Scientific Committee on Consumer Products

SCCP

OPINION ON

N-PHENYL-P-PHENYLENEDIAMINE

COLIPA N° A9

The SCCP adopted this opinion at its 10th plenary of 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:
Scientific Committee on Consumer Products (SCCP), 19 December 2006, Opinion on N-Phenyl-p-phenylenediamine COLIPA N° A9

TABLE OF CONTENTS

1. BACKGROUND

Submission I of N-Phenyl-*p*-phenylenediamine (CAS 101-54-2) was submitted in September 2003 by COLIPA[1, 2].

The substance is currently regulated as an oxidative hair dye by the Cosmetics Directive (76/768/EC), Annex III, part 1 under entry 8 on the List of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission II was submitted by COLIPA in July 2005. According to this submission N-Phenyl-*p*-phenylenediamine is used in oxidation hair dyes at a maximum concentration of 0.4%. After mixing in the ratio 1:1 with hydrogen peroxide, the concentration on the head is 0.2%. Submission I should be evaluated together with this submission.

Submission II 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 N-Phenyl-p-phenylenediamine safe for use in oxidative hair dye formulations up to a concentration of 0.2% on the head taken into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of N-Phenyl-p-phenylenediamine in oxidative hair dye formulations besides the existing labelling requirements for allergic reactions?*

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

N-Phenyl-p-Phenylenediamine

3.1.1.2.	Chemical names

N-Phenyl-p-Phenylenediamine
4-Aminodiphenylamine
N-Phenyl-1,4-Phenylenediamine
N-Phenyl-1,4-Benzenediamine
p-Aminodiphenylamine
p-Anilinoaniline

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

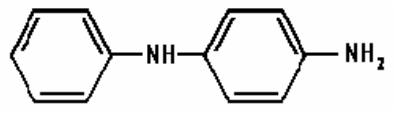
3.1.1.3. Trade names and abbreviations

Trade name Rodol Gray B Base (Lowenstein)
Rodol Gray BC (for the HCl salt), Rodol Gray BS (for the sulphate salt)
COLIPA N° A9

3.1.1.4. CAS / EINECS number

CAS:	101-54-2	2198-59-6 (hydrochloride)	4698-29-7 (sulfate)
EINECS:	202-951-9	218-599-4 (hydrochloride)	225-173-1 (sulfate)
CI number:	37240, 76085		

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{12}H_{12}N_2$

3.1.2. Physical form

Medium to dark grey flakes

3.1.3. Molecular weight

Molecular weight: 184.24

3.1.4. Purity, composition and substance codes

Purity: > 98% (given as specification)
Ash content (600°C): < 0.1% (w/w)
Moisture (Karl Fischer): < 0.22% (w/w)

Complete analysis and structure characterisation is provided only for Lot No. 217. It was characterised with different techniques, including GC-MS, LC-MS and NMR. These techniques were also used to demonstrate homogeneity and stability.

Using GC-MS, it was confirmed to be 99.6% N-phenyl-p-phenylenediamine (NPPD) as a % of total area. The identity was confirmed by spectral confirmation. It was also quantified versus a NPPD Sigma- Aldrich standard by GC-MS.

The chemical structure and molecular formula of the test substance was also confirmed to be NPPD by NMR and elemental analysis. All the results of the trace metal analysis were within specification set by the supplier.

The results are summarised in the following tables.

Analytical Results of Lot No. 217 by GC/MS

Component name	**% of total area ***
Rodol Gray B Base	99.61 - 99.54

Component name	% of total area *
Diphenylamine	0.05 - 0.08
Unknown Impurity 1	0.02 - 0.03
Unknown Impurity 2	0.01 - 0.02
Unknown Impurity 3	0.05 - 0.05
Unknown Impurity 4	0.02 - 0.03
MPPD **	0.17 - 0.16
Unknown Impurity 5	0.02 - 0.02
Unknown Impurity 6	0.05 - 0.08
Total unknown	**0.17 - 0.22**
Sum	**100**

* The second values were found after 6-months storage in the dark at ambient temperature.
** MPPD = 2-methoxy-N-4-phenyl-1,4-phenylene diamine

Results of Compositional Analysis Lot No. 217

Elemental analysis	
Carbon (C)	78.13% (w/w)
Hydrogen (H)	6.57% (w/w)
Nitrogen (N)	15.12% (w/w)
Sulphur (S)	< 0.3% (w/w)
Chlorine (Cl)	< 0.3% (w/w)

3.1.5. Impurities / accompanying contaminants

Water content: < 0.2 g/100 g
Loss on drying: /
Ash: < 0.1 g/100 g

Trace metal analysis

Arsenic (As): < 2 ppm
Iron (Fe): 1 ppm
Chromium (Cr): < 1 ppm
Cobalt (Co): < 1 ppm
Nickel (Ni): < 1 ppm
Cadmium (Cd): < 1 ppm
Antimony (Sb): < 1 ppm
Lead (Pb): < 1 ppm
Mercury (Hg): < 0.5 ppm

Organic solvents: /

Two minor impurities were identified as Diphenylamine (0.05% of total area) and 2-methoxy-N-4-phenyl-1,4-phenylene diamine (MPPD) (0.17% of total area). The NPPD and the two minor impurities were identified with mass spectrometry spectra confirmation versus National Institute of Standards and Technology (NIST) mass spectral library; version 1.6d and comparison of the spectra from Sigma-Aldrich standards.
A further six impurities were seen by GC-MS which were not identified (max. 0.05% of total peak area). The total amount of unknown impurities was 0.17% of total peak area by GC-MS (see table above).

3.1.6.	Solubility

Water: 0.55 g/l at 20 ± 0.5°C
Ethanol: /
DMSO: /

3.1.7.	Partition coefficient (Log P_{ow})

Log P_{ow}: 1.81 (free base)

3.1.8.	Additional physical and chemical specifications

- Melting point: 72.8 °C
- Flash point: 210 ± 2°C
- Vapour pressure: /
- Boiling point: /
- Density at 20 °C: /
- Viscosity: /
- pKa: 5.30 at 20.5 ± 0.5°C
- UV absorption spectrum: λ_{max} 296 nm, 247 nm
- Refractive index at 20 °C: /

3.1.9.	Stability

N-Phenyl-p-phenylenediamine oxidizes in air (3 month stability on raw material).
A method was validated to analyse NPPD at levels of 1mg/mL to 80 mg/ml in 0.5% CMC by RP-HPLC-UV/VIS. Homogeneity, dose confirmation and stability were performed at levels of 2.5, 5, 10 and 75 mg/ml NPPD in 0.5% CMC.
At dose levels 2.5, 5, 10 and 75 mg/ml of NPPD in 0.5% CMC, homogeneity of the suspension was demonstrated. All replicates were considered to be within a ± 10% error bar of the mean concentration both on Days 0 and 2. At dose levels 2.5, 5 and 10 mg/ml of NPPD in 0.5% CMC, the doses varied ± 20% of the nominal concentration. At these concentrations, 2-day stability varied ± 10% of the mean concentration on Day 0. At the dose level 75 mg/ml, the variation was ± 10% of nominal concentration. Stability analysis was not required for 75 mg/ml NPPD in 0.5% CMC.

General Comments on Physico-chemical characterisation

- Adequate physico-chemical characterisation has been performed for only one batch.
- The batches used in the mutagenicity studies were not characterised.
- Stability data on marketed formulation is not provided.

3.2. Function and uses

According to this submission N-Phenyl-p-phenylenediamine is used in oxidation hair dyes at a maximum concentration of 0.4%. After mixing in the ratio 1:1 with hydrogen peroxide, the concentration on the head is 0.2%.

Comments on Function and uses

- N-Phenyl-p-phenylenediamine is used also in the forms of the hydrochloride and sulphate salts. No data were provided for the two salts; apparently their toxicological

behaviour is expected to be similar to that of the free base. All the 3 forms are included in the EU Inventory of Cosmetic Ingredients, part I.

- N-Phenyl-p-phenylenediamine is included in Annex III, part 1 "List of substances which cosmetic products must not contain except subject to the restrictions and conditions laid down" of the Cosmetics Directive, entry n° 8 "m- and p-phenylenediamines, their N-substituted derivatives and their salts; N-substituted derivatives of o-phenylenediamines, with the exception of those derivatives listed elsewhere in this Annex".
  It is subjected to the following "*conditions of use and warnings which must be printed on the label*":

  a) for general use: *Can cause an allergic reaction. Contains phenylenediamines. Do not use to dye eyelashes or eyebrows*.
  b) for professional use: *For professional use only. Contains phenylenediamines. Can cause an allergic reaction. Wear suitable gloves.*

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Only cited rat LD_{50}s for N-Phenyl-p-phenylenediamine (purity unknown) were provided with scant experimental detail. However the range was between 464 - > 1000 mg/kg bw.

Ref.: 1, 2, 3

3.3.1.2. Acute dermal toxicity

Only cited LD_{50} for N-Phenyl-p-phenylenediamine (purity unknown) was greater than 5000 mg/kg in rabbits. There was scant experimental detail. It was concluded to have low potential for acute dermal toxicity in rabbits.

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	/
Species/strain:	male albino rabbit
Group size:	3
Test substance:	N-Phenyl-p-phenylenediamine
Batch:	/
Purity:	/
Vehicle:	0.5 g of N-Phenyl-p-phenylenediamine into a paste with water
GLP:	not in compliance

Test material was applied to gauze patch (semi-occlusive dressing) and applied to shaved intact skin on the flank for 4 hours. Patch was removed and the site washed with water. The animals were examined 1, 24, 48 and 72 hours after patch removal and on day 7. No skin irritation was observed.

Ref.: 5

Conclusion
This study is considered inadequate: it was not performed according to OECD guidelines or GLP; no data were given on batch number and purity.

