

## FINAL REPORT ON THE SAFETY ASSESSMENT OF HC YELLOW NO. 4<sup>1</sup>

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*HC Yellow No. 4 is a colorant for use mostly in hair dyes and colors, but also in a few hair tints. Concentrations at which the ingredient is used range from 0.1% to 1.0%. Confusion has existed regarding the proper structure for this ingredient, but was resolved through additional analysis; the correct CAS number is 59820-43-8. Commercially available HC Yellow No. 4 may contain a nitroaniline impurity. Percutaneous absorption studies using commercial products containing 1% HC Yellow No. 4 found little absorption. Body weight decreases were noted in short-term oral toxicity studies and in a subchronic oral toxicity study. HC Yellow No. 4 did not produce irritation, sensitization, or photosensitization in animal tests (primarily using guinea pigs). In some feeding studies, fetal toxicity was observed, but no such effect was found in other feeding studies. HC Yellow No. 4 was mutagenic in several assays, but no evidence of carcinogenesis was found in oral or dermal studies. Two repeated insult patch tests, totalling over 200 human volunteers, found no sensitization reactions. While there was concern expressed over the reproduction and developmental toxicity found in feeding studies, such adverse responses would not be expected from the use of this ingredient in hair coloring products because so little HC Yellow No. 4 is absorbed. The presence of a low level of nitroaniline derivative impurity (0.3 to 7%) is not considered to present a human health risk because the products containing HC Yellow No. 4 are used in a brief and discontinuous manner, followed by rinsing. On the basis of the available data, therefore, it is concluded that HC Yellow No. 4 is safe as a hair colorant in the present practices of use.*

HC Yellow No. 4 is a color additive that functions as a hair colorant in hair dyes and colors and hair tints (Wenninger and McEwen 1997). In the past, there has been confusion over the structure of HC Yellow No. 4 (NTP 1992). The second edition of the Cosmetic, Toiletry, and Fragrance Association (CTFA) Cosmetic Ingredient Dictionary (Estrin 1977) had a structure for HC Yellow No. 4 that had both hydroxyethyl groups on the amine and an assigned CAS No. of 52551-67-4; this structure was incorrect. Since the third edition of the CTFA Cosmetic Ingredient Dictionary (Estrin, Crosley, and Haynes 1982), the structure for HC Yellow No. 4, as determined through additional analysis, is correct and the assigned CAS No. is 59820-43-8.

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The data found in the published literature on HC Yellow No. 4 and identified by the CAS No. for the incorrect structure (52551-67-4) are presumed to pertain to what is currently known to be HC Yellow No. 4 (CTFA 1994). Studies summarized in this report in which HC Yellow No. 4 was identified with the CAS No. for the incorrect structure are distinguished by an asterisk, i.e., HC Yellow No. 4\*.

## CHEMISTRY

### Definition and Structure

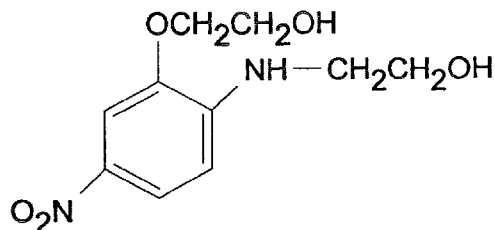
HC Yellow No. 4 (CAS No. 59820-43-8) is the hair colorant that conforms to the formula shown in Figure 1 (Wenninger and McEwen 1997). HC Yellow No. 4 is also known as 2-([2-(2-Hydroxyethoxy)-4-Nitrophenyl]Amino)Ethanol (Wenninger and McEwen 1997; Clairol 1995a; COLIPA 1995); Ethanol, 2-([2-(2-Hydroxyethoxy)-4-Nitrophenyl]Amino)-(Wenninger and McEwen 1997; RTECS 1994); *N,O*-Di(2-Hydroxyethyl)-2-Amino-5-Nitrophenol (NTP 1992; IARC 1993; Wenninger and McEwen 1997; RTECS 1994); 1-Nitro-3-(2-Hydroxyethyl)-Oxy-4-(2-Hydroxyethyl)Aminobenzene (Clairol 1995a; COLIPA 1995); 2-[3-Nitro-6-(*beta*-Hydroxyethylamino)Phenoxy]Ethanol (IARC 1993); and HC Yellow 4 (IARC 1993; RTECS 1994).

### Physical and Chemical Properties

The physical and chemical properties of HC Yellow No. 4 are described in Table 1.

### Manufacture and Production

The manufacturing process for HC Yellow No. 4 involves the treatment of 2-amino-5-nitrophenol with 2-chloroethanol in the presence of base (Clairol 1995a).



**Figure 1.** Chemical formula for HC Yellow No. 4.

**Table 1.** Physical and chemical properties of HC Yellow No. 4

Properties		Reference
Physical appearance	Fluffy yellow powder	NTP 1992
	Yellow-orange crystalline material	COLIPA 1995
Molecular weight	242.2	NTP 1992
Melting point	145–147°C	NTP 1992
	143–145°C	COLIPA 1995
Solubility	Partially soluble in water and in ethanol	COLIPA 1995
Specifications	95% (min) HC Yellow No. 4; 2% (max) ash	Clairol 1995a

## Analytical Methods

HC Yellow No. 4 has been identified by infrared, ultraviolet (UV)/visible, and nuclear magnetic resonance spectroscopy (NTP 1992). Purity has been determined by weight loss on drying, Karl Fischer water analysis, thin-layer chromatography (TLC), high-performance liquid chromatography, UV/visible spectroscopy, titration, and elemental analysis.

## Chemical Reactivity/Stability

The stability of HC Yellow No. 4 was determined using TLC and comparing the spectrodensitometric quantitation, as well as visible and UV bands, of a hair color containing 1% HC Yellow No. 4 (Clairol 1995a, b). TLC was done before and 30 min after the addition of hydrogen peroxide. There was no change in HC Yellow No. 4 following a 30-min reaction with hydrogen peroxide, and there were no changes in the visible or UV bands between the samples.

## Impurities

The purity of commercially available HC Yellow No. 4 was  $\geq 93\%$ , with the largest impurity being *N*-(2-hydroxyethyl)-2-hydroxy-4-nitroaniline (0.3–7%) (NTP 1992).

## USE

### Cosmetic

HC Yellow No. 4 is reported to function as a hair colorant in hair dyes and colors and hair tints (Wenninger and McEwen 1997). The product

**Table 2.** Product formulation data

Product category	Total no. formulations in category	Total no. containing ingredient
Hair dyes and colors	1437	78
Hair tints	45	3
<b>1995 total</b>		<b>81</b>

*Source.* FDA, 1995.

formulation data submitted to the Food and Drug Administration (FDA) in 1995 reported that HC Yellow No. 4\* was used in 81 cosmetic formulations (Table 2).

Concentration of use values are no longer reported to the FDA by the cosmetic industry (FDA 1992). However, data submitted to the Cosmetic Ingredient Review (CIR) by CTFA state that HC Yellow No. 4 is used in semipermanent hair colors at a concentration of 0.5% and in direct hair dye at a concentration of 0.01–1.0% (CTFA 1995). Information submitted by Clairol (1995c) states the HC Yellow No. 4 is used in oxidative and semipermanent hair colors at an on-head concentration of  $\leq 3.0\%$ . The product formulation data submitted to the FDA in 1984 stated that HC Yellow No. 4 was used in 36 hair dye/color formulations that required caution statements and in two coloring hair rinses at concentrations of  $\leq 1\%$  (FDA 1984) (Table 3).

Hair coloring formulations are applied to or may come in contact with hair, skin (particularly at the scalp), eyes, and nails. Individuals dyeing their hair may use such formulations once every few weeks, whereas hairdressers may come in contact with products containing these ingredients several times a day. Under normal conditions of use, skin contact with hair dye is restricted to 30 minutes.

The hair dyes containing HC Yellow No. 4, as coal tar hair dye products, 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 the label bears a caution

**Table 3.** Concentration of use data

Product category	Concentration (percent)		Total
	0.1–1	0–0.1	
Hair dyes/colors (requiring caution statements)	21	15	36
Hair rinses (coloring)	0	2	2
<b>Total</b>	<b>21</b>	<b>17</b>	<b>38</b>

*Source.* FDA, 1984.

statement and patch test instructions for determining whether the product causes skin irritation. The following caution statement should be displayed conspicuously on the labels of coal tar hair dyes:

**Caution**—This product contains ingredients that 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 eyelashes or eyebrows; to do so may cause blindness.

