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Final Report on the Safety Assessment of 2,4-Diaminophenoxyethanol Dihydrochloride

2,4-Diaminophenoxyethanol Dihydrochloride (2,4-DAPE) is an aromatic amine that is used as a coupler in permanent (oxidative) hair dyes at concentrations up to 1.0%.

2,4-DAPE was slightly toxic in rats and mice. No significant adverse changes were observed in a subchronic toxicity test. 2,4-DAPE was practically nonirritating when a 4% aqueous solution was instilled into the conjunctival sacs of the eyes of rabbits. 2,4-DAPE was slightly irritating to the skin of rabbits when tested at a concentration of 4.0%.

Based on animal studies, 2,4-DAPE was judged to produce low level sensitization in humans. Coal tar hair dyes, including those containing 2,4-DAPE, are exempt from the principal adulteration provision and the color additive provisions in Sections 601 and 706 of the Federal Food, Drug, and Cosmetic Act of 1938 when the label bears a caution statement and patch test instructions for determining whether the product causes skin irritation.

No teratogenic effects were observed due to administration of 2,4-DAPE. No mutagenic activity attributable to 2,4-DAPE was observed in 22 mutagenic studies. However, in a few studies, a marginal mutagenic response was reported.

In two oral carcinogenic studies, one in which mice were dosed with 0.04 and 0.07% 2,4-DAPE and the second in which rats were dosed with 0.05 and 1.0% 2,4-DAPE, no carcinogenic effects were produced.

On the basis of the data presented in this report, it is concluded that 2,4-Diaminophenoxyethanol Dihydrochloride is safe as a cosmetic ingredient in the present practices of use and concentration.

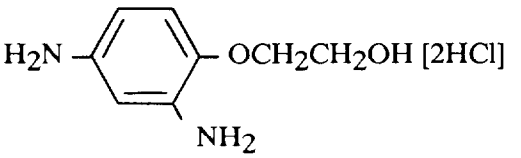
INTRODUCTION

2,4-Diaminophenoxyethanol Dihydrochloride is an aromatic amine used as a coupler mixed with primary intermediates in permanent (oxidative) hair dyes.[1]

CHEMISTRY

Definition and Structure

2,4-Diaminophenoxyethanol Dihydrochloride (CAS No. 66422-95-5) is the aromatic amine salt which conforms to the formula:[2]



2,4-Diaminophenoxyethanol Dihydrochloride

2,4-Diaminophenoxyethanol, Dihydrochloride (2,4-DAPE) is also know as 1-β-Hydroxyethyloxy-2,4-diamino-benzene, Dihydrochloride, (Diamino-2',4'-phenoxy)-2-ethanol, Dichlorhydrate, 2-(2',4'-Diaminophenoxy)ethanol, Dihydrochloride,[1] 1-(2-Hydroxyethyloxy)-2,4-diamino-benzene,[3] and Ethanol, 2-(2,4-Diaminophenoxy)-, Dihydrochloride.[2]

Properties

2,4-DAPE is an odorless white, slightly gray powder that is soluble in water and insoluble in acetone and 95% ethanol.[3] Physical and chemical properties of 2,4-DAPE are summarized in Table 1.

Impurities

1,3-Diaminobenzene, 2,4-diamino-1-methoxybenzene, and 2,4-diamino-1-ethoxy-benzene are not detected as impurities in 2,4-DAPE.[3]

TABLE 1. PROPERTIES OF 2,4-DIAMINOPHENOXYETHANOL HCl

Property	*Description*	*Reference*
Physical appearance	White powder, slightly gray	3
	Lavender-gray powder	4
Odor	None	3
Molecular weight	241	1
Empirical formula	$C_8H_{12}N_2O_2$ [2HCl]	2
Melting point	198-216°C	5
Free base		
Melting point	84°C	6
Solubility at 25°C	Water soluble	3
	95% ethanol insoluble	3
	Acetone insoluble	3
Spectrum absorbance	238 ± 5 - 0.630 ± 0.050	3
(λm - Absorbancy)	286 ± 5 - 0.260 ± 0.020	3
Decomposition point	198-217°C	1
Thermopan microscope		
Titer, potentiometry	≥ 99.5%	1
Assay, % Acid function	99 ± 1	3
Chloride concentration	28.0 - 31.0	3

USE

Cosmetic Use

The product formulation data submitted to the Food and Drug Administration[7] for 2,4-DAPE indicated that it is contained in 82 hair dye and coloring products at a concentration of ≤ 5%. Seventy-six of the formulations occur at a concentration of ≤ 0.1% (Table 2).

The FDA cosmetic product formulation computer printout[7] is compiled through voluntary filing of such data in accordance with Title 21 part 720.4 of the Code of Federal Regulations.[8] Ingredients are listed in preset concentration ranges under specific product type categories. Since certain cosmetic ingredients are supplied by the manufacturer at less than 100% concentration, the value reported by the cosmetic formulator may not necessarily reflect the actual concentration found in the finished product. The actual concentration would be a fraction of that reported to the FDA. Data submitted within the framework of preset concentration ranges provide the opportunity for overestimation of the actual concentration of an ingredient in a particular product. An entry at the lowest end of a concentration range is considered the same as one entered at the highest end of that range, thus introducing the possibility of a two- to ten-fold error in the assumed ingredient concentration.

When the amount of 2,4-DAPE added to an oxidative hair dye formulation is based on a desired shade, the concentrations are generally as follows: less than 0.025% in light tones, between 0.025 and 0.1% in medium tones, and between 0.1% and 4% in darker tones (which represent a smaller part of the market).[1] The maximum 4% concentration allowed for use is reduced to a final on-head concentration of 2% when combined with hydrogen peroxide on application.

The hair dyes containing 2,4-DAPE, as coal tar hair dye products, are exempt from the principal adulteration provision and from the color additive provision in sections 601 and 706 of the Federal Food, Drug, and Cosmetic Act of 1938 when their label bears a caution statement as well as patch test instructions to determine whether the product causes skin irritation.[9] The following caution statement should be displayed on all coal tar hair dye products.

> Caution—This product contains ingredients which may cause skin irritation on certain individuals and a preliminary test according to accompanying directions should be made. This product must not be used for dyeing the eyelashes or eyebrows; to do so may cause blindness.