3.3.2.2. Mucous membrane irritation

No data submitted

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA), study 1

Guideline:	OECD 429
Species/strain:	mouse – CBA/CaOlaHsd
Group size:	4 females (8-12 weeks) per dose group
Test substance:	Rodol Gray B Base
Batch:	Lot 217, SEAC sample number S2582101
Purity:	not specified in the report
Doses:	0.1, 0.25, 0.5, 1.0 or 2.5% (w/v)
Positive control	alpha-hexylcinnamaldehyde (5, 10 and 25%)
Vehicle:	Acetone/Olive oil (4:1, V/V)
GLP:	in compliance

N-Phenyl-p-phenylenediamine in acetone:olive oil (4:1) was applied at concentrations of 0.1, 0.25, 0.5 , 1 and 2.5% to the dorsal surface of each ear lobe for three consecutive days. This was followed by a rest period of three days. The animals were treated with 3H-methyl thymidine and then sacrificed five hours later. A cell suspension of the lymph nodes was prepared and the incorporation of 3H-methyl thymidine was measured.

	Test item concentration % (w/v)	**S.I.**
Group 2	0.1	8.0
Group 3	0.25	8.8
Group 4	0.5	8.9
Group 5	1	12.2
Group 6	2.5	10.5
An EC3 value could not be determined because this calculation requires an S.I. value of less than 3.		

Although an EC3 was not derived because all responses were higher than a stimulation index (SI) of 3, N-Phenyl-p-phenylenediamine was shown to be a sensitiser.

Ref.: 42

Local Lymph Node Assay (LLNA), study 2

Guideline:	OECD 429
Species/strain:	mouse – CBA/CaOlaHsd
Group size:	4 females (8-12 weeks) per dose group
Test substance:	Rodol Gray B Base
Batch:	Lot 217, SEAC sample number S2582101
Purity:	not specified in the report

Doses:	0.005, 0.01, 0.025, 0.05 or 0.09% (w/v)
Positive control	alpha-hexylcinnamaldehyde (5, 10 and 25%)
Vehicle:	Acetone/Olive oil (4:1, V/V)
GLP:	in compliance

N-Phenyl-p-phenylenediamine in acetone:olive oil (4:1) was applied at concentrations of 0.005, 0.01, 0.025, 0.05 and 0.09% to the dorsal surface of each ear lobe for three consecutive days. This was followed by a rest period of three days. The animals were treated with 3H-methyl thymidine and then sacrificed five hours later. A cell suspension of the lymph nodes was prepared and the incorporation of 3H-methyl thymidine was measured.

	Test item concentration % (w/v)	**S.I.**
Group 2	0.005	1.8
Group 3	0.010 *	2.7 *
Group 4	0.025 *	3.3 *
Group 5	0.050	9.1
Group 6	0.090	9.6
EC3 = 0.0175% (w/v)		
A clear dose-response relationship was observed. * This value was used in calculation of EC3		

An EC3 value of 0.0175% was determined for N-Phenyl-p-phenylenediamine. Rodol Gray B Base is an extremely potent skin sensitizer in the LLNA test.

Ref.: 43

Human studies

A number of human patch testing studies have been conducted using N-Phenyl-p-phenylenediamine on a wide range of dermatology patients including generally sensitised individuals, hairdressers and clients of hairdressers.

Ref.: 47, 48, 49, 50, 51, 52, 53

Conclusion
N-Phenyl-p-phenylenediamine has been shown to be a skin sensitizer in the LLNA. This confirms the findings of the published studies. The substance has repeatedly been demonstrated to be an allergen for humans.

3.3.4. Dermal / percutaneous absorption

***In Vitro* Percutaneous Absorption**

Guideline:	OECD 428 (2004)
Tissue:	Five Landrace/Large White pigs (4 male, 1 female), from frozen pig skin library, spilt thickness skin 1300 / 1900 µm
Tissue integrity:	/
Method:	Flow through Teflon diffusion cells, exposed membrane area - 0.64 cm² flux: 1.5 ml/h at 32 °C.
Test substance:	N-phenyl-p-phenylenediamine hydrochloride [^{14}C]-NPPPD SEAC sample number S2585701, Rodol Gray B
Batch:	Base Lot No 217 (unlabelled NPPPD) SEAC sample number S2582101,
Purity:	99.6%

Purity of the formulation:	Due to the reactive nature of hair dyes, the radiolabelled test item will exist in many different forms once the oxidative reactions are under way
Dose applied:	10 µl / cm² N-Phenyl-p-phenylenediamine was included at 0.4% in both dye bases. Complete Dye Base C mixed with developer at 1:1 w/w giving an "on skin" concentration of 0.23% w/w (2.33mg/g) N-Phenyl-p-phenylenediamine. Complete Dye Base U mixed with developer at 1:2 w/w giving an "on skin" concentration of 0.14% w/w (1.35mg/g) N-Phenyl-p-phenylenediamine.
Formulation:	Complete Dye Base C and developer Complete Dye Base U and developer
Receptor fluid:	Phosphate buffered saline containing 5% (V/V) new born calf serum.
Contact:	45 minutes, then washing of the skin surface, and monitoring of the diffusion during 24 hours.
No. of replicates:	10 cells (formulation C) 12 cells (formulation U), of which from 5 subjects
Assay:	Liquid scintillation counting, dpm
GLP:	in compliance

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 1.5mL/h with a skin surface temperature of ca 32°C. The skins were allowed to equilibrate under these conditions. [^{14}C]-NPPPD was applied in the two hair dye formulations (Complete Dye Base C and Complete Dye Base U) after mixing with developer. The two formulations were applied at a formulation application rate of 10µl/cm². Complete Dye Base C and developer giving an "on skin" concentration of 0.23% w/w N-Phenyl-p-phenylenediamine and Complete Dye Base U and developer giving an "on skin" concentration of 0.14% w/w N-Phenyl-p-phenylenediamine.

Absorption was assessed by collecting receptor fluid hourly from 0-24 hours post dose. At 45 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-Squame® 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.

	Complete Dye Base C (n=10)	**Complete Dye Base U (n=12)**
Application rate (µl/cm²)	10	10
Application rate (mg/cm²)	8.79	8.99
NPPPD concentration (mg/g)	2.33	1.35
Unavailable	18.37 (16.65 – 19.51)	11.57 (10.67 – 12.35)
Penetrated **Inner skin digest**	0.03 (0.01 – 0.06) 0.81 (0.19 – 2.29)	0.01 (0.00 – 0.02) 0.24 (0.08 – 0.46)
Tape strips	0.12 (0.04 – 0.20)	0.15 (0.08 – 0.24)
Total absorbed	0.84 (0.25 – 2.32)	0.26 (0.10 – 0.47)

Most of the applied dose was washed off at 45 minutes post dose; 90.38% and 89.79% for Complete Dye Base C and developer and the Complete Dye Base U and developer respectively. The absorbed dose and dermal delivery of [^{14}C]-N-Phenyl-p-phenylenediamine from the Complete Dye Base C and developer was 0.13% (0.03µg equiv./cm²) and 4.18% (0.84µg equiv./cm²) of the applied dose. The absorbed dose and dermal delivery of [^{14}C]-N-Phenyl-p-phenylenediamine from the Complete Dye Base U and developer was 0.10% (0.01µg equiv./cm²) and 2.10% (0.26µg equiv./cm²) of the applied dose. The total recovery was 99.0%.

Ref.: 41

Conclusion
The absorption and dermal delivery of N-Phenyl-p-phenylenediamine from the two hair dye formulations was approximately 3 fold greater in hair dye formulation "Complete Dye Base C and developer" with 0.23% w/w of N-Phenyl-p-phenylenediamine than in hair dye formulation "Complete Dye Base U and developer" with 0.14% w/w of N-Phenyl-p-phenylenediamine.

The maximum absorption value observed in the experiment, 2.32 µg/cm^2 can be used for calculating the MoS.

3.3.5. Repeated dose toxicity

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

Eight Week Range finding Toxicity Study

Guideline:	/
Species:	Rat, Fisher F344, mice B6C3F1
Route:	oral
Group sizes:	10 (5male /5 female)
Substance:	N-Phenyl-p-phenylenediamine
Batch:	0100700 GB
Purity:	approx. 90%
Dose Rat:	rat: 0, 2200, 3200, 4600, 6800 and 10,000 ppm *ad libitum* mice: 0, 3000, 4400, 6500, 9500, 14700, 21600 and 31500 ppm *ad libitum*
Exposure:	30 days - male, 31 days – female
GLP:	in compliance

An eight week range-finding oral, dietary feeding study was performed to establish dose levels for a subsequent chronic, 2-year carcinogenicity bioassay. Observations were limited to clinical, survival and growth parameters.

Result
Rat: a decrease in mean bodyweight of 72% (males) and 50% (females) compared with the controls was observed at a dose level of 2200 ppm in the diet (the lowest level tested). Deaths occurred in both sexes at dose levels of 6800 ppm and above.

Mice: no signs or symptoms of toxicity were observed at dose levels between 3000 and 9500 ppm in the diet. Female mice, but not males, had decreased body weight gains at higher doses.

Ref.: 8

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

Guideline: /

Species:	Rat, albino Wistar
Route:	oral
Group sizes:	12 male
Substance:	N-Phenyl-p-phenylenediamine
Batch:	/
Purity:	/
Dose:	0.1, 0.25, 0.5 and 0.75% in diet
Exposure:	90 days
GLP:	/

N-Phenyl-p-phenylenediamine was admixed with a powdered rodent diet (0.1%, equivalent approximately to 50 mg/kg). Body weight and food intake quantities were measured weekly. At the end of the treatment period, blood samples were taken for clinical chemistry analysis and biochemical parameters were measured in the liver and testes. Organs were excised, weighed and samples prepared for histopathology.

Results
A dose-response related reduction in body weight gain correlated with reduced food intake, beginning at week 10 was found in the 0.5% and 0.75% dye-treated groups. Relative liver weight increased by ~36%, though other organs were unaffected. Food intake, weight gain and organ weights in the 0.10% and 0.25% groups were similar to the controls.
Significantly increased serum levels of enzymes GOT and GPT were found in the 0.25% 0.5% and 0.75% dye-treated groups and alkaline and acid phosphatase, ALT and AST levels in the 0.5% and 0.75% dye-treated groups.
Haematological observations suggested normocytic normochromic anaemia following dietary administration of dye at 0.25% or higher. Statistically significantly decreased RBC count and haemoglobin and increased mean corpuscular volume in the top 2 doses.

