24 hours	16.8	5.7
Total faeces 0-96 hours	**27.0**	**5.1**
Cage wash	3.9	2.6
Total excreted	**95.5**	**1.9**
Blood	<0.05	<0.05
Remaining carcass	0.9	0.2
Subtotal blood/carcass	**0.9**	**0.2**
TOTAL	**96.4**	**1.8**

HPLC analysis of pooled urine from selected rats showed 7-8 radioactive fractions.
The major fraction, identified as N-acetyl-4-nitro-o-phenylenediamine accounted for 57.9% of the radioactivity on the HPLC trace (rel.%), corresponding to 9.4% of the administered radioactivity (adm.%) at 0-4 hours, and 74.2 rel.% (corresponding to 10.2 adm.%) at 4-8 hours.
The second fraction, identified as N-acetyl-acetylcysteine-4-nitro-o-phenylenediamine, accounted for 17.6 rel.% (corresponding to 2.9 adm.%) at 0-4 hours, and 4.8 rel.% (corresponding to 0.7 adm.%) at 4-8 hours. The third major fraction identified was unchanged 4-nitro-o-phenylenediamine occurring at 9.4 rel.% (corresponding to 1.5 adm.%) at 0-4 hours, but not distinctly separated in the 4-8 hour pool. Four other minor

metabolites were detected, but not identified. Together, the metabolites accounted for 30.2% of the administered dose after 8 hours.

Analysis of faecal extracts revealed the major metabolite to be N-acetyl-4-4-nitro-o-phenylenediamine, making up 27.5% of the radioactivity present in the faeces (rel.%), corresponding to 1.0% of the radioactivity administered (adm.%) at 0-24 hours and 18.2 rel.%, corresponding to 0.4 adm.% at 24-48 hours. The remainder of the radioactivity could not be identified.

Conclusion
It was concluded that at least 65% of the administered dose was absorbed, based on urinary output. Given that conjugated 4-nitro-o-phenylenediamine was found, at least part of that excreted in faeces had been absorbed. Thus absorption of 4-nitro-o-phenylenediamine may have approached 100%. Based on HPLC analysis of urine, 4-nitro-o-phenylenediamine was mostly converted into the conjugates N-acetyl-4-nitro-o-phenylenediamine and N-acetyl-actetylcysteine-4-nitro-o-phenylenediamine, and only low amounts of unchanged 4-nitro-o-phenylenediamine were present. Additionally, at least 4 unknown conjugates and/or metabolites occurred in minor amounts.

Ref.: 51

Whole body autoradiography of albino rat following oral administration of 4-nitro-o-[U-^{14}C]-phenylenediamine

Guideline:	/
Species/strain:	Rat, albino, Han Brl: Wist (SPF) Wistar
Group size:	5 Females
Test substance:	4-nitro-o-phenylenediamine
Vehicle:	1% carboxymethylcellulose solution
Batch:	4-nitro-o-[U-14C]-phenylenediamine dihydrochloride: Batch number: 1, SEAC sample number: S2600401, RCC sample number: 152064/A Unlabelled 4-nitro-o-phenylenediamine: Lot number: A9366, Batch no./SEAC sample number: S2575501, RCC sample number: 152019/A
Purity:	Radiochemical purity: >97 % by HPLC and TLC on June 14, 2004 and 98.8% by HPLC on June 30, 2004 Non-labelled: not specified
Dose levels:	45 mg/kg bw (containing approximately 63 kBq/mg or 1.7 µCi/mg radioactivity)
Dose volume:	5 ml/kg bw
Route:	Oral, by gavage
Dosing schedule:	Single administration
GLP:	In compliance

Five albino female Wistar rats were dosed orally by gavage with 45 mg/kg bw ^{14}C 4-nitro-o-phenylenediamine, and one rat was killed by CO_2 narcosis at 2, 8, 24, 48 and 96 hours post dosing. Frozen rat carcasses were embedded and frozen in wallpaper paste. Longitudinal sections were cut from head to tail at 25µm thickness to obtain the relevant organs and tissues. Sections were dehydrated for 48 hours. Five or six sections from each rat were applied to imaging plates (IP) and exposed to the IPs for 7 days. Following exposure the IPs individually scanned using a phosphorimager by the Image Plate Reader. The scan data were captured electronically by TINA v.2.09g. The areas of the tissues were defined using the tool in TINA for each section. The photostimulated luminescence per area measured was converted to nCi/g and background corrected. Mean and standard deviations were calculated where possible.