TABLE 2. PRODUCT FORMULATION DATA FOR 2,4-DIAMINOPHENOXYETHANOL HCl[7]

Product category	*Total no. of formulations in category*	*Total no. containing ingredient*	*No. of product formulations within each concentration range (%)*		
			>1–5	*>0.1–1*	*≤0.1*
Hair Dyes and colors	1073	82	1	5	76
1989 Totals		82	1	5	76

Consumers are strongly urged to patch test for sensitivity 24 h prior to every application of the hair dye. Instructions are to apply a few drops of the hair dye with a cotton swab to a small area (the size of a quarter) behind either ear or inside the elbow. This area is to be left uncovered and undisturbed for 24 h and then evaluated for irritation.[10]

International

Diaminophenols are accepted for use by the European Economic Community,[11] providing their concentration does not exceed 10% free base. Diaminophenols can be used singly or in combination, provided that the sum of the ratios of the concentrations of each diaminophenol used in the cosmetic product expressed with reference to the maximum concentration authorized for each of them does not exceed 1.

The Japan Ministry of Health and Welfare has approved 2,4-Diamino Phenol Hydrochloride for quasidrug use only, and proper ingredient labeling is required.[12]

GENERAL BIOLOGY

Dermal Absorption

Female hairless Wistar rats were used to determine the penetration of ^{14}C-2,4-DAPE (^{14}C uniformly on the ring—specific activity: 0.8 μCi/mg).[13] The penetration of ^{14}C-2,4-DAPE was determined as pure compound and in a complete commercial formulation consisting of ^{14}C-2,4-DAPE (0.40%), ^{14}C-2,4-diaminoanisole (0.33%), *p*-phenyldiamine hyddrochloride (1.8%), resorcinol (0.05%), and *m*-aminophenol (0.1%). Both the individual compound and the formulation were dissolved in a vehicle containing nonionic and amphoteric surfactants, alcohols, glycols, oleic acid, copra diethanoamide, antioxidants and complexing agents, water, and 10% aqueous ammonia. Immediately before use, the solution was mixed with an equal volume of 20% hydrogen peroxide solution. The animals were anesthetized, and 20 mg/cm^2 of compound was applied to a 25 cm^2 area of the dorsal region. The length of time the compound remained in contact with the skin was 40 min. Following the exposure period, excess test material was removed, and a stripping process was carried out on the site of application to avoid contamination of the excrement. For 4 days, the feces and total amount of urine excreted were collected at 24-h intervals and analyzed. The animals were then killed and necropsied in order to determine the quantity of compound that had been absorbed and not excreted. In the majority of cases, the visceral organs, carcasses, skin (except for the site of application), and, in certain cases, an additional number of selected organs were examined for residual radioactivity. The quantity of compound that penetrated was 5.05 nM ± 0.79 nM (0.84 μ ± 0.13 μ) of pure compound per cm^2 of skin and 2.83 nM ± 0.49 nM (0.47 μ + 0.08 μ) of compound in the commercial formulation per cm^2 of skin. Penetration of 2,4-DAPE in the commercial formulation was 40% of the penetration of the pure compound alone. The study was then carried out applying the hair dye solutions every 30 to 40 days (duration of testing not given) to simulate human hair-dying frequency. The livers and thyroids of the treated rats were examined for accumulation of test article. At the highest doses, with the animals being killed 4 days after treatment, no radioactivity appeared in the thyroid, and only trace amounts appeared in the liver.

Female hairless Wistar rats were used to measure the absorption of ^{14}C-2,4-DAPE dissolved in a commercial vehicle at concentrations of 0.40% (23.65 nM), 0.80% (47.30 nM), and 1.20% (70.95 nM).[14] The solution was mixed with an equal volume of 20% H_2O_2 before use. Twenty mg/cm^2 were applied to a 25 cm^2 area on the back of each rat, 6 rats per group, for a period of 40 min. Urine and feces were collected for 4 days after treatment, and the animals were then killed and necropsied to determine the amount of compound absorbed and not yet excreted. The penetration per cm^2 was between 5.03 nM ± 0.79 nM (0.84 μ ± 0.13 μ) for the lowest concentration and 9.42 nM ± 0.84 nM (1.58 μ ± 0.14 μ) for the highest concentration.

ANIMAL TOXICOLOGY

Acute Toxicity

Oral

Twenty albino Swiss mice (10 males and 10 females), approximate weight 20 g each, and 20 albino Wistar rats (10 males and 10 females), approximate weight 200 g each, were orally administered a 1 ml/100 g volume of solution containing 2,4-DAPE in the chlorhydrate form by gavage.[15] The animals were fasted for 12 h and then allowed to eat and drink normally for 2 h before dosing. Dose concentrations were calculated in geometric progression. The animals were observed for 3 h following administration and during the following week. Tearing, agitation then calm, piloerection, vasoconstriction, ptosis, discolored urine, difficult breathing, salivation, and convulsions were observed in some mice. Salivation, tearing, piloerection, loss of grip reflexes, difficult breathing, shaking, bloody snouts, vasodilation, diarrhea, discolored urine, and convulsions were observed in some rats. The oral LD_{50}, calculated using the method of Litchfield and Wilcoxon,[16] was 1760 mg/kg (confidence limits of 1595–1950 mg/kg) for male mice, 1739 mg/kg (confidence limits of 1356–2232 mg/kg) for female mice, 1745 mg/kg (confidence limits of 1539–1980 mg/kg) for male and female mice, 1191 mg/kg (confidence limits of 1075–1321 mg/kg) for male rats, 1040 mg/kg (confidence limits of 883–1225 mg/kg) for female rats, and 1113 mg/kg (confidence limits of 1037–1194 mg/kg) for male and female rats.

Six groups of 10 albino male Swiss mice, 25 to 30 g, were administered 2,4-DAPE by gavage.[17] The animals received 10 ml/kg of compound, either dissolved in water or suspended in methocel at 0.5% (pH nonmodified), at concentrations of 630, 790, 1000, 1580, 2000, or 2500 mg/kg. The mice were then observed for 14 days. The oral LD_{50} was calculated[16] and determined, as an average, to be 1160 mg/kg (95% confidence limits of 850–2100 mg/kg).