At its February 11, 1992, meeting, the CIR Expert Panel issued the following policy statement on coal tar hair dye product labeling:

The Cosmetic Ingredient Review (CIR) Expert Panel has reviewed the cosmetic industry's current coal tar hair dye product labeling, which recommends that an open patch test be applied and evaluated by the beautician and/or consumer for sensitization 24 hours after application of the test material and prior to the use of a hair dye formulation.

Since the recommendation on the industry's adopted labeling establishes a procedure for individual user safety testing, it is most important that the recommended procedure be consistent with current medical practice.

There is a general consensus among dermatologists that screening patients for sensitization (allergic contact dermatitis) should be conducted by the procedures used by the North American Contact Dermatitis Group and the International Contact Dermatitis Group (North American Contact Dermatitis Group 1980; Eiermann et al. 1982; Adams et al. 1985). These procedures state that the test material should be applied at an acceptable concentration to the patient, covered with an appropriate occlusive patch, and evaluated for sensitization 48 and 72 hours after application. The CIR Expert Panel has cited the results of studies conducted by both the North American Contact Dermatitis Group and the International Contact Dermatitis Group in its safety evaluation reports on cosmetic ingredients (Elder 1985).

During the August 26–27, 1991, public meeting of the CIR Expert Panel, all members agreed that the cosmetic industry should change its recommendation for the evaluation of the open patch test from 24 hours to 48 hours after application of the test material.

The industry was advised of this recommendation and asked to provide any compelling reasons why this recommendation should not be made by the Expert Panel and adopted by the cosmetic industry. No opposition to this recommendation was received. At the February 11, 1992, public meeting of the CIR Expert Panel, this policy statement was adopted.

## International

Clairol (1995c) reported that HC Yellow No. 4 is used in oxidative and semipermanent hair colors in Europe at an on-head concentration of  $\leq 3\%$ .

## Noncosmetic

Published data on the noncosmetic use of HC Yellow No. 4 were not found.

## GENERAL BIOLOGY

### Percutaneous Absorption

The percutaneous absorption of a commercial semipermanent dye base containing 1%  $^{14}\text{C}$ -HC Yellow No. 4 was determined using female cadaver abdominal skin (Clairol 1995d). A skin integrity test was first performed and only samples having a  $^3\text{H}_2\text{O}$  water flux  $<1.5 \text{ mg/cm}^2/\text{h}$  were used. After a 30-minute rinse-out period, a mean applied dose of  $11.1 \text{ mg/cm}^2$  of the formulation, corresponding to  $111 \text{ }\mu\text{g/cm}^2$  of dye, was placed on the skin for 30 minutes. Samples were withdrawn at intervals up to 48 hours after removal of the dye base; following the 48-hour sample, the skin was solubilized and counted using a Packard TR 1600 scintillation counter. The mean cumulative absorption of the applied dose was  $0.12 \pm 0.03\%$  and  $0.15 \pm 0.03\%$  after 24 and 48 hours, respectively. The mean cumulative flux was  $0.11 \pm 0.03 \text{ }\mu\text{g/cm}^2$  and  $0.15 \pm 0.03 \text{ }\mu\text{g/cm}^2$  at 24 and 48 hours, respectively. Mass balance determinations indicated the  $>95\%$  of the applied radioactivity was recovered; 94% of the applied amount was found in the rinsate and 1.66% of the applied dose remained in the skin after 48 hours.

## ANIMAL TOXICOLOGY

### Acute Toxicity

#### *Oral*

A 10% suspension of HC Yellow No. 4 in 3% acacia in water was administered orally to two groups of five male and five female Sprague-Dawley rats at a dose of 1250 or 5000 mg/kg and to a third group of five female rats at a dose of 2500 mg/kg (Clairol 1987a). None of the animals of the 1250 mg/kg dose group but all of the animals of the 5000 mg/kg dose group died. Three of the five female rats of the 2500 mg/kg group died within 24 h of dosing. The  $\text{LD}_{50}$  was between 1250 and 5000 mg/kg for male rats and between 1250 and 2500 mg/kg for female rats. Groups of five male and five female Sprague-Dawley rats were dosed orally with 500 or 2000 mg/kg HC Yellow No. 4 in 0.5% carboxymethylcellulose and observed for 14 days (Inveresk Research International 1995a). No evidence of toxicity and no deaths were observed for animals of the 500 mg/kg dose group. Seven animals of the 2000 mg/kg dose group were

killed in extremis. The LD<sub>50</sub> for Sprague-Dawley rats was between 500 and 2000 mg/kg.

## Short-Term Toxicity

### *Oral*

Groups of five male and five female Sprague-Dawley rats were fed diet containing 0.5, 1.0, 1.25, or 1.5% HC Yellow No. 4 for 4 wks and a control group was given untreated feed (Clairol 1987b). Observations were made daily for mortality and weekly for pharmacologic or toxicologic effects. Body weights and feed consumption were determined weekly. The hair coats and urine of the test animals were discolored. A significant decrease in male mean body weights was observed in the 0.5% dose group at week 1, in the 1.0% dose group during weeks 1–3, and in the 1.25 and 1.50% dose groups during weeks 1–4. Female mean body weights of all test groups were significantly decreased during week 4. Terminal body weights were significantly decreased for males of the 1.0% dose group and for males and females of the 1.25 and 1.5% dose groups. Relative liver weight for male rats of all test groups was significantly increased while relative and absolute liver weights were significantly decreased for female rats of the 0.5 and 1.0% test groups as compared to controls. Absolute kidney weight was significantly decreased for male rats of the 1.25 and 1.50% test groups and for female rats of all test groups.

Groups of five male and five female F344/N rats were fed diets containing 5000, 10,000, 20,000, 40,000, or 80,000 ppm HC Yellow No. 4 for 14 days (NTP 1992). A control group was given laboratory chow. At the termination of dosing, all animals were given laboratory chow and observed for 1 day. During the study, all animals were observed twice daily for signs of toxicity. Body weights were measured at study initiation, on days 7 and 14, and at the time of necropsy. Feed consumption per cage was determined weekly, with animals being housed five per cage. Selected organs were weighed at necropsy. Microscopic examination was performed on selected tissues of animals of the control, 20,000, 40,000 and 80,000 ppm groups. All animals survived until study termination. Final mean body weights and mean body weight gains were significantly decreased for male animals of the  $\geq 20,000$  ppm groups and female animals of the  $\geq 10,000$  ppm groups as compared to control values. Feed consumption for these animals was also significantly decreased during wk 1 of the study. During week 2, feed consumption by males of the 40,000 ppm group was lower than that of controls, whereas feed consumption by all other groups during this time period was similar to or greater than that of controls; it was concluded, due to decreased

body weight gains, that the feed consumption values were high, possibly due to scattering of feed. Significant differences in absolute and relative organ weights were observed but considered to be secondary to decreased body weights. No other signs of toxicity were attributed to dosing.

A study using B6C3F<sub>1</sub> mice, five per sex per group, was performed according to the same procedures as above (NTP 1992). The dose groups were fed diet containing 1250, 2500, 5000, 10,000, and 20,000 ppm HC Yellow No 4. Microscopic examination was performed on selected tissues of animals of the 20,000 ppm group. All animals survived until study termination. In the 20,000 ppm group, final mean body weights and mean body weight gains of females and final mean body weight gains of males were significantly decreased compared to control values. Feed consumption by animals of all test groups were similar to controls during wk 1; feed consumption by male and female animals of the 10,000 and 20,000 ppm groups were increased compared to controls during week 2. No biologically significant differences in absolute or relative organ weights were observed. No signs of toxicity were attributed to compound administration.