In addition, the following observations were seen:
In the testes, there was a statistically significant decrease in hyaluronidase levels in the 0.5% and 0.75% dye-treated group. LDH was also decreased by 25% at these doses. The other measured biochemical indices were similar to the controls.
Histopathology of the testes showed degeneration of the seminiferous tubules in patches in the high dose animals only (0.75%); interstitial tissue was normal.
In the liver, the top doses (0.5% and 0.75%) showed degenerative changes and prominent plasma cell reactions in the portal areas of the liver.

Conclusions
N-Phenyl-p-phenylenediamine in diet, in the two highest doses groups (0.5% and 0.75%), caused decreased weight gain and food intake. Histopathological effects in the liver with accompanying biochemical abnormalities (significant increase in serum alkaline and acid phosphatases and raised ALT and AST) suggestive of degenerative changes were seen at these doses. The testes showed impaired spermatogenesis. The lowered LDH and hyaluronidase indicated partial arrest of spermatogenesis. This was supported histopathologically.

Ref.: 2

Comment
The study was inadequate. This was not a study report, but a journal publication. It did not follow conventional guidelines and was not GLP.
Data in this publication was only for males, though the implication was that males and females were investigated in the 90 day study.
No NOAEL could be derived from this study.
A new 90 day study is required with special attention to the effects on the reproduction, since fertility in the male would seem to be effected. The effects of N-Phenyl-p-phenylenediamine on females need to be addressed.

Dye formulation studies

Rabbit, 13-week toxicity study

Guideline:	/
Species:	Rabbit, New Zealand White
Route:	Dermal
Group sizes:	12 (6/sex)
Substance:	Formulation 7404, (2.0% N-Phenyl-p-Phenylenediamine hydrochloride, 1.0% resorcinol 1.5% m-phenylenediamine, 1.0% o-phenylenediamine, 1.0% N-Methyl-p-aminophenol sulfate)
Batch:	/
Purity:	/
Dose:	2% with an equal volume of 6% H_2O_2
Exposure:	1-h twice a week for 13 weeks 90 days
GLP:	/

An oxidative hair dye formulation containing 4 hair dyes including 2.0% N-Phenyl-p-phenylenediamine was mixed with an equal volume of 6% H_2O_2 just prior to topical dosing. In addition to N-Phenyl-p-phenylenediamine, this formulation contained 4 other oxidative hair dyes (see submission I, Appendix III). The dose applied was 1 ml/kg. The application sites were abraded on the first treatment day of each week in 3 rabbits in each sex group. Three independent control groups of 12 rabbits each were treated identically, except that no dye was applied [29].
No product-related death occurred during the study. Body weight gain in the dye-treated group was similar the controls.
There were no statistically significant differences in clinical chemistry values between treated and control groups. Urinalysis results were within the control range and no dye discoloration was seen. In males, a statistically significant increase in methaemoglobin in the dye-treated group compared with the combined control group (2.86 + 0.94 vs 1.42 + 1.11) was observed. It was not considered to be of toxicological significance.

Conclusions
Topical exposure of a hair dye formulation containing 2% N-Phenyl-p-phenylenediamine, when repeatedly applied for 13-weeks to shaved and abraded skin of the rabbit, did not cause any discernible signs of toxicity.

Ref.: 29

3.3.5.3. Chronic (> 12 months) toxicity

See 3.3.7

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial gene mutation assay (1)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537.
Replicates:	/
Test substance:	p-aminodiphenylamine
Solvent:	/
Batch:	/
Purity:	/

Concentrations: probably 10, 100, and 500 or 1000 µg/plate with and without S9
Treatment: direct plate incorporation method
GLP: not in compliance

The present assay is reported in a paper from the open literature in which 300 chemicals were tested in the *Salmonella*/microsome assay. The aim of the paper is to demonstrate the correlation between the *Salmonella*/microsome assay and carcinogenicity. The paper was published before the implementation of OECD guidelines.

p-Aminodiphenylamine was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the direct plate incorporation method. Liver S9 fraction from Aroclor 1254-induced rats was probably used as exogenous metabolic activation system.
Toxicity was not indicated. Positive controls were lacking.

Results
Both in the absence and presence of S9 metabolic activation p-aminodiphenylamine did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the *Salmonella* strains. The number of revertants per nmol was < 0.01, representing the mutagenic potency of p-aminodiphenylamine in reverting the *Salmonella* strains used.

Conclusion
Under the experimental conditions used p-aminodiphenylamine is not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 9

Comment
Since this test has several deficiencies compared to the minimal current requirements for a genotoxicity test and due to the poor reporting, this study is considered inadequate and not suitable for evaluation.

Bacterial gene mutation assay (2)

Guideline: /
Species/strain: *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537
Replicates: at least duplicates in 2 independent experiments
Test substance: N-phenyl-p-phenylenediamine
Solvent: ethanol 95%
Batch: /
Purity: /
Concentrations: 10 - 3333 µg/plate with and without S9
Treatment: pre-incubation method
GLP: not in compliance

The present assay is reported in a paper from the open literature in which 270 chemicals were tested in the Ames test. The aim of the paper is to demonstrate the correlation between the Ames test and carcinogenicity.

N-phenyl-p-phenylenediamine was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the pre-incubation method. Doses were chosen after a preliminary dose-setting experiment with strain TA100 in the absence and presence of S9. Chemicals were tested up to a high dose which exhibited some degree of toxicity.
Toxicity was evidenced by the appearance of his^- pinpoints colonies, reduced numbers of revertant colonies per plate or thinning or absence of the bacterial lawn. Liver S9 fraction from Aroclor 1254-induced rats and hamsters was used as exogenous metabolic activation system. Negative and positive controls were included.

Results
In all *Salmonella* strains tested both without and with S9 toxicity was observed at 1000 µg/plate.
Both in the absence and presence of S9 metabolic activation N-phenyl-p-phenylenediamine did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the *Salmonella* strains.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine is not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 10

Comment
Since the reporting of the test is limited and may have deficiencies compared to the minimal current requirements for a genotoxicity test, this study only has supportive value.

Bacterial gene mutation assay (3)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, and TA1537
Replicates:	duplicates
Test substance:	N-phenyl-p-phenylenediamine
Solvent:	/
Batch:	/
Purity:	/
Concentrations:	3 -3333 µg/plate with and without S9
Treatment:	pre-incubation method
GLP:	not in compliance

The present assay is from the NTP website (http://ntp-server.niehs.nih.gov/htdocs/Overviews/SaDoc.html).

N-phenyl-p-phenylenediamine was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the pre-incorporation method. Liver S9 fraction from rats and hamsters was used as exogenous metabolic activation system. Toxicity was not indicated. Negative and positive controls were used.

Results
Without S9 toxicity was at 1000 µg/plate in all *Salmonella* strains tested; with S9 in strains TA98 and TA1537 and occasionally in strains TA100 and TA 1535. At 3333 µg/plate toxicity was found in all *Salmonella* strains both without and with S9.
Both in the absence and presence of S9 metabolic activation N-phenyl-p-phenylenediamine did not induce an increase in the number of revertant colonies as compared to concurrent vehicle controls in any of the *Salmonella* strains.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine is not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 10a

Comment
The poor reporting reduces the value of this gene mutation test in bacteria, and, therefore, it can only be used as supportive evidence.

Bacterial gene mutation assay (4)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538.
Replicates:	/
Test substance:	4-aminodiphenylamine
Solvent:	DMSO
Batch:	/
Purity:	/
Concentrations:	15, 50, 150, 500 and 1000 µg/plate without S9 metabolic activation 1, 4, 20, 100, 300 and 1000 µg/plate with S9 metabolic activation
Treatment:	plate incorporation method
GLP:	not in compliance

4-Aminodiphenylamine was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test) using the plate incorporation method. Test concentrations were based on the level of toxicity in a toxicity range finding experiment with TA1538 and TA100. Based on these toxicity results, a dose level of 1500 µg/plate was chosen as the highest dose with and without S9. Liver S9 fraction from Aroclor 1254-induced rats was probably used as exogenous metabolic activation system. Toxicity was probably evaluated on the basis of a reduction in the number of revertant colonies and/or a thinning of the bacterial lawn.
Concurrent negative and positive controls were included.

Results
Both in the absence and presence of S9 metabolic activation 4-aminodiphenylamine did not induce an increase in the number of revertant colonies in any of the *Salmonella* strains. Since 4-aminodiphenylamine was toxic at 1500 µg/plate 2 repeat tests were performed. An increase in the number of revertants was not observed in these repeat tests.

Conclusion
Under the experimental conditions used 4-aminodiphenylamine is not genotoxic (mutagenic) in the gene mutation tests in bacteria.

Ref.: 11

Comment
Only a study summary delivered by Monsanto was available. The results were only described; the actual data (raw data) were not delivered. Consequently, the conclusions of the authors could not be checked. Therefore, value of this study is limited and, consequently, can only be used as supportive evidence.

Bacterial gene mutation assay (5)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98
Replicates:	/
Test substance:	N-phenyl-p-phenylenediamine
Solvent:	/
Batch:	/
Purity:	/
Concentrations:	15, 50 and 150 µg/plate with and without S9
Treatment:	/
GLP:	not in compliance

The present assay is from an abstract of a paper from the open literature. The abstract as well as the tables are in English, the text is in Japanese.

N-phenyl-p-phenylenediamine was investigated for the induction of gene mutations in *Salmonella typhimurium* strain TA98. A liver S9 fraction from was used as exogenous metabolic activation system. Toxicity was not indicated. Concurrent untreated controls were lacking.

Results
With S9 metabolic activation N-phenyl-p-phenylenediamine did induce a dose dependent increase in the number of revertant colonies in TA98 from 15 up to 150 µg/plate. This increase was also seen in the presence of hydrogen peroxide. Without S9 N-phenyl-p-phenylenediamine did not induce an increase in the mutation frequency.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine is genotoxic (mutagenic) in Salmonella TA98 in the presence of S9.

Ref.: 12

Comment
Due to the very poor reporting (only abstract available) and the fact that only one *Salmonella* strain was used, this study is considered inadequate and not suitable for evaluation.
The paper was published before the implementation of OECD guidelines.

