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August 20, 1976

Mr. Armond Welch, Panel Administrator
Division of OTC Drug Evaluation (HFD-510)
Bureau of Drugs
Food and Drug Administration
5600 Fishers Lane
Rockville, Maryland 20852

Dear Mr. Welch:

Mr. C. Burnett, Associate Director Research, Toxicology, of Clairol Research Laboratories, forwarded to me toxicological data on resorcinol which he wanted submitted for review by the Antimicrobial II Panel when appropriate. As such, I am enclosing six published papers relating to toxicological studies conducted with resorcinol.

Perhaps you would arrange to have the data distributed to the Panelists.

With all best wishes,

Sincerely yours,

G Hildick-Smith

Gavin Hildick-Smith, M.D., F.R.C.P.
Director, Medical Affairs

GHS/tm

cc: Mr. C. Burnett

OTC 070182

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Study of Long-term Percutaneous Toxicity and Carcinogenicity of Hair Dyes (Oxidizing Dyes) in Rats*

H. J. KINKEL and S. HOLZMANN

Biosciences Department, Battelle-Institut e.V., 6000 Frankfurt (Main) 90, Am Römerhof 35, Bundesrepublik Deutschland

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Abstract—The long-term percutaneous toxicity and carcinogenicity of *p*-toluenediamine, resorcinol and *m*-diaminoanisole, which are all important constituents of hair dyes, were studied in rats. The substances examined, *p*-toluenediamine alone in an appropriate vehicle or a mixture of all three compounds similarly formulated, were applied to the shaved dorsal skin of the rats twice weekly for 2 yr, in doses considerably greater than those used in practice. One group of control animals remained untreated while a second was treated with the vehicle alone. After the application period of 2 yr, surviving animals were observed for another 6 months. There were no indications that the substances examined had any adverse effects. Behaviour, feed intake, body-weight changes, blood counts, activity of serum glutamic-pyruvic transaminase and results of the bromosulphthalein test for liver function were comparable in the control and experimental groups. No pathological formation of Heinz bodies or methaemoglobin was associated with application of the dye components. Similarly, experimental and control animals showed no difference in respect of their mean lifespan or the type and incidence of tumours. No tumours or other skin reactions occurred at the site of application of the substances and histopathological studies of the liver, kidneys and lungs provided no evidence of degenerative change or functional disturbance. Acute studies involving sc or ip injection of the substances confirmed the lack of effect on erythropoiesis and methaemoglobin formation.

INTRODUCTION

Of the aromatic diamines, aminophenols and phenols commonly included in preparations used for dyeing human hair, the most important is the oxidizing dye, *p*-toluenediamine. *p*-Phenylenediamine is another major component in some countries, while other para and the corresponding meta compounds are normally used in much lower concentrations as shading dyes. Reports of tumour induction by some dyes administered to experimental animals by various routes (Saruta, Yamaguchi & Matsuoka, 1962) has provoked some suspicion about the carcinogenicity of substances of this type. Ito, Hiasa, Konishi & Marugami (1969) reported the formation of hepatic carcinomas in Wistar rats fed diets containing 0·1 or 0·06% *m*-toluenediamine.

In the work reported here, the percutaneous toxicity and possible carcinogenic effects of *p*-toluenediamine and of a mixture of *p*-toluenediamine, resorcinol and *m*-diaminoanisole in concentrations used in practice in hair-dyeing to achieve particularly dark shades were studied in rats over almost the whole of their lifespan.

*These investigations were conducted on behalf and with the assistance of Elida-Gibbs GmbH, Hamburg, L'Oreal, Haarkosmetik und Parfumerien GmbH, Karlsruhe, Hans Schwarzkopf GmbH, Hamburg, Thera-Chemie GmbH, Düsseldorf, and Wella AG, Darmstadt.

EXPERIMENTAL

Materials. The substances examined were prepared by incorporating *p*-toluenediamine at a concentration of 4% (model substance T) or levels of 3% *p*-toluenediamine, 0·75% resorcinol and 0·75% *m*-diaminoanisole (substance TRA) in a carboxymethylcellulose (CMC) slime (4% Tylose HT supplied by AKU, Arnhem, The Netherlands), to which 0·5% pure sodium sulphite was added to prevent premature atmospheric oxidation of the amino components. The two dye bases were available commercially as the sulphates from Farbwerke Hoechst AG, Frankfurt a.M., were therefore partly dissolved and converted with 25% ammonia solution in the absence of iron and, with stirring, dissolved completely in the CMC slime to form slightly brown, transparent jellies with a pH of about 10·1. These were filled into aluminium tubes coated inside with a protective lacquer, in which form they were stable for several years. A colourless control jelly (substance O) was similarly prepared, part of the ammonia being replaced by ammonium sulphate in an amount equivalent to that formed in the experimental preparations by neutralization of the basic dye sulphates (Table 1). All three formulations were therefore comparable in free ammonia content and pH, although in each case control analyses showed that part of the free ammonia was lost during preparation and the filling of the tubes (Table 1).