Subchronic Toxicity

Oral

Six groups of BDF_1 mice and six groups of F344 rats, 10 males and 10 females per group, were administered 2,4-DAPE at concentrations of 0, 0.01, 0.03, 0.05, 0.1, and 0.2% in tap water, *ad libitum,* for a period of 12 weeks.[18] No clinical signs were observed during treatment. The survival rate was 90% for the male mice in the 0.1 and 0.2% dose groups, 100% for all remaining mice, and 100% for all rats. Two mice that

had greatly reduced body weights died while on study. Their deaths were attributed to malnutrition due to inability to drink water. Both were necropsied, and atrophy of various organs was observed. In the surviving mice, males in the 0.1 and 0.2% dose groups had reduced body weight gains. Females displayed satisfactory growth throughout the course of the experiment. Feed consumption was reduced in these two dose groups for both male and female mice. For the rats, there was a dose-dependent decrease in mean body weight gain for all treated groups; feed consumption was reduced at the 0.2% concentration. Water intake was reduced for all treatment groups, both mice and rats, when compared to the control group values. Tissue specimens from 3 males and 3 females from each group, both mice and rats, were evaluated microscopically. Abnormalities of the kidneys were found in 2 mice, lesions of pneumonia were found in 7 mice, and pigment deposits were observed in the epithelial cells of thyroid follicles in a mouse from the 0.2% group. All male and female rats in the 0.2% dose group had pigment deposits in the epithelial cells of the thyroid follicles. There was no evidence of silver or iron in the deposits in either the mice or rats.

2,4-DAPE in a 5% Tween suspension was administered by oral intubation to 20 Sprague-Dawley rats (10 males and 10 females) for a period of 3 months.[19] The dose was 56 mg/kg/day (1/20 LD_{50}) at a volume of 10 ml/kg/day. A control group of 20 rats (10 males and 10 females) received vehicle alone. Clinical observations included a dull appearance of the pelage and light brown aureolas, pelage being soiled with urine, and a brown discoloration of the urine. Body weight gain of the treated group was slightly reduced, but the difference, as compared to the controls, was not statistically significant. At necropsy, a brown discoloration of the thyroid and of the trachea at the level of the thyroid was due to the hair dye. The results of histological and clinical examination of the treated animals were normal, with the exception of an increased serum glutamic-oxaloacetic transaminase (SGOT) activity and a slight increase in serum glutamic-pyruvic transaminase (SGPT) activity, alkaline phosphatase activity, and uric acid values. However, the SGPT, alkaline phosphatase, and uric acid values were within the normal limits. The significance of the increased SGOT value was relative in comparison to the control group, and it also did not exceed the normal limits of variation. The only mortality reported was the accidental death of 1 animal while on study.

Ocular Irritation

A volume of 0.1 ml of a 4% aqueous solution (pH 2.5) of 2,4-DAPE was instilled into the conjunctival sac of one eye of 6 albino Bouscat rabbits (3 males and 3 females) and was not rinsed after administration. The other eye was untreated and served as a control.[20] This solution was considered "practically not irritating" to the eyes of rabbits, with the ocular irritation index estimated to be 1.66/110 after 24 h, 0.33/110 after 48 h, and 0/110 after 72 h, 4 days, and 7 days.

Dermal Irritation

2,4-DAPE was applied to the shaved intact and abraded skin of 6 albino Bouscat rabbits (3 males and 3 females) as a 4% solution in distilled water (pH 8.5).[21] Each animal received 5 ml of solution. This solution was determined to be "slightly irritating" to rabbit skin with a primary irritation index of 0.08/8.

Sensitization

Ten female Hartley guinea pigs were used to determine the sensitizing potential of 2,4-DAPE following a modified Magnusson and Kligman technique.[22] Before administration of the compound, two 0.2 ml injections of 50% Freund's adjuvant were administered intradermally to the site of application. The compound was moistened with a few drops of distilled water for better adherence and applied epicutaneously to a 3 cm^2 area of deeply abraded skin. The test site was covered with an occlusive patch for 48 h. On day 7, a second epicutaneous application of 25% test article in petroleum jelly was administered, and an occlusive patch was applied for 48 h. On day 21, a challenge was performed by applying 25% 2,4-DAPE in petroleum jelly to a 5 cm^2 shaved, previously untreated area of skin. This area was covered with an occlusive patch for 24 h. Five female nonsensitized guinea pigs also were treated with a 25% application of 2,4-DAPE in petroleum jelly, and the site was covered with an occlusive patch for 24 h. This group served as the control group. The excess test substance was removed after patch removal, and sensitization readings were taken 48 and 72 h after the challenge application. Erythema was observed in 3 of the 10 animals, all of which recovered within 5 days. The authors suggested, based on the results of this study, that 2,4-DAPE could have a low sensitizing potential in humans.

Reproductive Effects/Teratogenicity

A dose range-finding study was conducted to determine the concentrations of 2,4-DAPE to be used in a teratology study. 2,4-DAPE was administered by intragastric intubation on days 1 to 10, inclusive, of pregnancy to four groups (6 rats per group) of pregnant specific pathogen-free rats.[23] The dose concentrations were 0, 125, 250, and 500 mg/kg/day, and the dose volumes were calculated on days 1, 4, and 7 to adjust for change in body weight. Control animals received vehicle, distilled water, only. Clinical signs in the 500 mg/kg dose group included severely increased postdose salivation, elevated gait, stained coats, fur loss in 1 animal, and discolored urine. Similar signs were observed in the 250 mg/kg dose group, with the exceptions that they had a later onset, were not as severe, less animals were affected, and more animals exhibited fur loss. At the 125 mg/kg dose, the only signs observed were slight or moderate postdose salivation and discolored urine. Feed consumption was reduced on days 1 to 3 of dosing in the 250 and 500 mg/kg dose groups and continued throughout dosing in the 500 mg/kg group. On days 1 to 4 of dosing, there was a weight loss in the high-dose group, retarded weight gain in the middose group, and minimal retardation of weight gain in the low-dose group. During days 4 to 11, weight gains were reduced in the 250 and 500 mg/kg dose groups, and, despite some recovery, weight gain was reduced in the postdose period when compared to controls. Animals were killed on day 15, and a necropsy was performed. Except for discolored fur on 4 animals, no compound-related changes were observed.