## Subchronic Toxicity

### *Dermal*

Groups of 12 New Zealand white rabbits, six males and six females per group, were used to determine the percutaneous toxicity of a semipermanent hair dye formulation (P-24) containing 0.4% HC Yellow No. 4 in water (Burnett et al. 1976). In this report, the incorrect name and compound was given in the list of ingredients in studies referring to semipermanent hair dye formulation P-24; it should be noted that in all papers referring to P-24, *N,N*-bis(2-hydroxyethyl)-2-amino-5-nitrophenol should be HC Yellow No. 4 (COLIPA 1995). One ml/kg of the mixture was applied undiluted twice weekly for 13 weeks to clipped sites on the dorsolateral aspect of the thoracolumbar area (one on each side of the midline), with the sites being alternated to minimize dermal irritation. The application sites on three animals per sex per group were abraded for the first dose of each week. The animals were restrained for 1 hour following dosing, and the test site was then washed and rinsed. Three groups of 12 negative control animals were treated in the same manner as the test animals with the exception that no dye was applied. All animals were weighed weekly. Hematologic, clinical chemistry, and urinary determinations were made at study initiation and after 3, 7, and 13 weeks. All animals were killed after 13 weeks and examined grossly. Various organ-to-body weight ratios were determined and a number of tissues



were examined microscopically. No evidence of compound-induced toxicity was observed, no gross abnormalities were seen at necropsy, and no test article-related microscopic lesions were reported. No discoloration of the urine due to administration of the hair dye formulation was observed.

### *Oral*

Groups of 40 male and 45 (control and low-dose) or 55 (mid- and high-dose) female Sprague-Dawley rats were fed diets containing 0, 0.1, 0.3, or 1.0% HC Yellow No. 4 for  $\leq 6$  months (Clairol Research and Development Laboratories 1989a). These doses were determined in a previously described 4-week pilot study (Clairol 1987a). After 13 weeks of dosing, 10 animals per sex per group were killed for necropsy. Ten males and 10 females of each group were then maintained on their respective diets for a total of 26 weeks of dosing. Another 10 females of both the mid- and high-dose groups, serving as a "recovery group," were maintained on their respective diets for a total of 21 weeks and then fed untreated feed for 5 weeks. The remainder of the female animals were used in a teratology study and the remainder of the male animals were used in a dominant lethal study (both studies are summarized later in this report). Daily observations were made for mortality, and weekly observations were made for pharmacologic or toxicologic effects. Body weights and feed consumption were determined weekly. During weeks 1–13 of the study, sporadic increases and decreases in body weight and feed consumption were observed for test males and females as compared to control values; mean body weights were significantly decreased for females of the high dose group for all 13 weeks. Changes that were observed in clinical chemistry and hematology values did not appear to be clinically important and significant changes in organ and terminal body weights did not appear to be test article related. No HC Yellow No. 4-related microscopic alterations were noted for weeks 1–13. At the 6-month necropsy, the hair coat of most animals of the mid- and high-dose groups were discolored. Males and females of the high-dose group weighed less than control animals throughout the study, but this difference was only significant for females of the high-dose group during week 25; the females of the low- and mid-dose groups also weighed significantly less than control females during this week. No significant difference was observed in the body weights of female test and control recovery animals, but feed consumption was significantly decreased for females of the low-dose group during week 23. Significant decreases in absolute and relative testes weights were observed for males of the high-dose group after 6 months. Relative liver weights were increased for animals of all test groups as compared to controls. Because significant findings were not observed after 13 weeks, blood was

not collected from and microscopic examination was not performed on tissues of the animals maintained on their respective diets for a total of 6 months.

F344/N rats, 10 per sex per group, were fed diet containing 2500, 5000, 10,000, 20,000, or 40,000 ppm HC Yellow No. 4 for 13 wks (NTP 1992). A control group was given untreated diet. During the study, all animals were observed twice daily and clinical observations were recorded once daily. Body weights were measured at study initiation and then weekly. Feed consumption per cage was determined weekly, with five animals housed per cage. Selected organs were weighed at necropsy. Microscopic examination was performed on all animals that died or were killed in moribund condition and on all animals of the 40,000-ppm group, whereas the kidneys, thyroid gland, and uterus of animals of the other dose groups were examined. All animals survived until study termination. Final mean body weights and mean body weight gains were significantly decreased for male animals of the  $\geq 10,000$ -ppm groups and female animals of the 40,000-ppm group as compared to control values; final mean body weights were significantly decreased compared to controls for females of the 20,000-ppm dose groups. Feed consumption by males of all dose groups and females of the 40,000-ppm dose group was generally greater than that of controls, whereas feed consumption by females of all other dose groups was lower than controls. The extremely high values for feed consumption for animals of the 40,000-ppm group were probably due to spillage of unpalatable diet. Significant differences in absolute and relative organ weights were observed but considered to be secondary to decreased body weights. No significant signs of toxicity were observed. A statistically significant increase in dose-related thyroid gland pigmentation was observed for male rats of the 40,000-ppm dose group. The lesion appeared as a golden brown, granular pigment scattered within the cytoplasm of follicular epithelial cells; occasionally a sloughed cell containing pigment was observed within the colloid. Also in male rats of the 40,000-ppm dose group, a statistically significant increase in mineralization of the renal papilla, consisting of small numbers of minute basophilic crystalline foci diffusely scattered within the papilla and usually located within the tubular lumens, was observed. A statistically significant increase in uterine atrophy was observed in female rats of the 20,000- and 40,000-ppm groups, with the severity of lesions ranging from minimal to mild and mild to moderate, respectively. The atrophy was characterized by a decrease in uterine size, a decrease in the myometrium and endometrium, and a decrease in the size and number of endometrial glands as compared to the uteri of control females.

A study using B6C3F<sub>1</sub> mice, 10 per sex per group, was performed according to the same procedures as above (NTP 1992). The dose groups

were fed diet containing 5000, 10,000, 20,000, 40,000, or 80,000 ppm HC Yellow No. 4. Microscopic examination was performed on all animals that died or were killed moribund and on all animals of the 80,000-ppm group. The thyroid gland from animals of the other dose groups was examined. Eight males and seven females of the 80,000-ppm dose group and one male of the 40,000-ppm dose group died on study. In the 80,000-ppm dose group, nine of the deaths occurred during week 1, five during week 2, and one during week 11; the animal of the 40,000-ppm dose group died during week 7. Final mean body weights and mean body weight gains were significantly decreased for male and female animals of the  $\geq 10,000$ -ppm dose groups. Feed consumption by males and females in the dosed groups, particularly the three highest doses, was generally greater than that by controls; these high values were probably due to spillage of unpalatable diet. Significant differences in absolute and relative organ weights were observed but considered to be secondary to decreased body weights. No significant signs of toxicity were observed. A statistically significant increase in dose-related thyroid gland pigmentation that appeared as a golden brown granular pigmentation within the cytoplasm of follicular epithelial cells was observed for male and female mice of the 5000–40,000-ppm dose groups. A significant increase in pigmentation was not observed in animals of the 80,000-ppm group, presumably because most animals of this group died within the first 2 weeks of the study. However, a significant increase in mild-to-moderate depletion of lymphoid tissue and subsequent atrophy of the spleen and thymus were observed in males and females of the 80,000-ppm dose group; this observation was considered secondary to decreased body weights. A statistically significant increase in minimal to mild uterine atrophy was observed in female mice of the 40,000- and 80,000-ppm groups; the atrophy was characterized by thinner myometrium and endometrium and a decrease in the size and number of endometrial glands as compared to controls.

## Chronic Toxicity

### *Oral*

Six male and six female purebred beagle dogs were fed a composite material representative of a series of commercially available hair coloring products which included the greatest concentration of each dye and each base component present in any of the formulations used for 24 mos; HC Yellow No. 4\* composed 0.31% of the formulation (Wernick, Lanman, and Fraux 1975). Two groups were fed 19.5 or 97.5 mg/kg/day of the test material; a control group was fed laboratory feed. All animals were observed daily for toxicologic and pharmacologic effects. Body weights and

feed consumption were determined weekly and daily, respectively. Physical examinations were conducted at study initiation and after 3, 6, 18, and 24 months. Hematologic, clinical chemistry, and urinalysis parameters were determined on all dogs of the control and high-dose groups and on three male and three female dogs of the low-dose group at the same time. One male and one female animal of each group was selected for necropsy after 6, 12, and 18 months; all surviving animals were necropsied after 24 months. Selected organs were weighed, and organ-to-body weight ratios calculated. At the 24-month necropsy, liver and urinary bladder sections were taken from all animals for microscopic examination. No significant toxicologic or pharmacologic observations were made. No statistically significant differences were observed in body weight gain or in hematologic or clinical chemistry values between the treated and control groups. All animals in both test groups excreted blue-brown colored urine daily; however, urinalysis did not report any remarkable findings. There were no significant differences in organ-to-body weight ratios between the treated and control groups, and no gross or microscopic lesions attributable to dosing were noted. All animals survived until study termination.