Bacterial gene mutation assay (6)

Guideline:	/
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538
Replicates:	/
Test substance:	4-aminodiphenylamine
Solvent:	DMSO or distilled water
Batch:	/
Purity:	/
Concentrations:	/
Treatment:	direct plate incorporation method with S9
GLP:	not in compliance

The present assay is reported in a paper from the open literature in which 60 azo dyes were tested in the Ames test. The aim of the paper was to describe a strategy for the development of non-mutagenic dyes by identifying and elaborating some non-genotoxic precursors.

4-aminodiphenylamine was investigated for the induction of gene mutations in *Salmonella typhimurium*. Liver S9 fraction from Aroclor 1254-induced was used as exogenous metabolic activation system. Test concentrations were not reported. Toxicity was not indicated. Positive and negative controls were lacking.

Results
With S9 metabolic activation 4-aminodiphenylamine did not induce an increase in the number of revertant colonies in the *Salmonella* strains.

Conclusion
Under the experimental conditions used 4-aminodiphenylamine is not genotoxic (mutagenic) in gene mutation test in bacteria.

Ref.: 14

Comment
Due to the very poor reporting and the many short comings compared to the minimal requirements described in the OECD guideline, this study is considered inadequate and not suitable for evaluation.

In vitro Gene Mutation Assay (mouse lymphoma assay, $tk^{+/-}$ locus)

Guideline:	/
Species/strain:	mouse lymphoma L5178Y cells
Replicates:	duplicates
Test substance:	N-phenyl-p-phenylenediamine
Solvent:	DMSO
Batch:	/
Purity:	/
Concentrations:	first experiment: 0.16 – 4.0 µg/ml second experiment: 0.25 – 3.0 µg/ml
Treatment	4 h treatment with 2 days recovery followed by an expression period of 10-12 days
GLP:	not in compliance

The present assay is from the NTP website (http://ntp-server.niehs.nih.gov/htdocs/Overviews/SaDoc.html)

N-phenyl-p-phenylenediamine was assayed for gene mutations at the $tk^{+/-}$ locus in mouse lymphoma cells in the absence of S9 metabolic activation. Cells were treated for 4 h followed by a recovery period of 2 days. Mutation frequencies were determined 10-12 after the end of the treatment. The study is exclusively conducted in the absence of S9 due to the positive response that was obtained in each of two trails under this condition. Toxicity was determined as cloning efficiency and relative growth. Negative and positive controls were included.

Results
On the basis of relative growths the appropriate level of toxicity was (almost) reached pointing to sufficient exposure of the cells. In both experiments a dose dependent increase in the mutation frequency at the $tk^{+/-}$ locus of mouse lymphoma cells was observed.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine induced an increase in the mutation frequency at the $tk^{+/-}$ locus of mouse lymphoma cells and, consequently, is mutagenic in mouse lymphoma cells *in vitro*.

Ref.: 15

Comment
The results were delivered in a separate E-mail. There is no evidence that these results belong to this experiment. Due to this and several other shortcomings together with the poor reporting, this study is considered inadequate and not suitable for evaluation.

In vitro gene mutation assay in mammalian cells (*hprt* locus)

Guideline:	/
Species/strain:	CHO cells
Replicates:	triplicate cultures
Test substance:	4-aminodiphenylamine
Solvent:	/

Batch:	/	
Purity:	/	
Concentrations:	initial assay:	10, 33 and 100 µg/ml without S9 33, 100 and 330 µg/ml with S9
	definitive assay:	5, 10, 33, 67 and 100 µg/ml without and with S9
Treatment:	5 h treatment with 19 h recovery followed by an expression period of 7 days.	
GLP:	not in compliance	

4-aminodiphenylamine was assayed for gene mutations at the *hprt* locus of CHO cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a cytotoxicity study and a preliminary mutation assay. 4-aminodiphenylamine was cytotoxic at 333 µg/ml without S9 and at 1000 µg/ml with S9.
In the main test, cells were treated for 5 h and 19 h recovery followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Toxicity was measured as percentage relative survival of the treated cultures relative to the survival of the solvent control cultures. Negative and positive controls were included.

Results
In the definitive mutation assay relative survival ranged from 10-100% indicating sufficient cellular exposure. Both in the initial assay and in the definitive mutation assay, an increase in the mutation frequency, as compared to concurrent negative controls, was not observed. Exclusively a slight increase was seen at 5 µg/ml without S9 in the definitive assay. However, this increase was not reproducible and is considered not biologically relevant.

Conclusion
Under the experimental conditions used, 4-aminodiphenylamine was not mutagenic in CHO cells at the *hprt* locus.

Ref.: 18

Comment
Only a study summary delivered by Monsanto was available. The results were only described the actual data (raw data) were not delivered. Consequently, the conclusions of the authors could not be checked. Therefore, value of this study is limited and, consequently, can only be used as supportive evidence.

In vitro chromosome aberration test (1)

Guideline:	/	
Species/strain:	CHO-W-B1 cells	
Replicates:	/	
Test substance:	N-phenyl-p-phenylenediamine	
Solvent:	DMSO	
Batch:	/	
Purity:	/	
Concentrations:	without S9:	first experiment: 0.5 – 10.0 µg/ml second experiment: 2.5 – 10.0 µg/ml
	with S9:	10.0 – 75.0 µg/ml
Treatment:	8-14 h treatment without S9, 2 h treatment with 10 h recovery with S9.	
GLP:	not in compliance	

The present assay is from the NTP website (http://ntp-server.niehs.nih.gov/htdocs/Overviews/SaDoc.html)

N-phenyl-p-phenylenediamine has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Cells were

treated for 8-14 h in the absence or 2 h in the presence of S9 metabolic activation. Harvest time was 12-14 hours after the beginning of treatment. Two hours before harvest, each culture was treated with colcemid solution. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Toxicity was not reported. Chromosome (metaphase) preparations were stained with Giemsa and examined microscopically for chromosomal aberrations. Chromosomal aberrations were recorded as "simple aberrations" (breaks and terminal deletions) or "complex aberrations" (rearrangements and translocations). Negative and positive controls were included.

Results
Without S9 metabolic activation, a clear dose dependent increase in the number of cells with chromosomal aberrations was observed. At lower doses (experiment 1) the aberrations found were predominantly simple aberrations. With S9 metabolic activation an increase in cells with chromosomal aberrations was also found, however, without a clear dose response relation.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine induced an increase in chromosomal aberrations and, consequently, is genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 15b

Comment
The study has several shortcomings. It is unclear whether the results are included or excluded gaps. Moreover, data on cytotoxicity are lacking. Due to these shortcomings and the poor reporting, this study is considered inadequate and not suitable for evaluation.

In vitro chromosome aberration test (2)

Guideline:	/
Species/strain:	CHO cells
Replicates:	duplicate cultures
Test substance:	4-aminodiphenylamine
Solvent:	/
Batch:	/
Purity:	/
Concentrations:	5, 25, 50 and 75 µg/ml without S9 5, 10 and 20 µg/ml with S9
Treatment:	5 h treatment with 18-19 h recovery without and with S9
GLP:	not in compliance

4-aminodiphenylamine has been investigated in the absence and presence of metabolic activation for the induction of chromosomal aberrations in CHO cells. Dose selection was based on a preliminary cytotoxicity test. The highest dose selected was the dose which resulted in about 80% increase in proliferation kinetics in the cytotoxicity assay. Cells were treated for 5 h in the absence or presence of S9 metabolic activation. Harvest time was 23-24 hours after the beginning of treatment. Two to 3 hours before harvest, each culture was treated with colcemid solution to block cells at metaphase of mitosis. The way toxicity was determined was not reported. Negative and positive controls were included.

Results
In the main experiment 4-aminodiphenylamine was toxic at 75 µg/ml without S9. 4-aminodiphenylamine did not induce an increase in cells with structural chromosome aberrations at any dose level both in the presence and absence of S9 compared to the concurrent untreated controls.

Conclusion
Under the experimental conditions used 4-aminodiphenylamine did not induce an increase in chromosomal aberrations and, consequently, is not genotoxic (clastogenic) in CHO cells *in vitro*.

Ref.: 16

Comment
Only a study summary delivered by Monsanto was available. The results were only described and the actual data (raw data) were not delivered. Consequently, the conclusions of the authors could not be checked. Therefore, this study is not suitable for evaluation. Consequently, it can only be used as supportive evidence.

In vitro Micronucleus Test

Guideline:	OECD draft guideline 487 (2003)	
Cells:	human lymphocytes of 2 healthy, non-smoking, male volunteers	
Replicates:	duplicate cultures in 2 independent experiments with and without S9	
Test substance:	Rudol Gray B base Lot No. 217	
Solvent:	DMSO	
Batch:	S2582101	
Purity:	99.6 %	
Concentrations:	experiment 1:	18.86, 26.10 and 36.13 µg/ml without S9 28.15, 68.72 and 134.2 µg/ml with S9
	experiment 2:	16.03, 22.19 and 30.71 µg/ml without S9 96.0, 120.0 and 150.0 µg/ml with S9
Treatment	experiment 1:	24 h PHA stimulation, 20 h treatment and 28 h recovery without S9 24 h PHA stimulation, 3 h treatment and 45 h recovery with S9
	experiment 2:	48 h PHA stimulation, 20 h treatment and 28 h recovery without S9 48 h PHA stimulation, 3 h treatment and 45 h recovery with S9
GLP:	In compliance	

Rudol Gray B base Lot No. 217 has been investigated in 2 independent experiments in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Treatment periods were 24 h without S9 and 3 h with S9. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. The final 27-28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was determined by measuring the reduction in replication index (RI). In every separate experiment various dilutions of Rudol Gray B base Lot No. 217 were tested. However, only 3 doses were analyzed. The top dose for analysis was to be the one with at least 60% reduction in RI. The lower doses were chosen such that a range from maximum to little or none cytotoxicity is covered. Micronucleus preparations were stained with Giemsa and examined microscopically for RI and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9 indicated that Rudol Gray B base Lot No. 217 had no effect on osmolarity or pH as compared to concurrent vehicle controls.