Results
^{14}C blood levels (measured in the heart only) were highest 2 hours after dosing, dropping rapidly 22 hours post dosing and more slowly in the following 72 hours. At 2 hours after dosing there was widespread distribution of ^{14}C throughout all the organs and tissues of the body, following absorption from the GI-tract (with exception of the white fat and bone). By 8 hours the levels in the tissues were falling and were generally significantly lower by 24 hours. Elimination was fairly rapid from the body and by 24 hours and a significant proportion of the dose excreted by the faecal route. At 8 hours the key organs and tissues, which demonstrated localisation of ^{14}C labelled material were the thyroid, kidney, liver, lacrimal glands, Meibomian glands (in the eyelid), fore-stomach lining (a rat specific tissue) and in the brain and spinal cord. Levels in most tissues fell by 24 hours however complete elimination of ^{14}C labelled material (parent or metabolites) particularly from the key tissues i.e. the thyroid, liver, kidney, brain, and spinal cord was not achieved by 96 hours after dosing.

Conclusion
4-nitro-o-phenylenediamine or radiolabelled metabolites were widely distributed throughout the body following absorption after oral administration. Although elimination via the urinary and faecal routes ensured rapid removal from the body by 24 hours of a significant proportion of the absorbed dose, complete elimination of ^{14}C labelled material (parent or metabolites) from key tissues was not achieved by 96 hours after dosing. Key tissues exhibiting localisation included the thyroid gland, lacrimal glands, liver, kidney, Meibomian glands, brain (and spinal cord) and fore-stomach lining (a rat specific tissue). Localisation in the brain (and spinal cord) indicated that the material was able to pass the blood brain barrier (BBB) and be retained in the nervous tissue up to 96 hours.

Ref.: 46

Comment
Although this study is not performed according to a guideline, it is useful for evaluation.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

Phototoxicity

Guideline:	OECD 432
Test strain:	Balb/c 3T3 mouse fibroblast cells
Substance:	4-nitro-o-phenylenediamine
Batch:	S2510001
Purity:	/
Dose levels and Controls:	Maximum of 100µg/ml 4-NOPD tested (8 concentrations tested). Chlorpromazine (positive control) Vehicle control (untreated cells) Blanks (vehicle only without cells)
GLP:	in compliance

96-well plates were seeded with 10000cells/100µl heat-inactivated newborn calf serum (DMEM) and incubated overnight.
After culture medium was removed from the cells they were washed with phosphate buffered saline (PBS). 100µl of each test concentration was put into eight replicate wells with blanks and vehicle controls on each plate. Duplicate plates were prepared, with the test compound and positive control on separate plates. Following a one hour incubation, one of the duplicate plates was irradiated by UVA/visible light for 50 minutes using the Hone SOL 500 lamp and the other replicate plate was kept in the dark. Plates were then washed,

media reapplied and incubated overnight. After medium was removed cells were washed with PBS and 100µl DMEM of 50µg/ml neutral red (NR) was added to the wells. Plates were incubated for a further 3 hours.
Medium was removed, cells washed with PBS and 150µl of destain solution added and cells were agitated at room temperature for 10 minutes. The absorbance was measured at 540nm against an air blank by Datacapture RMS v12. The concentration of test item causing a 50% reduction in neutral red uptake from control cell level (EC50) was estimated in the presence and absence of UV light using the PHOTOTOX (version 1) software. The software was used to calculate the photoirritation factor (PIF) and Mean Photo Effect (MPE).

Results
Chlorpromazine had a mean PIF of 47 and MPE of 0.853 indicating phototoxic potential.
4-NOPD had a PIF and MPE of 0.019.

Conclusion
Chlorpromazine was shown to be phototoxic and the results were consistent with historical positive control data.
4-NOPD was not phototoxic under the conditions of this study.