### **Dermal Irritation**

Four male and two female rabbits were used to evaluate the primary skin irritation potential of HC Yellow No. 4 under nonocclusive conditions (Clairol Research and Development Laboratories 1987a; COLIPA 1995). HC Yellow No. 4 was applied as an aqueous slurry to a shaved 1-sq-in area for 24 hours. No erythema or edema was observed at 24 or 72 h.

A preliminary dose-range-finding study was performed using four Dunkin-Hartley guinea pigs to determine induction and challenge dose concentrations for a sensitization study (described in the next section) (Inveresk Research International, Ltd. 1995b). HC Yellow No. 4 in 0.5% aqueous carboxymethylcellulose was applied to the flanks of two guinea pigs for 48 h at concentrations of 1, 2, or 5% and to the flanks of the remaining two guinea pigs at concentrations of 10, 25, 50, or 75%. No irritation was observed at any concentration.

### **Dermal Sensitization**

A Buehler sensitization study was performed using 20 female Dunkin-Hartley guinea pigs to determine the sensitization potential of HC Yellow No. 4 (Inveresk Research International, Ltd. 1995b). The induction

consisted of applying 0.5 ml of 75% HC Yellow No. 4 in 0.5% aqueous carboxymethylcellulose to the shaved backs of the animals under an occlusive patch for 6 hours, 1 day per week for 3 weeks. A control group of 10 guinea pigs was treated similarly but patched with vehicle only. The application sites were evaluated for irritation 24 hours after dosing. The challenge was performed 2 weeks after the last induction application by applying 75% HC Yellow No. 4 to a previously unpatched site on both the test and control animals for 6 hours. (Induction and challenge concentrations were based on the results of a previously described dose-range-finding study.) Observations were made 24 and 48 hours after patch removal. Irritation was not observed during induction, and no evidence of erythema or edema was seen upon challenge. The researchers concluded "there is no evidence to suggest that HC Yellow No. 4 is a delayed contact allergen in guinea pigs."

### Photosensitization

The photosensitization potential of HC Yellow No. 4 was evaluated using eight male and eight female Hartley albino guinea pigs (Clairol Research and Development Laboratories 1986). Prior to induction, the Minimal Erythematous Dose (MED) for UVA and UVB was determined using a 150-W xenon lamp. During induction, 0.1 ml of 10% HC Yellow No. 4 in 80% DAE 433/20% physiologic saline was used. The challenge was performed 2 wks after the last induction application using 5% HC Yellow No. 4. A positive control group of four male and four female guinea pigs were dosed with 5% musk ambrette for both induction and challenge. The study procedure was as follows. Induction: week 1—0.1 ml of the test article was applied to a site 1.8 cm in diameter on the shaved nuchal area of the animals for 4 consecutive days; 1/2 MED of UVA only using a WG-354 glass filter to remove UVB; week 2—same dose as week 1; 1 MED of UVB on all 4 days; given intradermal injections of Freund's Complete Adjuvant in physiologic saline at four different sites surrounding the application area on days 1 and 3; week 3—same as week 2. Challenge: site 1—0.1 ml of test material was applied to a site on the left lumbar area for 3 consecutive days; 1/2 MED of UVB 1 h after test material application; site 2—same dose as above applied below site 1; 1/2 MED of UVA 1 hour after test material application; site 3—same dose as above applied adjacent to site 1; no exposure to UV. The MEDs for UVA and UVB were 14 minutes and 90 seconds, respectively. Erythema and edema were not observed during induction; however, a yellow discoloration was observed at the application site of the test animals. No irritation was observed at the irradiated or nonirradiated test sites for animals of the test group at the challenge 72-hour observation.

For the positive control group, irritation was observed for 7 of 8 animals with irradiation and 0 of 8 animals without irradiation. The researchers concluded that "HC Yellow No. 4 showed no evidence of causing either a photoallergic or contact allergic reaction in the guinea pig."

### **Ocular Irritation**

One hundred mg HC Yellow No. 4 was applied to the conjunctival sac of the left eye of three female rabbits and one male rabbit; the eyes of the one male and one female were rinsed 20 seconds after application (Clairol Research and Development Laboratories 1987b). Conjunctival redness, lid swelling, and discharge were observed for all animals 1 hour after instillation. On day 1 after dosing, the eye of one rabbit was normal; the eyes of the remaining three animals were normal on day 2.

## **REPRODUCTIVE AND DEVELOPMENTAL TOXICITY**

### **Dermal**

Groups of 20 gravid Charles River CD rats were used to evaluate the teratogenic potential of a semipermanent hair dye formulation (P-24) containing 0.4% HC Yellow No. 4 (Burnett et al. 1976). The formulation was applied topically at a dose of 2 ml/kg to a shaved dorsoscapular area on days 1, 4, 7, 10, 13, 16, and 19 of gestation. (Pilot studies demonstrated that potential skin irritancy would not permit more frequent application.) Three negative-control groups of rats were shaved but not dosed and rats of a positive-control group were dosed orally with 250 mg/kg acetylsalicylic acid on days 6–16 of gestation. Feed and water were available ad libitum. All animals were weighed on the days of dosing and they were killed on day 20 of gestation. The only reported observation was a change in color of the skin and hair at the site of application. No signs of toxicity were reported. Body weight gains and mean feed consumption were similar for animals of the treated and negative-control groups. The increase in embryotoxicity observed in the positive-control group was reportedly in accord with the other studies of the effects of aspirin upon fetal rat development. It was concluded that dermal administration of a semipermanent hair dye formulation containing 0.4% HC Yellow No. 4 "every third day of the gestation period produces no embryotoxic or teratogenic effects" in Charles River CD rats.

A multigeneration reproduction study was conducted using groups of 40 male and 40 female Sprague-Dawley rats that received topical applications of a semipermanent hair dye formulation (P-24) containing

0.4% HC Yellow No. 4 (International Research and Development Corporation 1977). A dose of 0.5 ml was applied twice a week to a shaved area of the back that was approximately 1 inch in diameter. (The initial dose, 0.2 ml per application, was increased by 0.1 ml per application increments weekly until reaching 0.5 ml per application.) Successive applications were made to adjacent areas to minimize dermal irritation. Three negative-control groups of rats were shaved but not dosed. When the rats were 100 days old, they were mated to produce an  $F_{1a}$  generation which was eventually used in a carcinogenicity study (that is summarized later in this report in its respective section). The  $F_0$  generation was then reduced to 20 animals per group, remated to produce an  $F_{1b}$  generation, and then killed following weaning of the  $F_{1b}$  litters. Twenty male and 20 female rats per group were chosen from the  $F_{1b}$  litters and mated after 100 days to produce  $F_{2a}$  and  $F_{2b}$  litters. Five male and five female  $F_{1b}$  parents were necropsied after weaning of the  $F_{2b}$  litters. Again following the same procedures, 20 male and 20 female  $F_2$  parents per group were selected from the  $F_{2b}$  litters and mated to produce  $F_{3a}$ ,  $F_{3b}$ , and  $F_{3c}$  litters. After weaning the  $F_{3b}$  litters, one weanling per litter per group was necropsied; the pups of the  $F_{3a}$  and  $F_{3c}$  litters were killed after weaning. Parental generations were observed daily for changes in general behavior and appearance, and detailed observations were recorded weekly. Body weights and feed consumption were measured weekly. The pups were counted and weighed as a litter on days 0, 4, and 14 of lactation. On day 21 of lactation, the pups were counted, sexed, and examined for pharmacologic effects. Dermal reactions consisting of mild scabbing, fissuring, atonia, and a leathery texture occurred intermittently throughout the treatment period in each generation. No dose-related pharmacotoxicologic signs were observed, and body weight gains, feed consumption, and survival were comparable for treated and control rats in each generation. During week 61, sialoadenitis was observed for some test and control animals; this regressed at week 63 but was followed by increased incidence of respiratory congestion in both test and control animals. The respiratory congestion persisted in the  $F_2$  parents during the production of successive litters. Litter size and pup body weights were similar for test and control groups. Fertility, gestation, survival, and live birth indices were comparable between test and control animals for the  $F_0$ ,  $F_1$ , and  $F_2$  parents. The  $F_2$  parents had markedly reduced fertility indices for the three separate matings, but there were no significant differences between the control and test group with respect to fertility. The researchers did not report that the respiratory congestion was a significant factor in the reduction of fertility indices. The results of a special study established that the decreased fertility was due to reproductive tract changes in both the treated and control rats. No gross or microscopic treatment-related lesions were observed in  $F_{1b}$  parental

rats or F<sub>3b</sub> weanling rats. The topical application of a semipermanent hair dye formulation containing 0.4% HC Yellow No. 4 did not affect the reproductive performance of rats.