Based on the results of the previous preliminary study performed by Bottomley et al.,[23] dose concentrations of 0, 50, 100, and 200 mg/kg/day were chosen for a complete teratology study.[23] 2,4-DAPE was administered by gavage to four groups (20 per group) of pregnant Specific Pathogen-Free rats on days 6 to 15, inclusive, of pregnancy. The controls received vehicle, distilled water, only. Each rat received 10 ml/kg of solution, and the dose volume administered was calculated on days 6, 10, and 14 to adjust for changes in body weight. Clinical signs included increased postdose

salivation and discolored urine in all dose groups and fur loss in the later stages of dosing and in the postdose period in the 200 mg/kg dose group. Body weight gain was reduced at the 100 mg/kg dose and even more at the 200 mg/kg dose. On day 20 of pregnancy, the dams were killed, and the litters were examined. With the exception of a discoloring of or loss of fur for some animals, no dose-related changes were observed at necropsy. No statistically significant changes in litter size were observed. Marginal reductions in litter size were evident in the 100 and 200 mg/kg dose groups, but were considered to be unrelated to treatment. No statistically significant differences were observed in litter and fetal mean weight values. However, lower litter and fetal mean weight values in the 200 mg/kg dose group were considered to be treatment-related due to maternal effect and effects on skeletal development. In the 200 mg/kg dose groups, there was a significant dose-related increase in the incidence of skeletal anomalies and skeletal variants. This increase was thought to be "probably related to a general nonspecific retardation of embryo/fetal development during gestation." The incidence of major malformations and minor visceral anomalies was comparable among all groups.

Pregnant C57B1/6 mice (crossed with T stock males) were used to determine the teratogenic potential of 2,4-DAPE dissolved in corn oil.[24] There were 10 animals in the low and middose groups and 18 animals in the high-dose group. The solution was topically administered to a shaved area on the back of each mouse. The volume applied was 0.2 ml at concentrations of 15, 150, or 1500 mg/kg. Negative and positive control tests were performed. Sixteen negative control animals received dermal applications of corn oil only, and 19 positive control animals were administered benzo[a]pyrene on day 10.5 of pregnancy by intraperitoneal injection. Following fetal evaluation, "no teratogenic effect" nor "any significant difference in skeletal development" was found when comparing the dose groups to the negative controls.

MUTAGENICITY

In Vitro

Mutagenicity study results are summarized in Table 3.

Chinese hamster ovary (CHO) cells were used to evaluate the mutagenic potential of 2,4-DAPE at concentrations of 0.6 and 1.2 mg/ml.[25] The tests were performed with and without metabolic activation. A positive control using 2,4-diaminoanisole dihydrochloride (2,4-DAA) was included in the study. The results obtained did not give any evidence of increase in chromosomal aberration due to 2,4-DAPE either with or without metabolic activation.

Escherichia coli strains WP2, WP2uvrA, and WP2uvrA/recA were used to test for the genotoxic potential of 2,4-DAPE.[26] Concentrations of 30, 75, 189, 754, and 2000 μg/plate were tested both with and without metabolic activation by S9 mix. An *E. coli* reversion test was performed using strains WP2 and WP2uvrA. A modified Ames test was performed in which the soft top agar contained 0.25 μg/ml of L-tryptophan instead of the histidine/biotin mixture. No increase in revertants was found with or without metabolic activation. Strains WP2, WP2uvrA, and WP2uvrA/recA were used in a DNA damage/repair test. A modified Ames test similar to the test by Darroudi et al.[25] was performed, with the exceptions that the L-tryptophan concentration was increased to 1 μg/ml, and the test solution (0.1 ml) was pipetted into a hole (1 cm in diameter) cut into the center of each plate rather than being incorporated into the soft agar. There was no

TABLE 3. 2,4-DIAMINOPHENOXYETHANOL HCl MUTAGENICITY STUDIES

Test	*Organism*	*Strain*	*Method*	*Results*	*Reference*
Chromosomal aberration test	Chinese hamster ovary cells	—	Concentrations of 0.6 and 1.2 mg/ml tested in the presence and absence of metabolic activation	Negative	25
Escherichia coli reversion test	*E. coli*	WP2, WP2uvrA	Modified Ames test in which the soft top agar contained 0.25 μg/ml of L-tryptophan instead of the histidine/ biotin mixture. Concentrations of 30, 75, 189, 754, and 2000 μg/plate were tested in the presence and absence of metabolic activation	Negative	26
DNA damage/repair test	*E. coli*	WP2, WP2uvrA, WP2uvrA/recA	Modified Ames test in which the soft top agar contained 1 μg/ml of L-tryptophan instead of the histidine/ biotin mixture. 0.1 ml of test solution was placed in a hole in the center of the plate instead of being incorporated in the agar and tested in the presence and absence of metabolic activation	Negative	26
Chromosomal aberration test	Human lymphocytes	—	Concentrations of 10^{-3}, 10^{-4}, and 10^{-5} tested in medium with a fixation time of 1 h	Negative	6
Reverse mutation assay	*S. typhimurium*	TA1538, TA98	Ames test at concentrations of 0, 0.65, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/plate tested in the presence and absence of metabolic activation promoted by phenobarbitol + β-naphthoflavone S9 mix	Negative	27

TABLE 3. *Continued*

Test	*Organism*	*Strain*	*Method*	*Results*	*Reference*
Reverse mutation assay	*S. typhimurium*	TA1538	Ames test at concentrations of 0, 1, 10, 50, 100, 500, and 1000 μg/plate tested in the presence and absence of metabolic activation promoted by Aroclor 1254 S9 mix	Negative	27
Forward mutation assay	*Schizosaccharomyces pombe*	SP ade-60/rad10-198h$^-$	Cells treated with concentrations of 0, 10, 15, 20, 30, and 40 mM tested in the presence and absence of metabolic activation promoted by phenobarbitol + β-naphthoflavone S9 mix	Negative	27
Forward mutation assay	Chinese hamster ovary cells	V79	6-Thioguanine-resistant mutant colonies were scored using concentrations of 0, 5, and 20 mM in the presence and absence of metabolic activation promoted by phenobarbitol + β-naphthoflavone S9 mix	Negative	27
Mitotic gene conversion assay	*Saccharomyces cerevisiae*	D4, Genotype α/a; ga12/+; ade2-2/ade2-1; trp5-12/trp5-27; leu1/+	Treated during growth with concentrations of 0, 10, 20, and 40 mM in the presence and absence of metabolic activation promoted by phenobarbitol + β-naphthoflavone S9 mix	Negative	27
Unscheduled DNA synthesis test	HeLa cells	Human cells obtained from a cervical carcinoma	Cells were exposed to concentrations of 0, 0.02, 0.06, and 0.2 mM	Negative	27