In experiment 1 without S9 metabolic stimulation Rudol Gray B base Lot No. 217 did not induce an increase in the number of micronuclei compared to the concurrent untreated controls. In experiment 2 statistical significant increases were found at the lowest and the highest dose. However, only at the lowest dose the micronucleus frequency exceeded the historical control values. These positive findings were, therefore, not considered biologically relevant.
With S9 metabolic stimulation in experiment 1 statistically significant increased frequencies of micronuclei compared to the concurrent controls were found at all doses tested without apparent dose-response relationship. The micronucleus frequencies exceeded the historical control values at the mid and high dose. In experiment 2 statistically significant increased frequencies of MNBC compared to the concurrent controls were found at the two highest doses tested with a weak dose-response relationship. However, only one culture exceeded the historical control values.

Conclusion
Under the experimental conditions used Rudol Gray B base Lot No. 217 induced micronuclei in the presence of S9 metabolic activation and, consequently, is genotoxic (clastogenic and/or aneugenic) in human lymphocytes *in vitro*.

Ref.: 39

In vitro unscheduled DNA synthesis test

Guideline:	/
Species/strain:	primary rat hepatocytes
Replicates:	triplicate cultures
Test substance:	4-aminodiphenylamine
Solvent:	acetone
Batch:	/
Purity:	/
Concentrations:	preliminary assay: 0.5 – 1000 µg/ml
	replicate assay: 0.5 - 100 µg/ml
Treatment:	20 h treatment
GLP:	not in compliance

4-aminodiphenylamine was investigated for the induction of unscheduled DNA synthesis (UDS) in primary hepatocytes of rats. Hepatocytes were exposed simultaneously to 4-aminodiphenylamine and 10 µCi/ml ^{3}H-thymidine for 20 h. Evaluation of autoradiography was done 7 days after exposure.
UDS was measured by counting nuclear grains and subtracting the average number of grains in 2 nuclear sized areas adjacent to each nucleus; this value is referred to as net nuclear grain count. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 3 replicate cultures. Negative and positive controls were included.

Results
Cytotoxicity was observed at 100 µg/ml in the preliminary assay and at 50 µg/ml in the replicate assay. Precipitation of 4-aminodiphenylamine was seen at 50 µg/ml. Occasionally, an increase in net nuclear grain count was seen, however, this increase was completely attributed to a decrease in cytoplasmic labelling.
None of the treatment groups in both experiments had net nuclear grain counts above zero or a notable increase in cells in repair (cells with 5 or more nuclear grains per cells).

Conclusion

Under the experimental conditions used 4-aminodiphenylamine did not induce unscheduled DNA synthesis in primary rat hepatocytes and, consequently, is not genotoxic in the *in vitro* UDS test.

Ref.: 19

Comment
Only a study summary delivered by Monsanto was available. The results were only described and the actual data (raw data) were not delivered. Consequently, the conclusions of the authors could not be checked. Therefore, value of this study is limited and, consequently, can only be used as supportive evidence.

In vitro Sister Chromatid Exchange Assay

Guideline:	/	
Species/strain:	CHO-W-B1 cells	
Replicates:	/	
Test substance:	N-phenyl-p-phenylenediamine	
Solvent:	DMSO	
Batch:	/	
Purity:	/	
Concentrations:	without S9:	first experiment: 0.16 – 5.0 µg/ml second experiment: 0.5 – 4.0 µg/ml
	with S9:	5.0 – 500.0 µg/ml
Treatment:	26 h treatment with 2 h recovery without S9, 2 h treatment with 26 h recovery with S9.	
GLP:	not in compliance	

The present assay is from the NTP website (http://ntp-server.niehs.nih.gov/htdocs/Overviews/SaDoc.html).

N-phenyl-p-phenylenediamine has been investigated in the absence and presence of metabolic activation for sister chromatid exchanges in CHO cells. Cells were treated for 26 h in the absence or 2 h in the presence of S9 metabolic activation. Two h after the start of treatment 5-bromodeoxyuridine was added. Harvest time was 28 hours after the beginning of treatment. Two hours before harvest, each culture was treated with colcemid solution. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system.
Toxicity was not reported. Chromosome (metaphase) preparations were stained with Hoechst 33258 and Giemsa and examined microscopically for sister chromatid exchanges. Negative and positive controls were included.

Results
Without S9 metabolic activation, a dose dependent increase in the number of sister chromosomes exchanges per chromosome was observed at doses from 1.0 µg/ml and above (experiment 2). At lower concentrations no increase in sister chromatid exchanges were found (experiment 1, except for the highest dose). With S9 metabolic activation a dose dependent increase in the number of sister chromosomes exchanges per chromosome was also found.

Conclusion
Under the experimental conditions used N-phenyl-p-phenylenediamine induced an increase in sister chromatid exchanges per chromosome and, consequently, is genotoxic in CHO cells *in vitro*.

Ref.: 15b

Comment

Data on cytotoxicity are lacking. This shortcoming together with the poor reporting, reduces the value of this sister chromatid exchange test, and, therefore, it can only be used as supportive evidence.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Bone marrow micronucleus test in rats

Guideline:	OECD 474
Species/strain:	Sprague Dawley rats
Group size:	7 male rats
Test substance:	Rudol Gray B base Lot No. 217
Lot no:	S2582101
Purity:	(99.6%) not mentioned in the report
Dose level:	25, 50 and 100 mg/kg bw
Route:	*i.p.*
Vehicle:	0.5 % aqueous carboxymethylcellulose
Sacrifice times:	24 h for all concentrations, 48 h for the vehicle control and the highest dose.
GLP:	In compliance

Rudol Gray B base Lot No. 217 has been investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on the level of mortality and toxicity in a pilot toxicity study and a supplemental toxicity study. In these dose finding studies all rats treated with 250 mg/kg bw or more died between 1 h to 24 h after treatment. Since no significant differences were found between male and female rats the micronucleus study was conducted with male rats only. Rats were exposed to single *i.p.* doses of 0, 25, 50 and 100 mg/kg bw. 24 h or 48 h (highest dose and concurrent vehicle control only) after dosing bone marrow cells were collected. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/EC). Two satellite groups of 3 male rats, treated with vehicle and 100 mg/kg bw, were used in a toxicokinetic study. These animals were bled 1, 2, 8 and 24 h after treatment and plasma was collected. Bone marrow preparations were stained acridine orange and examined microscopically for the PCE/EC ratio and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
In the micronucleus study no mortality occurred. Clinical signs following exposure were lethargy at all doses and piloerection and prostration at 100 mg/kg bw indicating to systemic toxicity in exposed animals. Measurement of plasma levels in the toxicokinetics study confirmed the systemic exposure; plasma levels of Rudol Gray B base Lot No. 217 were found 1 and 2 h after exposure to 100 mg/kg bw. The decrease of 13 % in the PCE/EC ratio found after the highest dose compared to the concurrent controls also indicates to exposure of bone marrow cells.
A statistically significant increase in the number of micronucleated PCEs compared to the concurrent vehicle controls was only found at the 24 h sampling time for the highest dose. However, this increase was clearly within the range of the historical controls and is, therefore, considered not biologically relevant. At the 48 h sampling time (highest dose only) no increase in the number of micronucleated PCEs compared to the concurrent vehicle controls was found.

Conclusion
Under the experimental conditions used Rudol Gray B base Lot No. 217 did not induce micronuclei in bone marrow cells of treated rats and, consequently, Rudol Gray B base Lot No. 217 is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.

Ref.: 40

In vivo alkaline single cell gel electrophoresis (Comet) assay in mice

Guideline:	/
Species/strain:	ddY mice
Group size:	4 mice, no indication about sex
Test substance:	N-phenyl-1,4-phenylenediamine
Lot no:	/
Purity:	> 95%
Dose level:	300 mg/kg bw
Route:	single oral gavage.
Vehicle:	olive oil
Sacrifice times:	0, 3, 8 and 24 h after treatment
Organs studied:	stomach, colon, liver, kidney, urinary bladder, lung, brain, bone marrow
GLP:	not in compliance

The present assay is reported in a paper from the open literature in which 30 aromatic amines were tested in the *in vivo* alkaline single cell gel electrophoresis (Comet) assay. The aim of the paper is to demonstrate the correlation between the Comet assay and carcinogenicity.

N-phenyl-1,4-phenylenediamine has been investigated for the induction of DNA damage in the alkaline single cell gel electrophoresis (Comet) assay in various tissues of mice. Test concentrations were based on the results of a simple acute toxicity test in which the maximum tolerated dose (MTD, about half the LD50) was determined. As a result the mice were exposed by single oral gavage to the MTD which was for N-phenyl-1,4-phenylenediamine 300 mg/kg bw. Mice were treated for 0, 3, 8 and 24 h. Since in previous experiments no significant differences were observed between vehicle control group and untreated groups (0-time groups) at any sampling time for any organ, concurrent vehicle controls groups were not used in this experiment.
At necropsis tissues were examined macroscopically and prepared for histopathological examination. Cytotoxicity as well as evidence for exposure of the tissues were not reported.
Per slide per organ 50 nuclei were examined for migration being the difference between the length of the comet and diameter of the head. The use of positive controls was not reported.

Results
N-phenyl-1,4-phenylenediamine did not show clinical signs under the conditions of the test. Treatment with N-phenyl-1,4-phenylenediamine did not result in an increase in DNA damage in any tissue studied at any time point compared to the untreated mice (0-time groups)

Conclusion
Under the experimental conditions used N-phenyl-1,4-phenylenediamine did not induce DNA damage in various tissues of mice and, consequently, N-phenyl-1,4-phenylenediamine is not genotoxic in the Comet assay with mice.

Ref.: 23

Comment
Data on cytotoxicity are lacking. This shortcoming together with the poor reporting, reduces the value of this test, and, therefore, it can only be used as supportive evidence.