## Oral

Twelve female Sprague-Dawley rats were mated and fed 1.0% HC Yellow No. 4 from day 0 of gestation until they were killed at day 18 or 20 of gestation; a control group of 10 female rats was mated and given untreated feed (Clairol 1988). Maternal body weight changes, live and dead fetuses, early and late resorptions, and the number of corpora lutea were observed; statistical analyses were not conducted. Eleven of 12 test females and 5 of 10 control females were gravid. The mean number of corpora lutea/dam was comparable between the treated (14.0) and control (15.2) dams, the mean number of live fetuses/dam was decreased (12.2 vs. 8.4), and the number of dams with resorptions (3 of 5 vs. 8 of 11) and dead fetuses (0 of 5 vs. 10 of 11) was increased. The total numbers of resorptions (21 of 11 dams vs. 3 of 5 dams) and dead fetuses (47 of 11 dams and 0 of 5 dams) were also increased in treated females. The researchers concluded that "administration of HC Yellow No. 4 at a level of 1.0% to dams throughout the entire period of gestation resulted in fetotoxicity."

Four groups of 25 female Sprague-Dawley rats (from a previously described subchronic toxicity study) that had been fed 0, 0.1, 0.3, or 1.0% HC Yellow No. 4 in the diet for 13 weeks were maintained on their respective diets for another 2 weeks until being mated (Clairol Research and Development Laboratories 1989a). All animals were fed untreated feed during mating, and were then fed their original diet beginning at day 0 of gestation. (The males used for mating were fed only untreated feed.) Females were observed daily for mortality and signs of toxicity, body weights were determined on days 0, 6, 9, 12, 15, and 20 of gestation, and feed consumption was determined on days 11–12 and 19–20 of gestation. All females were killed on day 20 of gestation. Mean body weights were significantly increased during days 0–15 of gestation as compared to controls. No significant differences in feed consumption were observed between test and control animals. The number of total resorptions and dead fetuses was significantly increased for the high-dose group as compared to controls. The incidence of the number of litters with fetuses with sternebra(e) no. 5 and/or no. 6 unossified was significantly increased in the mid- and high-dose groups, and there was a single instance of severely maligned sternebrae and of anophthalmia in the high-dose group. It was concluded that HC Yellow No. 4 was possibly fetotoxic at a dose of 0.3% and severely fetotoxic at a dose of 1.0%; it did not produce fetal toxicity at a dose of 0.1%. HC Yellow No. 4 was not teratogenic.



Because the low-dose concentration of 0.1% used in the previous study was not an unequivocal no-effect concentration, a second study was performed (Clairol Research and Development Laboratories 1989b). Groups of 25 female Sprague-Dawley rats were fed diet containing 0.03 or 0.10% HC Yellow No. 4 or untreated feed for 6 weeks prior to the initiation of mating with untreated males. During mating, all animals were fed basal chow. On day 0 of gestation, all females were again maintained on their respective treated feed until being killed on day 20 of gestation. Females were observed daily for mortality and toxicity, and body weights were determined on days 0, 6, 9, 12, 15, and 20 of gestation. Body weight gains were comparable for test and control animals both prior to mating and during gestation. The number of gravid animals, preimplantation and postimplantation losses, and live pups were similar for all groups, and no gross malformations were observed. Visceral and skeletal variations and malformations were not evaluated. It was concluded that  $\leq 0.10\%$  HC Yellow No. 4 did not appear to produce a teratogenic effect or fetotoxicity under the conditions of this study. Groups of 12 female New Zealand white rabbits were dosed by gavage on days 6–18 of gestation with the hair dye composite previously described in a chronic toxicity study that contained 0.31% HC Yellow No. 4\* at a dose of 19.5 or 97.5 mg/kg/day, with the composite without the dyes at a dose of 97.5 mg/kg/day, or with 0.5% of aqueous methylcellulose (vehicle) (Wernick, Lanman, and Fraux 1975). The dose volume for all groups was 1 ml/kg. All rabbits were killed on day 30 of gestation and various parameters were observed. No teratogenic effects were observed in any of the groups. Fetal survival was not adversely affected by the dye-containing composite. Neither grossly abnormal fetuses nor soft tissue defects were observed. Animals of the high-dose group excreted blue-brown colored urine within an hour of dosing; urine color was normal the next day prior to dosing.

Groups of CFE-S rats, 20 males and 20 females per group, were mated, and gravid females were fed diet containing 1950 or 7800 ppm of the previously described dye composite that contained 0.31% HC Yellow No. 4\* on days 6–15 of gestation; a control group was fed untreated feed throughout the study (Wernick, Lanman, and Fraux 1975). The female rats were weighed biweekly and killed on day 19 of pregnancy. Various reproductive and fetal parameters were examined. No compound-associated adverse effects were observed for rats or the fetuses. No statistically significant dose-related effects were observed in the average number of implantation sites, live pups, early or late absorptions per litter, or number of females with one or more resorption sites. No gross abnormalities related to dosing were observed. The rats fed the test diet excreted blue-brown colored urine.

Groups of 10 male and 20 female Sprague-Dawley CD rats were fed the previously described dye composite that contained 0.31% HC Yellow

No. 4\* at concentrations of 1950 or 7800 ppm; a control group was fed untreated feed (Wernick, Lanman, and Fraux 1975). The study was divided into two parts. In part I, the females received the basal diet for 8 weeks prior to mating through weaning, and the males were fed the test diet for 8 weeks prior to and during mating. In part II, the females were fed the test diet 8 weeks prior to mating, during gestation, and 21 days of lactation, while the males were fed untreated feed prior to and during mating. The remainder of the test procedure was the same for both parts of the study. One gravid female of each group was killed for examination on day 13 of gestation. The remaining gravid dams were allowed to deliver; necropsy was performed on all dams that did not deliver to determine whether pregnancy had occurred. The pups were weighed at birth and after 4 and 21 days. At 21 days, all pups were killed and examined macroscopically. No statistically significant dose-related differences in male or female fertility, length of gestation, number of females with resorption sites, live pups per litter, pup body weight, or pup survival were observed between the test and control groups in either part of the study. No significant differences in body weight gain or feed consumption were observed. No abnormal pups were noted. The rats dosed with the composite excreted blue-brown colored urine.

## MUTAGENICITY

### In Vitro

A *Salmonella*/mammalian microsome test was used to evaluate the mutagenic potential of HC Yellow No. 4\* using *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98 with and without metabolic activation (Mortelmans et al. 1986). HC Yellow No. 4\* (93.4% analyzed purity) in dimethylsulfoxide was tested at doses ranging from 3 to 10,000  $\mu\text{g}/\text{plate}$ , based on results from a preliminary dose-setting experiment. Three plates were used per dose and all assays were repeated. A number of concurrent positive and negative controls were also tested. HC Yellow No. 4\* produced positive mutagenic results in all strains tested.