Ames test	*S. typhimurium*	TA1538, TA98	Concentrations of 0, 60, 120, 300, 600, and 1200 μg/plate tested in the presence and absence of metabolic activation	Negative	28
Ames test	*S. typhimurium*	TA1537, TA1538, TA98, TA1535, TA100	Concentrations of 5, 10, 20, 50, 100, 250, 500, and 1000 μg/plate tested in the presence and absence of metabolic activation	Negative	29
Ames test	*S. typhimurium*	TA1535, TA100, TA1538, TA1537, TA98	Concentrations of 5, 10, 20, 50, 100, 250, 500, and 1000 μg/plate tested in the presence and absence of metabolic activation	Negative	30
Gene reversion test	*Saccharomyces cerevisiae*	XV185-14C	Concentrations of 1000, 2000, 3000, 4000, 5000, and 6000 μg/ml tested in the presence and absence of metabolic activation following the methods of Shahin and von Borstel[31,32]	Negative	30
Gene conversion test	*Saccharomyces cervisiae*	D4	Concentrations of 100, 250, 500, 1000, 1500, 2000, and 4000 μg/ml tested in the presence and absence of metabolic activation, except 100 μg/ml was only tested without metabolic activation and 4000 μg/ml was only tested with it	Negative	30
Ames test	*S. typhimurium*	TA1535, TA1537, TA1538, TA100, TA98	A concentration range of 5–1000 μg/plate, 3 plates/dose, was tested in the presence and absence of metabolic activation	Negative	5

TABLE 3. *Continued*

Test	*Organism*	*Strain*	*Method*	*Results*	*Reference*
Ames test	*S. typhimurium* *E. coli*	TA100 WP2uvrA (pKM101)	A concentration range of 5–100 μg/plate, 3 plates/dose, was tested in the presence and absence of metabolic activation induced by 0, 4, 10, and 30% S9 mix	Negative	33
Ames test	*S. typhimurium*	TA1538, TA97, TA98	A concentration range of 5–100 μg/plate, 3 plates/dose, was tested in the presence and absence of metabolic activation induced by 0, 4, 10, and 30% S9 mix	Positive in the presence of at least 10% S9 mix	33
Ames test	*S. typhimurium*	TA1538, TA98	Concentrations of 5–100 μg/plate were tested using 30% Aroclor 1254 S9 mix	10-fold (TA1538) and 14-fold (TA98) increases at 80 μg/ml	33
Fluctuation test	*S. typhimurium*	TA1538, TA98	20 μg of an overnight Lab-M nutrient broth No. 2 shake culture, a known amt. of 2,4-DAPE, and 3 ml of 2% S9 mix added to 12 ml of Vogel-Bonner salts mix medium. Tests also run by substituting the S9 mix with 3 ml Vogel-Bonner medium. After 18 h, 1 ml supplemented Vogel-Bonner medium was added, and it was incubated for 3 days	Positive for both strains in the presence of S9 and negative for both in the absence of S9	33

Sex-linked recessive test	*Drosophila melanogaster*	Berlin K males In(1) sc^{SIL} sc^{8R}+S, sc^{S1} sc^{8} w^{a} B females (Basc)	1-2-day-old males received 2,4-DAPE in m/30 phosphate buffer via the adult feeding method[37] for 3 days. They were then mated to 3-5-day-old females. Sex-linked recessive lethals were scored in the F_2 generation, with all suspected lethals being retested	Negative	36
Mouse dominant-lethal assay	Mouse	T-strain males C57B1/6 females	Males received dermal applications at concentration range of 15-1500 mg/kg and a volume no greater than 0.5 ml/day for 5 days and were then placed with virgin females for 7-day intervals. Fourteen days after mid-week of mating, the females were killed and their uteri examined	Negative	4
Micronucleus test	Mouse	CD-1	Mice received two 24 h apart oral doses of 2,4-DAPE in distilled water at a volume of 0.1 ml/10 g body weight and concentrations of 25, 50, and 100 μg/ml. Animals were killed 6 h after second dose, and direct bone marrow smears were made and examined to determine the presence of micronucleated cells in 2000 polychromatic erythrocytes per animal, and the ratio of normchromatic to polychromatic erythrocytes was determined	No increase in micronucleated cells. Ratio of normchromatic to polychromatic erythrocytes was significantly reduced	39

TABLE 3. *Continued*

Test	*Organism*	*Strain*	*Method*	*Results*	*Reference*
Mouse spot test for somatic mutation	Mouse	T-strain males C57B1/6 females	Animals were mated. On days 8, 9, and 10 of gestation, the females received dermal applications at concentrations ranging from 15 to 1500 mg/kg. Newborns were scored for nonwhite spots on days 12 and 24 of lactation	Negative	4
Plate microbial assay	Mouse *S. typhimurium*	CD-1 TA1535, TA1537, TA98, TA100	Animals received dermal applications at concentrations ranging from 15 to 1500 mg/kg for 3 days at a volume no greater than 0.5 ml/day. Urine was collected for a 16 h period, and the bacteria received either 0.1, 0.2, 0.3 ml, or deconjugated urine. A standard Ames test was performed on the TA1538 and TA98 strains	Negative	4
Salmonella/ microsome test	Rat *S. typhimurium*	Wistar TA1538, TA98, TA100	Male rats were either dermally administered 120 mg 2,4-DAPE in 4 ml phosphate buffer for 20 min, orally administered 10 mg/kg 2,4-DAPE in distilled water, or intraperitoneally administered 100 mg/kg in 0.9% NaCl. 100, 200, or 300 μg/plate were used	Negative	29

indication of differential damage. A positive and negative control, 0.5 μg of 2-aminoanthracene and 200 μl of phosphate buffer, respectively, were included. *Salmonella typhimurium* strain TA1535 was included in the test because the *E. coli* strain WP2uvrA does not significantly react with such a low dose of 2-aminoanthracene.

A chromosomal aberration test using human lymphocytes was completed. 2,4-DAPE was administered at concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M in the medium.[6] The fixation time was 24 h, and the results obtained were negative.