In vivo unscheduled DNA synthesis (UDS) test (1)

Guideline:	/
Species/strain:	Sprague Dawley rats

Group size:	at least 3 rats
Test substance:	4-aminodiphenylamine
Lot no:	/
Purity:	/
Dose level:	50, 100 and 250 mg/kg bw
Route:	oral gavage
Vehicle:	corn oil
Sacrifice times:	16 h and 48 h after dosing
GLP:	not in compliance

4-aminodiphenylamine was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Rats were treated *in vivo*. Hepatocytes for UDS analysis were collected at 16 h and 42 h after administration of 4-aminodiphenylamine. Ninety to 120 minutes after plating the cells incubated for 4 h with 10 µCi/ml ^{3}H-thymidine followed by 15-17 h coincubation with unlabelled thymidine. Evaluation of autoradiography was done after 7 days thymidine exposure.
UDS was measured by counting nuclear grains and subtracting the average number of grains in 2 nuclear sized areas adjacent to each nucleus; this value is referred to as net nuclear grain count. Unscheduled synthesis was determined in 30 randomly selected hepatocytes on 3 replicate cultures per rat. Negative and positive controls were included.

Results
Both for the 16 h and the 42 time-points after treatment none of the individual groups showed an increased mean net nuclear grain count as compared to the untreated control. 250 mg/kg bw 4-aminodiphenylamine resulted in about 2.6% of the cells in S-phase, indicative for proliferative effect.

Conclusion
Under the experimental conditions used 4-aminodiphenylamine did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref.: 20

Comment
Only a study summary delivered by Monsanto was available. The results were only described. The actual data (raw data) were not delivered. Consequently, the conclusions of the authors could not be checked. Therefore, this study is not suitable for evaluation. Consequently, it can only be used as supportive evidence.

In vivo unscheduled DNA synthesis (UDS) test (2)

Guideline:	/
Species/strain:	Sprague Dawley rats
Group size:	3 male rats
Test substance:	Rudol Gray B base Lot No. 217
Lot no:	S2582101
Purity:	(99.6%) not mentioned in the report
Dose level:	187.5, 375.0 and 750.0 mg/kg bw
Route:	oral gavage, once
Vehicle:	0.5 % aqueous carboxymethylcellulose
Sacrifice times:	2-4 h and 12-16 h after dosing
GLP:	In compliance

Rudol Gray B base Lot No. 217 was investigated for the induction of unscheduled DNA synthesis (UDS) in hepatocytes of rats. Rats were treated *in vivo*. Test concentrations were based on the level of mortality and toxicity in a toxicity study. The highest dose selected for this UDS assay was the dose that produced toxicity such that higher doses would be expected to cause mortality or unacceptable severe clinical signs. In this dose range finding study 2 out of 3 females died in the 1000 mg/kg bw group. The highest dose selected was the maximum tolerated dose 750 mg/kg bw. Next 2 additional dose levels were selected using dilutions of the highest dose.
Hepatocytes for UDS analysis were collected at 2 - 4 h and 12 - 16 h after administration of Rudol Gray B base Lot No. 217. Ninety to 180 minutes after plating the cells incubated for 4 h with 10 µCi/ml ^{3}H-thymidine. Evaluation of autoradiography was done after 5-12 days exposure.
UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 nuclear sized areas adjacent to each nucleus; this value is referred to as mean net nuclear grain count. Unscheduled synthesis was determined in 50 randomly selected hepatocytes on 3 replicate cultures per rat.
Two groups of 3 rats were used for determination of plasma concentrations of Rudol Gray B base Lot No. 217 (vehicle and highest dose only). Blood samples were collected via the retro-orbital sinus 1, 2 and 12 h after treatment.
Negative and positive controls were in accordance with the OECD guideline.

Results
In the UDS test mortality was not observed. At the 2-4 h time point clinical signs were limited to piloerection in the two highest dose groups. At the 12-16 h time point clinical signs were only observed in the highest dose group; all animals showed lethargy and piloerection whereas 2 out 3 animals showed diarrhoea. Measurement of plasma levels confirmed the systemic exposure; high plasma levels of Rudol Gray B base Lot No. 217 were found 1 and 2 h after exposure to 750 mg/kg bw.
Both for the 2-4 h and the 12-16 time-points after treatment none of the individual groups showed an increased mean net nuclear grain count as compared to the untreated control. Also the number of cells with 5 or more nuclear grains per cells (in the tables referred to as "cells in repair") never reached the necessary criterion of 10% above the percentage found for the untreated control.

Conclusion
Under the experimental conditions used Rudol Gray B base Lot No. 217 did not induce unscheduled DNA synthesis and, consequently, is not genotoxic in rats in the *in vivo* UDS test.

Ref. 45

3.3.7.	Carcinogenicity

Oral administration, mice

Guideline:	/
Species/strain:	B6C3F$_1$ mice
Group size:	50 animals per sex and dose, matched controls 20 animals per sex
Test substance:	N-Phenyl-p-phenylenediamine in the diet
Batch:	0100700 GB
Purity:	90 %
Dose level:	Male: 0, 2,500 or 5,000 ppm; Female: 0, 5,000 or 10,000 ppm for 31 weeks. Because of toxicity of the chemical, the doses were lowered at that time and terminated at 48 weeks. The animals were then observed for 43 additional weeks. Time-weighted average doses during the period of administration were 2,057 or 4,114 ppm for the males and 3,672 or 8,170 for the females
Route:	Oral, in diet

Exposure: 48 weeks
GLP: in compliance

Groups of 50 mice of each sex (8 weeks old**)** were initially administered N-phenyl-p-phenylenediamine at one of the following doses, either 2,500 or 5,000 ppm for the males and either 5,000 or 10,000 ppm for the females, for 31 weeks. Because of toxicity of the chemical, the doses were lowered at that time and terminated at 48 weeks. The animals were then observed for 43 additional weeks. At least 85% of the males survived until the end of the study. Among the females, 58% (29/50) of the high dose group and 68% (34/50) of the low dose group and 85% of the matched control groups survived until the end of the study. All surviving mice were killed at 91 weeks.
Mean body weights of the dosed mice were appreciably lower than those of the matched controls, and mortality was high in the dosed groups prior to the reduction of the doses, particularly in the females. The shortened period used for administering N-phenyl-p-phenylenediamine to the mice may not have been adequate for determining the carcinogenic potential of the test chemical.
In the male mice, the incidence of combined hepatocellular adenomas and carcinomas was significantly higher (P=0.022) in the low-dose group than in the controls, but there was no significant dose-related trend (controls 2/20 (10%), low-dose 18/49 (37%), high-dose 10/50 (20%)). Furthermore, since at this laboratory the overall historical incidences of these combined lesions in male mice have been 53/340 (15.6%) and have been as high as 7/20 (35%), these neoplasms could not be established as being compound related. Unusually extensive hepatic inflammation occurred in large numbers of the dosed males (controls 0/20, low-dose 23/49, high-dose 24/50) and in lesser numbers of the dosed females (controls 1/20, low-dose 8/49, high-dose 2/48).
It is concluded that under the conditions of this bioassay, N-phenyl-p-phenylenediamine was not carcinogenic for $B6C3F_1$ mice.

Ref.: 8

Oral administration, rats

Guideline: /
Species/strain: Fischer 344 rats
Group size: 50 animals per sex and dose, matched controls 20 animals per sex
Test substance: N-Phenyl-p-phenylenediamine in the diet
Batch: 0100700 GB
Purity: 90%
Dose level: 0, 600 or 1,200 ppm
Route: Oral, in diet
Exposure: 78 weeks
GLP: in compliance

Groups of 50 rats of each sex (8 weeks old**)** were administered N-phenyl-p-phenylenediamine at one of two doses, either 600 or 1,200 ppm, for 78 weeks and were then observed for 26 additional weeks. At least 94% of the males and more than 85% of the females survived until the end of the study. All surviving rats were killed at 104 weeks.
Mean body weights of the dosed rats were only slightly lower than those of the matched controls during the bioassay.

In the male and female rats, the incidences of neoplasms in the groups receiving the test chemical were not significantly different from those in the corresponding control groups.
It is concluded that under the conditions of this bioassay, N-phenyl-p-phenylenediamine was not carcinogenic for Fischer 344 rats.

Ref.: 8

Comment

A sufficient numbers of rats of each sex survived in order to identify the risk for the development of late-appearing tumours.
It is concluded that under the conditions of this bioassay, N-phenyl-p-phenylenediamine was not carcinogenic for Fischer 344 rats or B6C3F$_1$ mice. However, the shortened period used for administering N-phenyl-p-phenylenediamine to the mice and the low survival of the females may not have been adequate for determining the carcinogenic potential of the test chemical.

Topical application, mice

Guideline:	/
Species/strain:	Swiss-Webster mice
Group size:	50 animals per sex and dose
Test substance:	one hair dye formulations containing 2.0% N-Phenyl-p-phenylenediamine hydrochloride
Batch:	/
Purity:	not stated
Dose level:	0.05 ml of a solution containing 2% N-phenyl-p-phenylenediamine (dye formulation 7404) 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 9 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.

Nine males and 13 females survived to 21 months in the group receiving the oxidative formulation containing N-Phenyl-p-phenylenediamine. 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 and 9 months. There were no statistically significant differences in the distribution of tumours among treated and control groups.

Ref.: 31, A

Comment
2,4-Diaminoanisole (EU, carcinogenic Category 2) was tested in the same experiment and no response was found. No conclusion with regard to carcinogenicity can be made from the study.

Topical application, rats

Guideline:	/
Species/strain:	Male and female weanling Sprague Dawley rats
Group size:	60 animals per sex and dose
Test substance:	One hair dye formulation (7404) containing 2% N-phenyl-p-phenylenediamine hydrochloride

Batch:	/
Purity:	not stated
Dose level:	0.5 ml of a solution containing 2% N-phenyl-p-phenylenediamine (dye formulation 7404) 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:	/

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 24 males and 16 females for the formulation group. Survival in the control groups were 17 – 20 males and 22 – 26 females for the control groups. The mean body weights at week 114 in the treated group were 669 g in males and 471 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. No increased tumour incidences were found in any of the tissues examined.

Ref.: 30, A

Comment
2,4-Diaminoanisole (EU, carcinogenic, category 2) was tested in the same experiment and no response was found.
No conclusion with regard to carcinogenicity can be made from the study.