### In Vivo

In a dominant lethal study, four groups of 20 male Sprague-Dawley rats (from a previously described subchronic toxicity study) that had been fed 0, 0.1, 0.3, or 1.0% HC Yellow No. 4 in the diet for 13 weeks were fed their respective diets for a total of approximately 21 weeks, at which time they were then fed laboratory chow and mated to untreated females (Clairol Research and Development Laboratories 1989a). Two females

were placed with each male until there was evidence of mating or for a maximum of 7 days; if no evidence of mating occurred, the females were killed on day  $17 \pm 1$  from the midpoint of mating. This procedure was then repeated with two new females. The procedure was again repeated after an additional 7 weeks with four new females per apparently sterile male of the mid- and high-dose groups; these animals had been maintained on basal diet. All gravid females were killed on day  $17 \pm 1$  of gestation. The males were observed daily for mortality and weekly for pharmacologic or toxicologic effects, and body weights were determined weekly until week 27. Females were observed daily for mortality, and body weights were determined on days 0, 12, and 17 of gestation. Body weights were significantly increased for males of the low-dose group during wks 14–27 and of the mid-dose group during weeks 14–22 and 24–27 as compared to the controls. Three and seven males of the mid- and high-dose groups did not successfully mate with one of four females within the initial 2-week mating period. One of these three males of the mid-dose group and four of these seven males of the high-dose group also did not successfully mate with one of four females after the additional 7 weeks of being fed basal diet. Microscopic examination of the testes of these animals reported severe diffuse atrophy affecting the whole of both testes, leaving only Sertoli cells in the tubules and aspermatogenesis with total loss of spermatogonia, spermatids, and spermatocytes. In two of the high-dose animals, mineralization within the lumen, clumping of eosinophilic proteinaceous material, and degeneration and loss of Sertoli cells were also seen. There were no significant differences observed in mean body weight during gestation between gravid females of the test and control groups during the first or second mating. During the first mating, the number of gravid animals was significantly decreased in all test groups as compared to the controls. During the second mating, the number of gravid mid- and high-dose animals was significantly decreased, and the average number of corpora lutea/dam, implant sites/dam, and live fetuses/dam of the high-dose group was also significantly decreased when compared to controls. It was concluded that feeding  $\leq 1.0\%$  HC Yellow No. 4 to rats for 21 weeks did not have a dominant lethal effect but did cause a significant decrease in fertility.

Because the low-dose concentration of 0.1% used in the previous study was not an unequivocal no-effect concentration, a second dominant lethal study was performed (Clairol Research and Development Laboratories 1989b). Two groups of 20 male Sprague-Dawley rats were fed chow containing 0.03 or 0.10% HC Yellow No. 4 and a control group was given untreated feed. After 10 weeks of dosing, all males were given untreated chow and placed with two untreated females until there was evidence of mating or for a maximum of 7 days. After 7 days, the procedure was repeated with two new females. Males were observed daily

for mortality and signs of toxicity. Females were observed daily for mortality and weighed on days 0, 12, and 17 of gestation. Gravid animals were killed on day 17 of gestation; females that showed no evidence of mating were killed on day  $17 \pm 1$  the midpoint of mating. No significant differences in body weight gain were observed between treated and control animals during the 10 weeks of dosing. Body weight gains of females from the second mating were significantly decreased on day 17 of gestation. All animals, except one control male, mated successfully at least once. The number of gravid females, preimplantation and postimplantation loss, and the number of live pups were comparable among all groups. It was concluded that  $\leq 0.10\%$  HC Yellow No. 4 did not produce a dominant lethal effect or cause infertility in Sprague-Dawley rats.

The ability of HC Yellow No. 4 to induce DNA repair or unscheduled DNA synthesis (UDS) was determined using primary rat hepatocytes isolated from Fischer-344 rats that were dosed with  $\leq 1000$  mg/kg HC Yellow No. 4 (Pharmakon Research International, Inc. 1993). Carboxymethylcellulose and dimethylnitrosoamine were used as negative and positive controls, respectively. HC Yellow No. 4 did not induce UDS or DNA repair in this assay.

The mutagenic potential of HC Yellow No. 4\* was evaluated in germ cells of *Drosophila melanogaster* using the sex-linked recessive lethal mutation and the reciprocal translocation tests (Woodruff et al. 1985). HC Yellow No. 4\* (93.1% analyzed purity) in 10% ethanol was both fed and injected at a dose of 10,000 ppm in the sex-linked recessive lethal mutation test and injected in the translocation test. HC Yellow No. 4\* was negative for mutagenic activity upon feeding and positive upon injection in the recessive lethal test and negative in the translocation test.

## CARCINOGENICITY

### Dermal

F<sub>1a</sub> generation Sprague-Dawley rats from a previously described reproduction study (International Research and Development Corporation 1977) were used to determine the carcinogenic potential of a semipermanent hair dye formulation (P-24) containing 0.4% HC Yellow No. 4 (International Research and Development Corporation 1979). Twice a week a dose of 0.5 ml of the hair dye formulation was applied topically to a shaved area of the back, approximately 1 inch in diameter, of 120 rats, 60 per sex, for 12 months. The initial dose was 0.2 ml per application, which was increased by 0.1 ml per application increments weekly until reaching 0.5 ml per application. Successive applications were made to adjacent areas to minimize dermal irritation. Three negative-control groups of 120 rats were shaved but not dosed. The rats were observed daily

for signs of toxicity and mortality, detailed observations were recorded weekly. Body weights were determined weekly for the first 14 weeks and monthly thereafter; feed consumption was determined weekly. Biochemical measures were determined from blood and urine samples that were collected from five male and five female fasted rats per group at 3, 12, 18, and 24 months. Five male and five female rats per group were killed after 12 months. No signs of toxicity were observed. Test rats had a slightly greater incidence of skin lesions from various locations, including ulceration, scabbing, abscessation, and thickening, than did control rats. Coloration of the hair and skin at the application site was observed in several treated rats but was not considered to be pathologically significant. Body weight gains, survival, hematologic values, and biochemical measures were similar for rats of the treated and control groups. After 3, 12, and 24 months, the animals consistently had dark straw-colored urine, with three and nine rats having a dark brown urine at 12 and 18 months, respectively. The incidence of enlarged and/or firm livers was slightly greater in the test group as compared to the controls; this was considered "possibly compound related." Other lesions considered "possibly compound related" for males and females of the test group include a proportionately greater number of rats with parathyroid gland hyperplasia, greater frequency of hepatocellular hypertrophy or hyperplasia, and a considerably increased incidence of hyperkeratosis and dermatitis from a variety of locations. Several male test rats had hyperkeratosis and/or acanthosis involving the gastric mucosa, which was also "possibly compound related." The incidence of hematopoiesis in the livers of test rats was somewhat greater than that of all controls; the significance of this increase was not determined. For female test animals, the incidence of pituitary adenomas was significantly increased as compared to females from two of the three control groups, and the incidences of mammary adenocarcinoma/mammary carcinoma were significantly increased as compared to females in one of the three control groups; however, these differences were not considered biologically significant. Actuarial (life table) analyses did not indicate significant variations in indices of tumor bearing in the test animals as compared to the control groups by sex.

A 23-month skin painting study was performed using groups of 50 male and 50 female Eppley Swiss Webster mice to determine the carcinogenic potential of a semipermanent hair dye formulation (P-24) containing 0.4% HC Yellow No. 4 (Burnett et al. 1980). A 0.05-ml sample of the test solution was applied undiluted to a 1-cm<sup>2</sup> area of clipped skin of the interscapular region. A group of negative controls was shaved but not dosed. Observations were made daily and body weights were determined monthly. After 9 mos, 10 male and 10 female animals from each group were necropsied, with liver and kidney weights being determined. Gross and microscopic examinations were made for all animals

found dead, killed due to moribund condition, or killed at study termination.

After 9 months, relative and absolute liver and kidney weights were not significantly different from control values. No dose-related neoplasms were observed. A semipermanent hair dye formulation containing 0.4% HC Yellow No. 4 applied dermally for 23 months did not induce a carcinogenic effect.