Assays performed using 2,4-DAPE to determine its metabolic activity included: reverse mutation assays using *S. typhimurium* strains TA1538 and TA98, forward mutation assays using the *Schizosaccharomyces pombe* strain SP ade6-60/rad10-198h$^-$ and using the V79 cell line of Chinese hamsters, mitotic gene conversion assays using the yeast *Saccharomyces cerevisiae* strain D4, genotype α/a; gal2/+; ade2-2/ade2-1; trp5-12/trp5-27; leu1/+, and an unscheduled DNA synthesis (UDS) test using the HeLa human cell line from a cervical carcinoma.[27] Methyl methanesulfonate, ethyl methanesulfonate, cyclophosphamide, hycanthone, N-nitrosodimethylamine, and 2,4-DAA were used as positive controls. All tests were performed with and without metabolic activation provided by Aroclor-1254-treated or phenobarbital + β-naphthoflavone-treated S9 mix for the reverse mutation assays and using phenobarbital + β-naphthoflavone-treated S9 mix for all remaining assays. An Ames test was performed, with and without Aroclor-treated rat liver S9 mix, using *S. typhimurium* strain TA1538 at concentrations of 0, 1, 10, 50, 100, 500, and 1000 μg/plate. Concentrations of 0, 0.65, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/plate were administered to strains TA1538 and TA98 in the presence of phenobarbital + β-naphthoflavone-treated S9 mix. No positive results were observed using 2,4-DAPE. In the forward mutation assay using *S. pombe*, the cells were treated with 2,4-DAPE at concentrations of 0, 10, 15, 20, 30, and 40 mM. The forward mutation assay using the Chinese hamster V79 cell line was done by scoring 6-thioguanine-resistant mutant colonies using concentrations of 0, 5, and 20 mM. Negative results were obtained for both forward mutation assays. The *S. cerevisiae* cell suspensions were treated during growth in the same manner as *S. pombe* and were exposed to 2,4-DAPE at concentrations of 0, 10, 20, and 40 mM. The HeLa cells used in the UDS test were exposed to 2,4-DAPE at concentrations of 0, 0.02, 0.06, and 0.2 mM. Results were negative for both tests.

S. typhimurium strains TA1538 and TA98 were used to test for mutagenic activity of 2,4-DAPE both with and without metabolic activation.[28] Positive controls, used successfully, were 4-nitro-*o*-phenylenediamine (NOPD) and 2,4-DAA. 2,4-DAPE was tested at concentrations of 0, 60, 120, 300, 600, and 1200 μg/plate. Negative mutagenic results were obtained both with and without metabolic activation.

An Ames test was performed using five *S. typhimurium* strains. The results are an average of two independent experiments, each using three plates/dose.[29] The tests were run in the presence and absence of metabolic activation by S9 mix. Two positive controls, 1,2-diamino-4-nitrobenzene and 2-aminoanthracene, also were used. *S. typhimurium* strains TA1537, TA1538, TA98, TA1535, and TA100 were administered compound at concentrations of 5, 10, 20, 50, 100, 250, 500, and 1000 μg/plate. No mutagenic activity due to 2,4-DAPE was observed.

Mutagenic activity of 2,4-DAPE was determined using *S. typhimurium* strains TA1535, TA100, TA1537, TA1538, and TA98 and the yeast *S. cerevisiae* strains D4 and XV185-14C.[30] The tests were carried out with and without metabolic activation by S9

mix. An Ames test was performed using all five *S. typhimurium* strains at concentrations of 5, 10, 20, 50, 100, 250, 500, and 1000 μg/plate, three plates per dose. No mutagenic activity was observed. These results are averages of two independent experiments that used three plates each. The yeast *S. cerevisiae* strain D4 was treated with compound at concentrations of 100 (only without metabolic activation), 250, 500, 1000, 1500, 2000, and 4000 (only with metabolic activation) μg/ml. The *S. cerevisiae* strain XV185-14C was treated with 2,4-DAPE at concentrations of 1000, 2000, 3000, 4000, 5000, and 6000 μg/ml and followed the test methods of Shahin and von Borstel.[31,32] No mutagenic activity was detected in either yeast strain. Positive controls were run with all tests using 1,2-diamino-4-nitrobenzene and 2-aminoanthracene.

Salmonella typhimurium strains TA1535, TA1537, TA1538, TA100, and TA98 were used to test the mutagenic activity of 2,4-DAPE both with and without metabolic activation.[5] The bacteria were exposed to 2,4-DAPE at concentrations ranging from 5 to 1000 μg/plate. Three plates were used at each dose. A positive and negative control also were used. The control compounds were not named. 2,4-DAPE did not exhibit mutagenic activity either with or without metabolic activation.

The only positive results were obtained by Venitt et al.[33] when *S. typhimurium* strains TA1538, TA97, TA98, and TA100 and *E. coli* strain WP2uvrA (pKM101) were used to test for mutagenic potential of aqueous 2,4-DAPE. Three plates were run per dose using 0, 4, 10, and 30% Aroclor 1254-induced S9 mix. A positive control, 2,4-DAA, was used. An Ames test was performed on all strains at a concentration range of 5 to 100 μg/plate. Negative results were obtained with *S. typhimurium* TA100 and *E. coli* WP2uvrA (pKM101) at all dose concentrations and with all S9 mixes. A statistically significant increase in the number of *his*$^+$ revertants was obtained with *S. typhimurium* strains TA1538, TA97, and TA98 when in the presence of 10% S9 mix. The number of 2,4-DAPE-induced revertants was greater than 3.5 times the background concentration using 20 to 40% S9 mix. The number of induced revertants also was affected by the amount of NADP in the S9 mix. The number of *his*$^+$ revertants corresponded to the amount of NADP per plate. In the second set of tests using 30% S9 mix with strains TA1538 and TA98, 10-fold and 14-fold increases, respectively, were observed at 80 μg/plate. A commercial sample of 2,4-DAPE was tested before and after purification, and the unpure sample was much more active than the pure sample.

Fluctuation tests, using *S. typhimurium* strains TA1538 and TA98, were performed by adding 20 μg of an overnight Lab-M nutrient broth No. 2 shake culture of the appropriate organism, a known amount of 2,4-DAPE, and 3 ml of 2% S9 mix to 12 ml of Vogel-Bonner salts medium containing 1% glucose, 10 μg/ml biotin, and 1.5 μg/ml histidine.[33] The tests also were performed by substituting the S9 mix with 3 ml of Vogel-Bonner medium. After 18 h of incubation, 1 ml of Vogel-Bonner medium supplemented with 1% glucose and 10 μg/ml bromcresol purple was added. The wells were then incubated for another 3 days. In the presence of S9 mix, statistically significant dose-related increases in the number of positive wells were observed for both strains. Negative results were obtained in the absence of S9 mix.