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Guideline:	/
Species:	Rat, Sprague Dawley
Route:	oral by gavage
Group sizes:	11- 12
Substance:	N-Phenyl-PPD (Lowenstein Dyes NY)
Batch:	/
Purity:	/
Dose:	50, 100 and 200 mg/kg bw/day in volume of 10 mL/kg day
Exposure:	Gestation Days (GD) 6 -15
Vehicle:	in propylene glycol
Control groups:	Vehicle control (propylene glycol),
Positive control:	Vitamin A at 100,000 IU/animal on day 9 only.
GLP:	/

Mature male and female Sprague-Dawley (225-250 grams) rats were housed together, and day 0 of pregnancy was defined as the day on which a copulatory plug or vaginal sperm were detected. Females were then housed separately and dosed by gavage during the embryonic period GD 6 -15. Treated rats were observed daily and weighed on GD 0, 6, 16 and 20. On GD 20, dams were euthanised with CO_2, the uterine horns were removed and numbers and conditions of foetuses recorded. Half the foetuses were fixed in Bouin's solution for examination of soft tissue, and the other half were fixed in 95% isopropyl alcohol and stained with Alizarin Red S for examination of skeletal effects.

Results
All dams completed the study. The positive control was teratogenic with 100% of litters having at least one abnormal foetus. In the high dose group (200 mg/kg), dams displayed a significant decrease in mean body weight up to GD 16, compared with the control group, but showed an increase in weight gain during the non-treatment days (GD 16 -20). There were no significant foetal effects seen as a result of N-Phenyl-PPD administration. There were no differences in mean foetal weights, sex ratios, external, visceral or skeletal foetal abnormalities between the control and treated groups.

Conclusions
N-Phenyl-p-Phenylenediamine was not teratogenic when administered to pregnant rats during the foetal period of development.

Ref.: 24

Comment
This is a journal publication, not a study report. It appeared to be well conducted but a complete assessment was not possible. No NOAEL could be derived from this data.

Guideline:	/
Species:	Rat, Sprague Dawley
Route:	oral by gavage
Group sizes:	25
Substance:	4-ADPA (N-phenyl-p-phenylenediamine)
Batch:	110113
Purity:	98.6%
Dose:	0 and 150 mg/kg bw/day
Exposure:	Gestation Days (GD) 6 -15
Vehicle:	corn oil
Control groups:	Vehicle control (corn oil),
Positive control:	/
GLP:	in compliance

Mature male and female Sprague-Dawley (225-250 grams) rats were housed together, and day 0 of pregnancy was defined as the day on which a copulatory plug or vaginal sperm were detected. Females were then housed separately and dosed by gavage from GD 6 -15. Treated rats were observed daily and weighed periodically. On GD 20, dams were killed with CO_2, the uterine horns were removed and foetus numbers and conditions recorded. Half were fixed in Bouin's solution for examination of soft tissue, and the other half stained with Alizarin Red S for examination of skeletal effects.

Results
Moderate to severe maternal toxicity occurred at 150 mg/kg, (significantly lower maternal weight gain, both during the dosing and post-dosing period). Staining of the anogenital area occurred in the treated animals. The mean gravid uterine weights were significantly lower. Embryotoxicity/foetotoxicity was shown as decreased foetal weights, increased resorption sites, and delayed ossification with an increased incidence of external, skeletal, and visceral malformations in the foetuses of treated dams.

Conclusions
N-Phenyl-p-phenylenediamine was embryotoxic, foetotoxic and teratogenic when administered to Sprague Dawley rats at a dose that was maternally toxic.

Ref.: 25

Guideline:	/
Species:	Rat, Sprague Dawley
Route:	oral by gavage
Group sizes:	25
Substance:	4-ADPA (N-phenyl-p-phenylenediamine)
Batch:	11013
Purity:	98.6%
Dose:	0 10, 50 and 100mg/kg bw in volume of 5 ml/kg day
Exposure:	Gestation Days (GD) 6 -15
Vehicle:	corn oil
Control groups:	Vehicle control (corn oil)
Positive control:	/
GLP:	in compliance

Mature male and female Sprague-Dawley (225-250 grams) rats were housed together, and day 0 of pregnancy was defined as the day on which a copulatory plug or vaginal sperm were detected. Females were then housed separately and dosed by gavage from GD 6 -15. Treated rats were observed daily and weighed periodically. On GD 20, dams were killed with CO_2, the uterine horns were removed and foetus numbers and conditions recorded Half were fixed in Bouin's solution for examination of soft tissue, and the other half stained with Alizarin Red S for examination of skeletal effects.

Results
Maternal toxicity occurred in the dams treated with 100 mg/kg. Mean weight gain and food consumption was significantly lower in the high dose dams compared with the vehicle controls and the mid and low dose groups. Foetotoxicity occurred in the high dose foetuses (100 mg/kg), with a significant reduction in mean foetal weights and increases in ossification variations. The external, visceral and skeletal malformations observed in the foetuses from the high dose treatment (100 mg/kg), were attributed to maternal toxicity effects and not to direct teratogenic effects. No adverse effects were seen in the foetuses of animals treated with 10 and 50 mg/kg.

Conclusions
N-Phenyl-p-phenylenediamine, at doses lower than those associated with maternal toxicity, was not embryotoxic, foetotoxic or teratogenic in the Sprague Dawley rat when

administered during the foetal period of development. Foetotoxic effects were seen in the high dose group, mainly affecting ossification were consistent with the reduced foetal weight.

Ref.: 26

Comment
The NOAEL for maternal and embryotoxicity was set at 50 mg/kg bw.

Fertility study

Guideline: /
Species: Rat, Wistar
Route: intra-peritoneal for 180 days
Group sizes: 30
Substance: p-ADPA (Koch-Light Labs.UK)
Batch: /
Purity: /
Dose: 0 and 42.5 mg/kg
GLP: /

Food intake (pelleted diet) and body weight were recorded weekly. Testes, ventral prostate, dorso-lateral prostate, seminal vesicles & coagulating glands were recorded at the end of the experimental period.
Biochemical analyses included: Testicular enzymes and bioconstituents including lactate dehydrogenase, hyaluronidase and lactic acid levels. Alkaline phosphatase in seminal vesicles; acid phosphatase in ventral prostate; fructose content in coagulating glands and dorso-lateral prostate and protein estimation.
Histopathology included: Left testicular tissue, after fixation in 10% neutral formalin and staining (5μm sections) with haematoxylin and eosin stain.

Results
p-ADPA treatment caused a statistically significant reduction in a number of biochemical parameters of the testes. LDH and hyaluronidase decreased by 35 and 23% respectively and lactic acid was reduced by 23% compared with controls.

	Treated group	**Control group**
Lactate dehydrogenase[1]	0.11 + 0.004	0.17 + 0.007
Hyaluronidase[2]	57.71 + 2.58	75.18 + 2.78
Lactic acid[3]	0.36 + 0.013	0.47 + 0.023

[1]μmole NADH oxidised/min/mg protein; [2] IU/mg protein; [3] mg/g fresh tissue

Histopathology of p-ADPA treated rats showed patchy degeneration of seminiferous tubules. Tubules most severely affected had desquamation of the epithelium and formation of vacuoles with multinucleated giant cells within the lumen of these damaged tubules. Interstitial tissue had normal vascularity and Leydig cells. There were no significant changes seen in the accessory sex organs of p-ADPA treated rats compared with controls.

Conclusion
p-ADPA treated animals showed signs of testicular toxicity, as seen arrested spermatogenesis, maturation of the spermatocytes and impaired fertility, signalled by significantly reduced lactate dehydrogenase, hyaluronidase activities and lactic acid in the testes suggesting lower energy availability during the spermatogenesis. However, the normal levels of acid and alkaline phosphatase, as well as the histopathology of the interstitium and Leydig cells in p-ADPA exposed rats confirms the normal androgenic status of the testis.

Ref.: 27

Comment
This is a journal paper, not a study report. It appeared to be well conducted but a complete assessment was not possible.
There was an unsubstantiated statement in Submission 1 but no reference was supplied that treatment of male mice with N-Phenyl-p-phenylenediamine caused adverse effects on male fertility as a consequence of toxicity to the testes and spermatogenesis.

Formulation

Guideline:	/
Species:	Rat, Sprague-Dawley rats, age not stated, group.
Route:	topical
Group sizes:	40 animals per sex per dose
Substance:	Formulation 7404, (2.0% N-Phenyl-p-Phenylenediamine hydrochloride, 1.0% resorcinol 1.5% m-phenylenediamine, 1.0% o-phenylenediamine, 1.0% N-Methyl-p-aminophenol sulfate)
Batch:	/
Purity:	/
Dose:	2% with an equal volume of 6% H_2O_2
Exposure:	1-h twice a week for 114 weeks
GLP:	/

The text was taken from submission, as the publication was not provided.
Treatment was continuous through growth, mating, gestation, and lactation to weaning at the F_{1b}, F_{2b}, and F_{3c} litters of the respective generations.
Dye 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. An initial level of 0.2 ml per application was increased incrementally by 0.1 ml per application weekly until reaching the final level.
Application was made to the parental generation (F_0) until they reached 100 days of age after which they were mated. The females were permitted to deliver their young (F_{1a}) naturally and litter size was reduced to 10 pups on Day 4 of lactation. F_{1a} pups were assigned to a lifetime chronic toxicity study. F_0 parents were reduced to 20 males and 20 females per group and were rebred to produce F_{1b} litters. Twenty males and 20 females were selected, treated as for the F_0 rats, and were mated twice to produce the $F2_a$ and F_{2b} litters. Twenty males and 20 females from the F_{2b} litters were mated to produce the $F_{3a\ -c}$ litters.
Gross necropsies and microscopic examinations were performed on 5 males and 5 females from the F_1 generation and on one weanling from each litter of the F_{3b} generation. 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.

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 (mild scabbing, fissuring, atonia, and leathery texture).
Reproductive performance: Performance of F_0, F_1, and F_2 parental rats showed no difference for treated and control rats with respect to fertility, gestation, and live birth indices. Although there was reduced fertility for F_2 parents in producing the F_{3a-c} offspring, there were no significant differences among the groups.

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 or in F_{3b} weanlings that were sacrificed, necropsied, and evaluated microscopically.

Conclusion
Topical application of a hair dye formulation containing 2% N-phenyl-p-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.: 29

Comment
Ref 29, a publication, was not supplied.