## Oral

F344/N rats, 70 per sex per group, were used to determine the carcinogenic potential of HC Yellow No. 4 (NTP 1992). Male rats were fed diet containing 2500 or 5000 ppm, and female rats were fed diet containing 5000 or 10,000 ppm, HC Yellow No. 4 for 105 weeks (NTP 1992). The doses were determined based on the results of the previous toxicity testing. A control group of male and female rats was given untreated diet. During the study, all animals were observed twice daily and the findings were recorded monthly or as necessary. Body weights were measured at study initiation, weekly for 13 weeks, and monthly thereafter. Feed consumption per cage was determined monthly, with five animals housed per cage. Ten animals per sex per group were selected after 6 months and after 15 months for interim necropsy. At 6 months, clinical pathology studies were performed on rats of the control and high-dose groups and thyroid hormone concentrations were determined. After 15 months, clinical pathology studies were performed on rats of all dose groups and hematologic and clinical chemistry parameters were measured. The brain, right kidney, and liver of each animal necropsied after 15 months were weighed. All surviving animals were necropsied at study termination. The thyroids of animals killed after 6 months were examined microscopically. Upon necropsy at 15 months, tissues from all control and high-dose animals were examined by light microscopy. Microscopic examination was also completed on tissues from all animals that died or were killed moribund and all that survived until study termination. No dose-related biologically significant changes in thyroid hormone concentrations or microscopic lesions were observed after 6 months. No biologically significant changes or significant differences in clinical chemistry values were observed between test and control animals after 15 months. An apparent significant increase in blood urea nitrogen observed for males of the 5000-ppm group and females of both dose groups may have been an artifact of the assay, caused by the presence of HC Yellow No. 4 in the urine. Statistically significant differences in absolute and relative organ weights between control and dosed female animals after 15 months were considered secondary to differences in body weights, with

final body weights of female rats of the high-dose group being significantly less than control values. No neoplasms were attributed to dosing with HC Yellow No. 4 after 15 months. Mean body weights of the males and females of the low-dose group were similar to controls throughout the study. Mean body weights of dosed males were slightly greater than males of the control group after week 61; mean body weights of females of the high-dose group were lower than control values after week 29. Feed consumption by males of the high-dose group and females of both dose groups was decreased compared to controls through week 53; feed consumption for these groups was similar to controls after this time. No dose-related clinical findings were observed. Survival of test animals was similar to control animals. Adenomas and hyperplasia of the pars distalis of the pituitary gland occurred at greater incidences in dosed male rats than in controls, with the increases being statistically significant in the high-dose group. Since these lesions were observed in male rats after 15 months, the combined incidence of adenomas and hyperplasia from the 15-month and final necropsies was examined for high-dose males. The increase in combined incidence of adenomas in males of the high-dose group was not statistically significant, whereas the increase in combined incidence of hyperplasia in these animals was. Stromal polyps of the uterus occurred with a positive trend, with the incidence in the females of the high dose group being significantly greater than that in females of the control group. Fibroadenomas of the mammary gland occurred with a significant negative trend in dosed females. However, these difference may have been due to unusually low and unusually high incidences of these lesions in the controls, respectively; therefore, these trends were not dose-related. The investigators concluded that under the conditions of this study, "there was equivocal evidence of carcinogenic activity of HC Yellow No. 4 in male F344/N rats based on the increased incidence of pituitary gland adenomas and hyperplasia. The male rats may have been able to tolerate a slightly higher dose of the chemical. There was no evidence of carcinogenic activity in female F344/N rats given 5000 or 10,000 ppm [HC Yellow No. 4]."

A study using B6C3F<sub>1</sub> mice, 70 per sex per group, was performed according to the same procedures as above (NTP 1992). The dose groups were fed diet containing 5000 or 10,000 ppm HC Yellow No. 4. Male mice were housed individually after 15 months. At study termination, microscopic examination was performed on tissues from mice of the control and high-dose groups; the thyroid gland and ovaries of low-dose mice were also examined. Statistically significant changes in thyroid hormone concentrations were observed after 6 months, but the biologic significance of these findings was uncertain. No biologically significant changes or significant differences in clinical chemistry values were observed between test and control animals after 15 mos. Fine golden brown granular

pigmentation was observed within follicular epithelial cells in the thyroid glands of all dosed mice. The severity of pigmentation increased with dose and was more severe in males than in females. Pigmentation was minimal in low-dose females, mild in low-dose males and high-dose females, and moderate in high-dose males. After 15 months, no biologically significant differences in hematology or clinical chemistry values were observed. Statistically significant differences in absolute and relative organ weights between control and dosed animals were considered secondary to differences in body weights, with final body weights of male rats of the high-dose group and female rats of both dose groups being significantly less than control values. Golden yellow to golden brown granular pigmentation was observed within the follicular epithelial cells and within the colloid of the thyroid gland of all dosed male and female mice. Again, severity of pigmentation increased with dose and was more severe in males than in females in the same manner as at the time of the 6-mo necropsy. Additionally, pigmentation was more severe in the follicular epithelium than in the colloid. Minimal follicular cell hyperplasia, characterized by scattered follicles lined by columnar cells, which were often crowded together and sometimes protruded into the follicular lumen, was observed in 5 of 10 high-dose male mice. After 15 months, no neoplasms were attributed to dosing.

Mean body weights of the males and females of the low-dose group were generally less than those of the controls throughout the study. Mean body weights of the low-dose males and females, high-dose males, and high-dose females were more than 10% lower than control values after weeks 53, 17, and 14, respectively. Feed consumption by all dose groups was increased compared to controls throughout the study; this was attributed to scattered feed. No dose-related clinical findings were observed. Survival of test animals was similar to that of control animals. No neoplasms were attributed to treatment after 2 years. The incidences of pigmentation and follicular cell hyperplasia of the thyroid gland were greatly increased in all dose groups. The pigment, which was gold-yellow to gold-brown and varied from fine granules to large aggregates, was present in the follicular cell cytoplasm, in the follicular lumens, and within macrophages in the interstitium between follicles. Pigmentation severity increased slightly with increasing dose and generally ranged from minimal to mild in the low-dose groups and mild to moderate in the high-dose groups. The increase in hyperplasia, which involved multiple follicles lined by increased numbers of closely packed cells, was not accompanied by an increase in follicular cell neoplasms and was of minimal-to-mild severity in all dose groups; as severity increased, the follicular cells formed clusters that projected into the lumen. A statistically significant increase in chronic inflammation of minimal severity, consisting of scattered aggregates of small



numbers of lymphocytes in the glandular interstitium, was observed in male animals of both dose groups. The investigators concluded "there was no evidence of carcinogenic activity of HC Yellow No. 4 in male or female B6C3F<sub>1</sub> mice given 5000 or 10,000 ppm." However, there was an HC Yellow No. 4-related increase in the incidence of thyroid gland pigmentation and follicular cell hyperplasia in mice.

## **CLINICAL ASSESSMENT OF SAFETY**

### **Dermal Sensitization**

A repeated insult patch test (RIPT) was completed with 103 of 105 subjects (6 male and 97 female; age range 20–78; primarily Caucasian and Hispanic) to determine the sensitization potential of HC Yellow No. 4 (TKL Research Inc. 1987; COLIPA 1995). During induction, 0.2 ml of a slurry of 3.0% HC Yellow No. 4 was applied under occlusive patches to the infrascapular area of the back for 24 hours, three times per week for 3 weeks, for a total of nine applications; the application sites were scored after 48 or 72 hours. After a 14-day nontreatment period, a patch was applied to a previously untreated site for 24 hours; the site was scored 24 and 48 hours after patch removal. A sensitization reaction was not observed in a RIPT using 3% HC Yellow No. 4.

Another RIPT was completed with 104 of 115 subjects following the same procedure and using the same concentration as above (TKL Research Inc. 1989). Again, a sensitization reaction was not observed with 3.0% HC Yellow No. 4.

### **Epidemiology**

Between 35% and 45% of American women dye their hair, often at monthly intervals, over a period of years (CTFA 1993). This estimate is drawn from market research data on hair dye product use, generally from females aged 15 to 60.

A number of epidemiologic studies have investigated the association between cancer and occupation as a hairdresser or barber, or between cancer and personal use of hair dyes. The World Health Organization's International Agency for Research on Cancer (IARC) empaneled a Working Group on the Evaluation of Carcinogenic Risks to Humans to review all available data on these issues. The Working Group met October 6–13, 1992, in Lyon, France (IARC 1993). The charge to the IARC Working Group was to ascertain that all appropriate data had been collected and were being reviewed; to evaluate the results of the epidemiologic and experimental studies and prepare accurate summaries of the

data; and to make an overall evaluation of the carcinogenicity of the exposure to humans.

The IARC Working Group concluded that: "There is *inadequate evidence* that personal use of hair colourants entails exposures that are carcinogenic." Hence: "Personal use of hair colourants *cannot be evaluated as to its carcinogenicity (Group 3)*." The IARC Working Group also concluded that: "There is *limited evidence* that occupation as a hairdresser or barber entails exposures that are carcinogenic." Hence: "Occupation as a hairdresser or barber entails exposures that *are probably carcinogenic (Group 2A)*" (IARC 1993). The Expert Panel concludes that the relevance of the occupational data and conclusion to individuals using hair dyes is unclear.

## SUMMARY

HC Yellow No. 4 is a color additive that functions as a colorant in hair dyes and colors and hair tints. In the past, HC Yellow No. 4 had been depicted with an incorrect structure, which was given the CAS No. 52551-67-4. The structure, as determined through additional analysis, has been revised and HC Yellow No. 4 is now assigned the CAS No. 59820-43-8.