A commentary paper submitted by a number of researchers regarding the previous study by Venitt et al.[33] states that the results obtained do not significantly alter the conclusions reached by a collaborative study,[34] since the findings from the collaborative study are a result of a battery of tests, whereas Venitt's results are based on a single test.[35] This battery of tests involved a number of reputable laboratories, and no mutagenic activity of 2,4-DAPE was observed.

In Vivo

Mutagenic activity of 2,4-DAPE was evaluated by performing a sex-linked recessive lethal test using *Drosophila melanogaster*.[36] At 25°C, 2,4-DAPE was dissolved in m/30 phosphate buffer (pH 6.8) and fed by the adult feeding method[37] to 1- to-2-day-old Berlin K males for 3 days. These males were then mated individually to 3- to-5-day-old virgin In(1) sc^{SIL} sc^{8R} + S, sc^{S1} sc^{8} w^{a} B females (Basc). The mating scheme used was one 3-day brood followed by two 2-day brood periods. At the end of each period, the treated male was transferred to a new vial and mated with more 3- to-5-day-old virgin females. Six-linked recessive lethals were scored in the F_2 generation using standard procedures,[37] and all suspected lethals were retested. No increase in mutation frequency was obtained from brood-fractionating experiments.

A micronucleus test[38] was performed using CD-1 mice.[39] Two doses of 2,4-DAPE were administered orally in sterile distilled water at concentrations of 25, 50, and 100 μg/ml per administration. The doses were 24 h apart and at a volume of 0.1 ml/10 g body weight. A negative control, vehicle, was administered orally, and a positive control, mitomycin C, was administered by intraperitoneal injection. After administration, ptosis, hypopnea, and lethargy were observed in all dose groups, and all animals excreted brown-pigmented urine. The animals were killed 6 h after administration of the second dose, and direct bone marrow smears were made.[40] These slides were examined to determine the presence of micronucleated cells in 2000 polychromatic erythrocytes per animal. The ratio of normochromatic to polychromatic erythrocytes also was determined. There was no increase in incidence of micronucleated cells in any dose group, but the ratio of normochromatic to polychromatic erythrocytes was significantly increased, indicating a toxic effect.

A mouse dominant-lethal assay was conducted after dermal administration of a suspension of 2,4-DAPE in corn oil to T-strain male mice.[4] Concentrations ranging from 15 to 1500 mg/kg, at a volume no greater than 0.5 ml/day, were applied to a shaved patch on the dorsal surface of the mouse. There may have been ingestion because the application site was not covered. Two mice per group received dermal applications of either the test compound or the control, corn oil, for 5 consecutive days. Another 2 received an intraperitoneal injection of the positive control, triethylene melamine (TEM), 2 days before mating. Two days after being dosed, each male was housed with 2 virgin C57B1/6 female mice for 7 days. These females were then replaced with 2 new virgin females. This sequence was repeated for 7 weeks. Fourteen days after the midweek of mating, the females were killed, and their were uteri examined for viable and nonviable fetuses, resorption sites, and total embryos. The dominant lethality results were negative. No significant results were observed with respect to the fertility index. After 6 weeks of mating, a fertility rate of only 30% was reported in the high dose. This was not considered compound-related because the indexes for weeks 5 and 7 were normal values. The average number of embryos per pregnant female also was not significantly different.

A mouse spot test for somatic mutation was performed using C57B1/6 female and T-strain male mice.[4] The animals were mated, and a minimum of 50 females with semen plugs were used per group. On days 8, 9, and 10 of gestation, the dose groups received dermal applications of 2,4-DAPE in corn oil at concentrations ranging from 15 to 1500 mg/kg. The negative control group received vehicle only. The application site was an uncovered shave patch on the dorsal surface of the mouse. The positive controls received a single 150 mg/kg intraperitoneal injection of benz[a]pyrene on gestation day

10. All animals were allowed to deliver, and the newborns were scored for nonwhite spots on days 12 and 24 of lactation. The high-dose group reported a coat color spot frequency of 1.9%; while, the negative control reported a frequency of 0%. The historical control frequency is between 1 and 2%, Therefore, these results were considered negative. There was no reduction in fertility, and there were no midventral white spots observed. The 2,4-DAPE solution was not systemically toxic or irritating to the skin of the dosed female.

In Vivo/In Vitro

2,4-DAPE was evaluated for genetic toxicity in a plate microbial assay developed by Durston and Ames[41] using urine collected from treated male CD-1 mice.[4] The test compound, suspended in corn oil, was applied to the skin for 3 days. Concentrations ranged from 15 to 1500 mg/kg. The volume was no greater than 0.5 ml/day. The application site was uncovered so there may have been ingestion of compound. A negative control, corn oil, and positive controls, Tris(2,3 Dibromo-propyl)PO4 and 2-acetylamino-fluorene, were used. Urine was collected for an approximately 16-h period, as it was excreted into containers that were being kept at 0 to 4°C. The collected urine was divided into three portions for testing. *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 were exposed to 0.1, 0.2, or 0.3 ml of urine or to deconjugated urine. Nonactivation tests also were performed by adding urine to the appropriate tubes and pouring it over the suface of selected agar plates. Slight increases were observed when the TA100 strain was exposed to treated urine, but these increases were not statistically significant. No positive results were obtained. A standard Ames test was performed using *S. typhimurium* strains TA1538 and TA98, and negative results were obtained.

A *Salmonella*/microsome test was performed using the urine of rats that were administered 2,4-DAPE in order to determine its mutagenicity potential.[29] *Salmonella typhimurium* strains TA1538, TA98, and TA100 were used. Male Wistar rats, 3 rats per group, were either topically, orally, or intraperitoneally administered 2,4-DAPE. Topical administration was made by applying 4 ml of phosphate buffer containing 120 mg of 2,4-DAPE to a 55.4 ± 8.7 cm^2 area of the back for 20 min. The compound was then removed by shampooing and thorough rinsing. One group of rats was given orally 10 ml of distilled water containing 100 mg/kg 2,4-DAPE, and another group received 10 ml intraperitoneal injections of 0.9% NaCl containing 100 mg/kg 2,4-DAPE. Negative controls using no urine or urine from rats given oral doses of 10 ml distilled water/kg were run. Positive controls were treated with 2,4-diaminoanisole. Urine was collected at −40°C for 24 h. The volume of urine that was used for each group was 100, 200, and 300 µl/plate. No mutagenic activity was detected.