Ref.: 58

Guideline:	OECD 432
Test strain:	Balb/c 3T3 mouse fibroblast cells
Substance:	4-nitro-o-phenylenediamine
Batch:	A9366
Purity:	/
Dose and Controls:	Maximum of 100µg/ml 4-NOPD tested (8 concentrations tested) Chlorpromazine (positive control) Vehicle control (untreated cells) Blanks (vehicle only without cells)
GLP:	in compliance

96-well plates were seeded with 10000cells/100µl heat-inactivated newborn calf serum (DMEM) and incubated overnight.
After culture medium was removed from the cells they were washed with phosphate buffered saline (PBS). 100µl of each test concentration was put into eight replicate wells with blanks and vehicle controls on each plate. Duplicate plates were prepared, with the test compound and positive control on separate plates. Following one hour incubation, one of the duplicate plates was irradiated by UVA/visible light for 50 minutes using the Hone SOL 500 lamp and the other replicate plate was kept in the dark. Plates were then washed, media reapplied and incubated overnight. After medium was removed cells were washed with PBS and 100µl DMEM of 50µg/ml neutral red (NR) was added to the wells. Plates were incubated for a further 3 hours.
Medium was removed, cells washed with PBS and 150µl of destain solution added and cells were agitated at room temperature for 10 minutes. The absorbance was measured at 540nm against an air blank by Datacapture RMS v12. The concentration of test item causing a 50% reduction in neutral red uptake from control cell level (EC50) was estimated in the presence and absence of UV light using the PHOTOTOX (Version 1) software. The software was used to calculate the photoirritation factor (PIF) and Mean Photo Effect (MPE).

Results
Chlorpromazine had a mean PIF of 85 and MPE of 0.796 indicating phototoxic potential.
4-NOPD had an unconfirmed PIF and a mean MPE of –0.57.

Conclusion
Chlorpromazine was shown to be phototoxic and the results were consistent with historical positive control data.

4-NOPD was not phototoxic under the conditions of this study.

Ref.: 59

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

Summaries of three human patch test studies have been reported in the CIR for 4-NOPD.

A repeat insult patch was conducted on 206 subjects using a hair dye containing 0.027% 4-NOPD. An equal mix of dye and oxidiser was applied using a non-occlusive challenge patch at 0.1ml/cm2 on the backs of the subjects. Ten 48 to 72 hour consecutive applications were made to the backs of subjects followed by an 11-day rest period. A 48-hour non-occlusive challenge patch was applied to a naive site on the back of each subject and the reactions were read at 15 minutes and 24 hours. There were no positive reactions at any induction or challenge reading, it was concluded that there was no evidence that the hair dye/oxidiser mix caused either irritation or sensitisation under the conditions of the study.

A further repeated insult patch test was conducted with a hair dye containing 0.039% 4-NOPD on the same 206 subjects following the same procedure. There were 41 very mild erythema reactions which barely exceeded that of the untreated skin during induction. No positive reactions were seen at any induction or challenge reading. It was concluded that there was no evidence that the hair dye/oxidiser mix caused either irritation or sensitisation.

The same 206 subjects were again used for a further repeated insult patch test using a hair dye containing 0.049% 4-NOPD following the same procedure. There were no positive reactions at any induction or challenge reading, it was again concluded that there was no evidence that the hair dye/oxidiser mix caused either irritation or sensitisation.

Comment
These studies are considered unethical.

Ref.: 12

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY
(4-nitro-o-phenylenediamine)
(oxidative)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**3.6 µg/cm^2**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.52 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.042 mg/kg**
No observed adverse effect level (rat, oral, 90 day)	**NOAEL**	**=**	**15 mg/kg**

Margin of Safety	**NOAEL / SED**	**=**	**357**

3.3.14. Discussion

Physico-chemical specifications
4-nitro-*o*-phenylenediamine is used in oxidative hair dye formulations at a maximum concentration of 0.5%, after mixing with hydrogen peroxide. Stability of 4-nitro-o-phenylenediamine in marketed products is not reported.

General toxicity
The acute median lethal oral dose of 4-nitro-o-phenylenediamine reported in mice and rats varies from 680 to 2100 mg/kg bw.
The majority of the toxicology studies is old and not performed under current guidelines and GLP. However, in a new 90 day study the NOAEL is relatively low (15 mg/kg bw/day). The studies submitted on reproduction toxicity were publications in open literature and were only of limited use for evaluation. In a teratogenicity study (also publication in open literature) effects on maternal weight gain observed at the lowest observed effect level for teratogenic and embryotoxic effects (265mg/kg/day; NOAEL 128 mg/kg/day) were judged to be equivocal by the applicant. The authors of the publication, however, argued that the effects could be accounted for by the reduction in average foetal weight and concluded that this dose was not toxic to pregnant dams. Since no other measurements of maternal toxicity were conducted, no definite conclusion with respect to the maternal toxic dose could be drawn. Therefore, it can not be ruled out that teratogenic (increased incidence of cleft palate) and embryotoxic (reduced foetal weight) effects occurred at dose levels which were not toxic to the pregnant dams.
There are no indications from the old studies and the new 90 day study that e.g. new developmental and/or reproductive studies will identify a (much) low(er) NOAEL. Therefore, and for ethical reasons the SCCP does not ask for new animal studies.