In examining the stability of a semipermanent hair dye formulation containing 1% HC Yellow No. 4 using TLC to determine the spectrophotometric quantitation, as well as visible and UV bands, it was found that there was no change in HC Yellow No. 4 following a 30-minute reaction with hydrogen peroxide, and there were no changes in the visible or UV bands between the samples. The purity of commercially available HC Yellow No. 4 is  $\geq 93\%$ , with the largest impurity being *N*-(2-hydroxyethyl)-2-hydroxy-4-nitroaniline (0.3–7%).

In 1995, data submitted to the FDA reported that HC Yellow No. 4 was used in 81 cosmetic formulations; 78 formulations were hair dye and colors and 3 were hair tints. Concentration of use information submitted to CIR reported the maximum concentration of use in oxidative and semipermanent hair colors was 3.0% (on-head). Hair dyes containing HC Yellow No. 4, as coal tar hair dyes, 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 the label bears a caution statement and patch test instructions for determining whether the product causes irritation.

In a study in which the percutaneous absorption of a commercial semipermanent dye base containing 1%  $^{14}\text{C}$ -HC Yellow No. 4 was determined using female cadaver skin, the mean cumulative absorption of the applied dose was approximately 0.12 and 0.15% after 24 and 48 hours,

respectively, and the mean cumulative flux was approximately 0.11 and 0.15  $\mu\text{g}/\text{cm}^2$  at these times, respectively. More than 95% of the applied dose was recovered, with 1.66% of the dose remaining in the skin after 48 hours.

The  $\text{LD}_{50}$  of HC Yellow No. 4 for Sprague-Dawley rats was between 500 and 2000 mg/kg. In a short-term oral toxicity study, body weights were decreased for male and female rats fed  $\leq 1.5\%$  HC Yellow No. 4; relative liver weights of male rats of all test groups were significantly increased whereas relative and absolute liver weights of female rats fed 0.5 and 1.0%, and absolute kidney weights of male rats fed 1.25 and 1.50% HC Yellow No. 4, and female rats of all test groups were significantly decreased compared to controls. In another short-term oral study, no signs of toxicity attributable to treatment were observed when rats were fed 5000–80,000 ppm, and mice were fed 1250–20,000 ppm HC Yellow No. 4; in both studies, decreased mean body weights and body weight gains were observed and in the study using rats, significant differences in absolute and relative organ weights were observed but considered secondary to decreased body weights.

In a dermal subchronic toxicity study in which a semipermanent hair dye containing 0.4% HC Yellow No. 4 was applied to the skin of rabbits, with half of the sites abraded, no compound-induced toxicity or lesions were observed. In a subchronic oral study in which rats were fed  $\leq 1.0\%$  HC Yellow No. 4 for up to 6 months, sporadic increases and decreases in body weights as compared to controls were observed during weeks 1–13, but no compound-related observations were made at the 13-week necropsy; at necropsy after 6 mos, absolute and relative testes weights were significantly decreased for males fed 1.0% HC Yellow No. 4, and relative liver weights were increased for all animals of all test groups. In subchronic oral studies in which rats were fed 2500–40,000 ppm, and mice were fed 5000–80,000 ppm HC Yellow No. 4, the following were observed: a statistically significant decrease in mean body weight gains and final mean body weights for male rats and male and female mice of the  $\geq 10,000$ -ppm groups and for female rats of the 40,000-ppm group; a statistically significant increase in thyroid gland pigmentation for male rats of the 40,000-ppm group and for male and female mice of the 5000–40,000-ppm dose groups; and a statistically significant increase in uterine atrophy for female rats of the 20,000- and 40,000-ppm dose groups and for female mice of the 40,000- and 80,000-ppm groups. All rats survived until study termination, but eight male and seven female mice of the 80,000-ppm group and one male mouse of the 40,000-ppm group died on study.

No significant dose-related signs of toxicity were observed in a chronic oral study in which dogs were fed a hair coloring formulation containing 0.31% HC Yellow No. 4.

Hc Yellow No. 4 did not produce irritation under nonocclusive conditions using rabbits or guinea pigs. Using guinea pigs, HC Yellow No. 4 was not a delayed contact allergen in a sensitization study using a 75% solution during induction and challenge, and it did not cause a photoallergic or contact allergic reaction in guinea pigs in a photosensitization study in which a 10% solution was used during induction and a 5% solution was used during challenge. HC Yellow No. 4 caused conjunctival redness, lid swelling, and discharge upon application to the eyes of rabbits; all eyes were normal 2 days after dosing.

In dermal studies of formulations containing 0.4% HC Yellow No. 4, administration every third day of gestation produced no embryotoxic or teratogenic effects in rats and in a multigeneration study, no effects on reproductive performance were observed. In feeding studies, fetal toxicity was observed when 1.0% HC Yellow No. 4 was fed to rats, and it was possibly fetotoxic when fed at a concentration of 0.3%. In a follow-up study, feeding of  $\leq 0.10\%$  HC Yellow No. 4 to rats was not teratogenic or fetotoxic. In other oral studies, a hair dye formulation that contained 0.31% HC Yellow No. 4 was fed to rats, mice, and rabbits and did not produce any teratogenic, reproductive, or fetotoxic effects.

HC Yellow No. 4 produced positive results in a *Salmonella*/mammalian microsome test. HC Yellow No. 4,  $\leq 1.0\%$ , did not have a dominant lethal effect but did cause a significant decrease in fertility; in a follow-up study,  $\leq 0.10\%$  did not produce a dominant lethal effect or cause infertility. HC Yellow No. 4 did not induce UDS or DNA repair using primary rat hepatocytes. HC Yellow No. 4 produced negative results upon feeding and positive results upon injection in a sex-linked recessive lethal mutation test. Negative results were obtained in a translocation test.

In a 12-month dermal study using rats in which a semipermanent hair dye formulation containing 0.4% HC Yellow No. 4 was applied twice weekly, enlarged and/or firm livers, increased parathyroid gland hyperplasia, hepatocellular hypertrophy or hyperplasia, keratosis and dermatitis, increased incidence of hematopoiesis in the liver of all test animals, and hyperkeratosis and/or acanthosis involving the gastric mucosa of males were possibly compound related; the incidence of pituitary adenomas and mammary adenocarcinomas/mammary carcinomas were significantly increased for female test animals compared to some of the control groups, but not considered biologically significant, and no significant variations in indices of tumor bearing in test animals were reported using actuarial (life table) analyses. In a 23-month skin painting study using mice given the same hair dye as just described, no dose-related neoplasms were observed and a carcinogenic effect was not induced. In an National Toxicology Program (NTP) study, HC Yellow No. 4 produced equivocal evidence of carcinogenicity in male

F344/N rats, based on the increased incidence of pituitary gland adenomas and hyperplasia, and no evidence of carcinogenic activity in female F344/N rats. In an NTP study using B6C3F<sub>1</sub> mice, HC Yellow No. 4 did not produce any evidence of carcinogenicity in males or females; however, an HC Yellow No. 4-related increase in the incidence of thyroid gland pigmentation and follicular cell hyperplasia was observed.

No sensitization reaction to 3.0% HC Yellow No. 4 was observed in either of two RIPTs completed with 103 and 104 subjects, respectively.

## DISCUSSION

The Expert Panel was concerned that HC Yellow No. 4 produces developmental and reproductive toxicity when fed to animals. However, because little is absorbed across the skin, such adverse responses would not be expected from the use of this ingredient in hair dyes and coloring products. HC Yellow No. 4 can be mutagenic, but was negative in several carcinogenicity (both oral and dermal) tests, further supporting its safety.

Another concern was any nitroaniline impurity that could be present in HC Yellow No. 4. However, since HC Yellow No. 4 is reported to be used at  $\leq 3\%$ , this nitroaniline impurity could at most constitute 0.21% of the product. The low exposure combined with the fact that products with HC Yellow No. 4 are used in a brief and discontinuous manner, followed by rinsing, led the Expert Panel to discount the presence of nitroaniline impurity.

## CONCLUSION

On the basis of the available animal and clinical data presented in this report, the Expert Panel concluded that HC Yellow No. 4 is safe as a hair colorant in the present practices of use.

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