CARCINOGENICITY

Three groups of BDF_1 mice, 50 males and 50 females per group, were used to determine the carcinogenic effect of 2,4-DAPE.[18] 2,4-DAPE was administered in tap water, *ad libitum*, at concentrations of 0, 0.04, and 0.07% for a period of 104 weeks. These doses were chosen by having first performed the subacute toxicity test on mice,[18] which is described earlier in this report. There were no significant differences observed in body weight, organ weight, or survival rate between treated and control

mice. At the termination of the study, gross and histopathological examinations were performed. There was no significant difference observed in target organs or tumor incidence when comparing the treated and control groups. Pigment deposits in epithelial cells of thyroid follicles, which were histochemically negative for silver and iron and were unrelated to tumor incidence, were observed in both treated groups. The chronic administration of 2,4-DAPE produced "no carcinogenic effect in mice."

Three groups of F344 rats, 50 males and 50 females per group, received 2,4-DAPE in tap water, *ad libitum*, at concentrations of 0, 0.05, and 0.1% for a period of 104 weeks.[18] The concentrations were determined by having first performed the subacute toxicity test on rats that was described earlier in this report. No dose was administered to the males in the 0.1% dose group during weeks 12 to 16 and to neither males nor females in that same dose group during weeks 32 to 36 due to a marked decrease in weight gain when compared to the controls. Mean body weight gains for both treated groups, males and females, were reduced when compared to the controls. There was no significant difference in survival rate between treated and control groups. After termination of treatment, necropsy and microscopic evaluation was performed on organs of all rats. There were no differences observed in the incidence or type of neoplasms between treated and control rats. Rats in the 0.1% dose group had pigment deposits in epithelial cells of thyroid follicles. These deposits were histochemically negative for silver and iron and were not related to the incidence of neoplasms. The researchers determined that, in rats, 2,4-DAPE produced no carcinogenic effect.

SUMMARY

2,4-DAPE is an aromatic amine that is an odorless white, slightly gray, or lavender gray powder. 1,3-Diaminobenzene, 2,4-diamino-1-methoxybenzene and 2,4-diamino-1-ethoxybenzene are not detected as impurities in 2,4-DAPE.

2,4-DAPE is used as a coupler mixed with primary intermediates in permanent (oxidative) hair dyes. In 1989, it was reported to the FDA as being used in 82 hair dye and color formulations at concentrations up to 5%, with the predominant concentration of use being $\leq 0.1\%$.

Coal tar hair dyes, including those containing 2,4-DAPE, are exempt from the principal adulteration provision and the color additive provisions in sections 601 and 706 of the Federal Food, Drug, and Cosmetic Act of 1938 when the label bears a caution statement and patch test instructions for determining whether the product causes skin irritation. The following caution statement should be displayed conspicuously on the label of coal tar hair dyes:

> Caution—This product contains ingredients which may cause skin irritation on certain individuals and a preliminary test according to accompanying directions should be made. This product must not be used for dyeing the eyelashes or eyebrows; to do so may cause blindness.

Dermal absorption of radioactive 2,4-DAPE, as a pure compound and in a formulation, was determined. Twenty mg/cm^2 of compound was applied; 5.05 nM $\pm$ 0.79 nM of pure compound/cm^2 and 2.83 nM $\pm$ 0.49 nM of compound in the complete formulation/cm^2 penetrated. In another dermal absorption study, solutions of radioactive 2,4-DAPE at concentrations of 0.40 to 1.20% were applied at a volume of 20

mg/cm^2. Penetration per cm^2 ranged from 5.03 nM ± 0.79 nM to 9.42 nM ± 0.84 nm depending on concentration.

The results of two acute toxicity studies found 2,4-DAPE to be slightly toxic in rats and mice according to the methods of Hodge and Sterner.[42] No clinical signs were observed in a subchronic toxicity test using concentrations of 2,4-DAPE ranging from 0.01 to 0.2% in tap water. In a subchronic study in which 56 mg/kg/day were administered in solution at a volume of 10 ml/kg/day, a dull appearance of the pelage and light brown aureolas, pelage being soiled with urine, and a brown discoloration of urine were observed. 2,4-DAPE was practically nonirritating when a 4% aqueous solution was instilled into the conjunctival sacs of the eyes of rabbits. 2,4-DAPE was slightly irritating to the skin of rabbits when a 4% solution was used. When evaluating the sensitizing potential of 2,4-DAPE, erythema was observed for 30% of the guinea pigs following the challenge. All of the animals recovered within 5 days. No teratogenic effects were observed due to administration of 2,4-DAPE.

In 22 mutagenicity studies, no mutagenic activity attributable to 2,4-DAPE was observed. In a few mutagenic assays, some mutagenic activity of 2,4-DAPE was observed.

In 2 oral carcinogenic studies, 1 in which tap water containing concentrations of 0.04 and 0.07% 2,4-DAPE was administered to mice for 104 weeks and the other in which tap water containing concentrations of 0.05 and 1.0% 2,4-DAPE was given to rats for 104 weeks, no carcinogenic effects were produced by 2,4-DAPE.

DISCUSSION

2,4-DAPE was slightly irritating to the skin of rabbits. In a sensitization study using guinea pigs, erythema was observed in 3 of 10 guinea pigs, all of which recovered in 5 days. Based on the results of this study, investigators suggested that 2,4-DAPE could have a low sensitizing potential in humans. The Expert Panel used these results, due to a lack of human data, in its safety assessment of 2,4-DAPE. Hair dyes containing 2,4-DAPE are exempt from the principal adulteration provision and from the color additive provisions in sections 601 and 706 of the Federal Food, Drug, and Cosmetic Act of 1938 when cautionary statements and patch test instructions are conspicuously displayed on the labels. Prophetic patch testing of hair dye formulations with open patches in less predictive of skin reactions than patch testing with closed patches. False negative reactions may occur. Some persons may be sensitized, even under the proper conditions of use.

CONCLUSION

On the basis of the data presented in this report, the CIR Expert Panel concludes that 2,4-Diaminophenoxyethanol Dihydrochloride is safe as a cosmetic ingredient in the present practices of use and concentration.

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