Photo-toxicity
4-Nitro-*o*-phenylenediamine is considered to have no photo-toxic potential.

Toxico-kinetics
Following oral administration of 4-nitro-o-phenylenediamine, urine was the major route of excretion. At least 65% of the administered 4-nitro-o-phenylenediamine dose was absorbed

based on urinary excretion. Main metabolism of the parent compound was conjugation to N-acteyl-4- nitro-o-phenylenediamine and N-acetyl-acetylcysteine-4- nitro-o-phenylenediamine. At least four unknown conjugates and/or metabolites occurred in minor amounts along with small amounts of unchanged 4-nitro-o-phenylenediamine. Conjugated 4-nitro-o-phenylenediamine was found in the faeces (27%) indicating that part of that excreted via the faeces had been absorbed. Therefore it can be assumed that the overall absorption of 4- nitro-o-phenylenediamine approached 100%. In the subsequent whole body autoradiography study, 4- nitro-o-phenylenediamine or radiolabelled metabolites were widely distributed throughout the body.
Rapid elimination via the urinary and faecal routes was seen, with a significant proportion of the absorbed dose eliminated within 24 hours. Radioactive material was detected in the thyroid, liver, kidney, brain and spinal cord at 96 hours after dosing.

Irritation / sensitisation
Under the conditions of the experiment, 4-nitro-o-phenylenediamine was not irritant to the rabbit skin and mildly irritant to the rabbit eye. However, these studies are inadequate. The purity of the test substance was not known.
In one LLNA, the substance (purity 99.7%) was shown as a extremely potent sensitizer. However, in a second study, the test substance (purity unknown) did not show a sensitising effect at 0.2%.

Human data
There was no evidence that the hair dye/oxidiser mix caused either irritation or sensitisation. These studies are considered unethical.

Dermal absorption
The concentration used in the experiments was approximately half that of the use concentration in hair dye products. The total absorbed of 4-NOPD was equivalent to 0.83 ± 0.69 µg eq/cm² and 0.39 µg equiv/cm² in the two hair dye formulations tested. The highest value (Complete Dye Base H) observed was 2.15 µg eq/cm² since the formulation contained 0.29% 4-NOPD a value of (2.15 x 0.50/0.29) 3.6 µg eq/cm² will be used for the calculation of the margin of safety.

Mutagenicity
4-Nitro-o-phenylenediamine was found to be clearly genotoxic *in vitro*, inducing both gene mutations and cytogenetic alterations. One study suggested that light from a mercury vapour lamp could increase the mutagenicity of the compound. Although the only GLP study on micronuclei in human lymphocytes *in vitro*, was negative, a positive result from the *in vitro* studies appears to be consistent. *In vivo*, 4-nitro-o-phenylenediamine did not induce micronuclei in bone marrow erythrocytes of mice or rats, UDS in rat hepatocytes, or sperm abnormalities in mice. This suggests that 4-nitro-o-phenylenediamine is not genotoxic *in vivo*. However, only a mouse micronucleus test and rat liver UDS tests were performed in compliance with GLP.
Inadequate data exist on the genotoxic effects of 4-nitro-o-phenylenediamine in oxidative conditions.

Carcinogenicity
Conflicting results have been found in *in vitro* studies using the Syrian hamster embryo (SHE) cell transformation assay. 4-nitro-o-phenylenediamine did not induce tumours in mice or rats after oral administration under the conditions of an US National Cancer Institute study. No conclusions with regard to carcinogenicity can be drawn from animal

studies in which hair dye formulations were tested. IARC has classified 4-NOPD in Group 3 (Not classifiable as to carcinogenicity to humans).

4. CONCLUSION

Based on the information provided, the SCCP is of the opinion that the use of 4-Nitro-o-phenylenediamine itself as an oxidative hair dye substance at a maximum concentration of 0.5% in the finished cosmetic product (after mixing with hydrogen peroxide) does not pose a risk to the health of the consumer, apart from its sensitising potential.

4-Nitro-o-phenylenediamine itself is not genotoxic *in vivo*.
However, Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

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