3.3.9.	Toxicokinetics

Metabolism studies

Guideline: /
Species: Rat, male albino.
Substance: N-Phenyl-p-Phenylenediamine (Sigma Chemicals)
Batch: /
GLP: /

A single literature study has been reported investigating the uptake of N-Phenyl –p-phenylenediamine (N-PPDA) through skin and its consequent distribution and excretion via the systemic circulation.

Methods
Experiment 1
0.5g skin segment was placed in a 25ml Erlenmeyer flask containing 5ml of Kreb's ringer bicarbonate pH 7.4. N-PPD (4μmol ethanolic solution) was added and the flask shaken at 120strokes/min for 60-mins. Triplicate sets of skin segments were set-up for each observation point and the mean value used in subsequent analyses. Variables investigated included:
weight of skin segment used (simulating varying exposure area); concentration of N-PPDA (0.1 to 0.5 μmol); exposure duration (15–90 minutes); temperature (25 – 70°C); pH of vehicle (5-10);
effect of 'bioinhibitors': KCN, NaF, HgCl2, Na arsenate etc; effect of reducing agents: ascorbic acid, cysteine, Na dithionite and Na metabisulphite.
After treatment, skin segments were washed with Krebs and either (a) homogenised using water saturated with n-butanol to determine skin-bound N-PPDA, or (b) the buffer washings + incubation medium were combined and extracted twice with 2.5 vol. water saturated butanol and analysed for residual amine.

Experiment 2
Skin segments preincubated with N-PPDA were washed twice with Krebs and transferred to flasks containing 5ml rat serum and incubated at 37°C. Effects of serum dilutions (0, 2.5, 5, 7.5 and 10-fold) and contact time (15-180min.) on the mobilisation of N-PPDA were investigated. Skin segments were extracted with water saturated butanol as above for estimation of non-effluxed N-PPDA.

Experiment 3
Finely chopped skin (ratio 10ml solvent to 100mg skin) was soaked for 6h at 25oC in a mixture of chloroform:methanol (2:1 v/v). This extraction was repeated 3-times using fresh solvent on each occasion. Pooled extracts were then dried using a rotary evaporator under vacuum and the lipid fraction dispersed in 0.05M phosphate buffer pH 7.5 (1mg/ml buffer).

This sample was homogenised and then centrifuged to provide a supernatant for the source of the lipid micelles.
25-100μg lipid micelles were interacted with 500μg N-PPDA in a final volume of 3ml 0.05M phosphate buffer pH 7.5 and the absorption spectrum scanned between 375-600nm. The decrease in absorbance of N-PPDA at 465nm with incubation time (1-5min) was monitored. Analysis of N-PPDA: p-DMAC (0.5ml) reagent was added to a 0.5ml aliquot of water saturated n-butanol containing N-PPDA and the final volume made up to 3ml with methanol. The brown Schiff's base formed was read at 550nm after 10-mins.
Under experimental conditions a linear Beer's Law relationship was established between Absorbance (A) and concentration (C) over the range 0.1 – 2.00μg/ml.

Results
Experiment 1
Uptake of N-PPDA through skin was dependent on surface area exposed over the range 0.1-0.5g. There was a linear uptake of N-PPDA up to 2μmol, this became non-linear between 2.5-3.5μmol and then followed saturation kinetics. Km/Vmax values were 2.54 x 10^4M and 4.76μmol/g skin
The optimal contact time was 75-mins.
Binding profile was linear with increasing temperature up to 40°C, after which there was a gradual decline. Optimal pH was 7.5
Tested at up to 1mM final concentration no effect on overall uptake of dye was seen with the exception of a partial inhibition caused by $HgCl_2$.

Experiment 2
Efflux of skin-bound N-PPDA was influenced by both serum concentration and contact time. Undiluted serum could dissociate 82% of skin bound N-PPDA. This was reduced to 47 and 17% respectively with 5 and 10-fold dilutions of serum. Rate of mobilisation increased with contact time up to a maximum efflux at 120-mins.

Experiment 3
The λmax shifted from 465 to 450nm with increasing lipid micelle content

Conclusion
N-Phenyl-p-phenylenediamine is relatively hydrophobic and skin absorption and subsequent systemic availability is dependent upon its ability to cross skin lipid barrier and compete for binding with serum proteins. Uncouplers of oxidative phosphorylation and inhibitors of active transport did not change uptake kinetics of N-PPDA and therefore ability to penetrate skin appears to be 'passive' process with some element of uptake associated with lipid micelles. Skin bound N-PPDA was effluxed into serum and serum proteins appear to have some affinity for this amine.

The conditions of exposure do not simulate those that occur during actual exposure to a hair colorant and thus the results from this study are not suitable for use in the risk assessment.

Ref.: 28

3.3.10.	Photo-induced toxicity

No data submitted

3.3.11.	Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Physico-chemical properties
N-Phenyl-p-phenylenediamine is used in oxidation hair dyes at a maximum concentration of 0.2% (after mixing in the ratio 1:1 with hydrogen peroxide).
Adequate physico-chemical characterisation has been performed for only one batch. The batches used in the mutagenicity studies were not characterised. Stability data on marketed formulation is not provided.
N-Phenyl-p-phenylenediamine is used also in the forms of the hydrochloride and sulphate salts. No data were provided for the two salts; apparently their toxicological behaviour is expected to be similar to that of the free base.

General toxicity
Much of the toxicity data was old and did not satisfy the guidelines in force even at the times when performed.
A new 90 day study is required to meet modern guidelines with special attention to the effects on the reproduction. The current lack of data on the effects of N-Phenyl-p-phenylenediamine on females needs to be addressed since maternotoxicity was noted in the developmental studies.
In the male, fertility seems to be compromised with signs of testicular toxicity (arrested spermatogenesis and maturation of spermatocytes) signalled by significantly reduced enzyme levels implying reduced energy availability during spermatogenesis (publication in journal). In male rats, effects were also noted in the liver.
N-Phenyl-p-phenylenediamine, at doses lower than those associated with maternal toxicity, was not embryotoxic, foetotoxic or teratogenic in the Sprague Dawley rat when administered during the foetal period of development. Foetotoxic effects were seen in the high dose group, mainly affecting ossification were consistent with the reduced foetal weight. The NOAEL for maternal and embryotoxicity was set at 50 mg/kg bw.

Irritation, sensitisation
The study concerning the skin irritation is not respecting the actual guidelines and is considered inadequate. Nevertheless the results can be accepted and N-Phenyl-p-phenylenediamine is classified as a non irritant substance. Numerous publications demonstrate that N-Phenyl-p-phenylenediamine is a strong sensitizer in humans. The LLNA studies conducted according to the OECD guideline show that N-Phenyl-p-phenylenediamine is an extremely potent skin sensitizer.

Dermal absorption
The absorption and dermal delivery of N-Phenyl-p-phenylenediamine from the two hair dye formulations was approximately 3 fold greater in hair dye formulation "Complete Dye Base C and developer" with 0.23% w/w of N-Phenyl-p-phenylenediamine than in hair dye formulation "Complete Dye Base U and developer" with 0.14% w/w of N-Phenyl-p-phenylenediamine.

The maximum absorption value observed in the experiment, 2.32 µg/cm^2 can be used for calculating the MoS.

Mutagenicity
N-phenyl-p-phenylenediamine was investigated for its potency to induce gene mutations in bacteria and mammalian cells. The description of these studies, in part papers from the open literature, was limited. Mostly it was not possible to compare the study design with the minimal requirements described in the OECD guidelines for these tests. Therefore, the value of these studies is limited and the results can only be used as supportive evidence. With and without metabolic activation N-phenyl-p-phenylenediamine was negative in three bacterial gene mutation studies, did not induce gene mutations at the *hprt* locus of mammalian cells, and did not cause unscheduled DNA synthesis in primary hepatocytes. Positive results were reported in two bacterial and one mammalian ($tk^{+/-}$locus) gene mutation assays. However, the quality of these studies was poor and the results are not suitable for evaluation.
In 1 of 2 *in vitro* chromosome aberration tests N-phenyl-p-phenylenediamine was positive. The second one scored negative and this finding was confirmed in a sister chromatid exchange test. These 3 tests, however, suffered from an inadequate description of the study design and are at most supportive evidence. In a well performed *in vitro* micronucleus test N-phenyl-p-phenylenediamine treatment resulted in an increase in cells with micronuclei.

Under *in vivo* conditions the potency to induce chromosome aberrations or putatively gene mutations could not be confirmed. In a well performed in vivo micronucleus test N-phenyl-p-phenylenediamine did not induce an increase in micronucleated bone marrow cells in the rat nor did exposure result in unscheduled DNA synthesis. These *in vivo* results were confirmed in a less well performed comet assay and a second unscheduled DNA synthesis assay.

In conclusion, N-phenyl-p-phenylenediamine itself can be considered to have no *in vivo* genotoxic potential and additional tests are unnecessary. To reach a definitive conclusion, appropriate tests with N-phenyl-p-phenylenediamine in combination with hydrogen peroxide have to be provided.

Carcinogenicity
The possible carcinogenic effects of N-phenyl-p-phenylenediamine have been tested in a 2-year study with rats and mice. The rat study was negative. The mice study was not adequate for evaluation. A hair dye formulation containing N-phenyl-p-phenylenediamine has also been tested together with hydrogen peroxide by topical application to mice and rats without any tumour induction. However, also in cases where known carcinogens were present in the hair dye formulation, no tumours were induced. Thus, no conclusions with regards to carcinogenicity can be drawn from the skin painting studies.

4. CONCLUSION

A proper NO(A)EL for subchronic toxicity cannot be set due to the shortcomings of the experimental data provided.

Data is required to meet modern guidelines with special attention to the effects on fertility together with reproductive parameters. The current lack of data on the effects of N-Phenyl-p-phenylenediamine on females needs to be addressed since maternotoxicity was seen in the developmental studies. In addition, liver effects were noted in males.
In the male, fertility seems to be compromised with signs of testicular toxicity (arrested spermatogenesis and maturation of spermatocytes) signalled by significantly reduced enzyme levels implying reduced energy availability during spermatogenesis.

The substance has shown to be an extremely potent skin sensitiser.

N-phenyl-p-phenylenediamine itself has no mutagenic potential *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

6. REFERENCES

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