Table 1. *Composition of experimental formulations*

Component/Analysis	Concentration (% w/w) in substance		
	T	TRA	O
Component			
Tylose HT	4·0	4·0	4·0
Sodium sulphite	0·5	0·5	0·5
Ammonia, 25%	13·0	12·5	8·5
p-Toluenediamine*	4·0	3·0	—
Resorcinol	—	0·75	—
m-Diaminoanisole*	—	0·75	—
Ammonium sulphate	as formed by neutralization	as formed by neutralization	3·7
Deionized water	78·5	78·5	83·3
Total....	100·0	100·0	100·0
Analysis			
Ammonia: total (100%), titrated	2·95	2·90	3·03
free (100%)	1·96	1·96	1·99
pH	10·1	10·1	10·2

*Used as sulphate, calculated as free base.

Animals and diet. Sprague–Dawley rats from a closed randomized stock were obtained from Dr. K. E. Mollegard-Hansen, Havdrup-Kopenhagen, Denmark, for the skin-painting study. The rats (about 3 months old and with a mean body weight just under 100 g) were given a 2-wk adaption period before treatment was begun and were maintained throughout on a standard diet (Altromin-R) and water given *ad lib.* Injection studies were carried out on 6-month-old rats of the Wistar–Elberfeld strain bred at Battelle.

Experimental design and conduct

Skin application study. A preliminary 4-wk experiment established that twice-weekly application of the test substances to the skin of rats caused no local irritation. In the main experiment, three groups, each of 50 male and 50 female rats, were treated with substance T or substance TRA or given no treatment, while a fourth group of 25 males and 25 females was treated with the control substance O. To simulate conventional hair-dyeing techniques, substances T, TRA and O were mixed with an equal volume of 6% hydrogen peroxide immediately before application. This was done in batches to ensure that the material was applied to the skin within 10 min of mixing.

The solution (0·5 g) was applied to the shaved dorsal skin over an area of 3 × 3 cm and was left on the skin for 30 min, during which the animals were kept apart and fitted with a rubber collar to prevent any ingestion of the applied material. As under practical conditions, the painted area was then cleaned with a commercial agent and washed with water, and the animal was rubbed with a towel and dried under an infrared lamp. This procedure was repeated twice weekly for 2 yr, after which surviving rats were observed for a further 6 months.

The rats were weighed weekly and observed regularly for any change in behaviour. Red and white cell counts and haemoglobin and methaemoglobin levels were determined in blood samples from ten males from each group at 3-monthly intervals, and at 4-monthly intervals liver function was checked by means of a bromsulphthalein test and the determination of serum glutamic–pyruvic transaminase activity (using the μl-scale Biochemica-Test Combination, Boehringer AG, Mannheim), again in ten males from each group. Transaminase activity was also determined in random samples taken from tumour-bearing animals. At month 7 and 16, blood samples from three animals in each group were examined for Heinz bodies by two methods, the conventional Nile-blue sulphate staining and a fluorescence microscopic-detection method using morin. All rats that died during the experiment or were killed when moribund and all survivors, which were killed by decapitation, were autopsied and all tumours were examined histologically. Sections from the application site, liver, kidneys, lungs and any organ that appeared abnormal at autopsy from a total of nine or ten males and ten females from each group killed randomly and singly at intervals of 6, 9, 12 and 24 months during the study were stained with haematoxylin and eosin for microscopic examination.

Injection study. Heinz-body counts and methaemoglobin determinations were carried out in groups of 2–4 rats given a single sc injection of 0·75–24·0 mg *p*-toluenediamine sulphate/kg, or the same dose repeated daily for 3–5 days, a single ip injection of the same compound in doses of 4–32 mg/kg, or a single sc injection of substance TRA in a 1 : 1 mixture with hydrogen peroxide in a dose equivalent to 1·5 or 3 mg *p*-toluenediamine sulphate/kg (1 mg *p*-toluenediamine sulphate being equivalent to 0·555 mg free base). Sodium sulphite was added to the aqueous solution of *p*-toluenediamine sulphate as in the formulation of substance T, used in the skin painting study, and the pH of the preparation was adjusted to 7 by addition of ammonia.

RESULTS

Growth, food intake and mortality

The behaviour of all the rats was normal and there were no marked differences between the various groups in respect of body-weight changes (Table 2) and food intake. The mean

lifespan and mortality rate during the long-term experiment were comparable in all experimental and control groups.

Table 2. *Mean body weights of groups of control rats and of rats treated with* p-*toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Treatment	Body weight* (g) at wk			
	0†	52	104	130
		Males		
None (control)	86 ± 15 (50)	456 ± 49 (36)	437 ± 71 (26)	298 ± 35 (9)
Substance O (control)	91 ± 13 (25)	452 ± 45 (18)	421 ± 64 (9)	361 (3)
Substance T	88 ± 15 (50)	443 ± 47 (43)	409 ± 64 (27)	343 ± 63 (8)
Substance TRA	90 ± 17 (50)	446 ± 53 (41)	411 ± 59 (27)	328 ± 35 (9)
		Females		
None (control)	83 ± 13 (50)	289 ± 40 (44)	287 ± 50 (21)	248 ± 41 (7)
Substance O (control)	83 ± 11 (25)	269 ± 33 (19)	258 ± 41 (10)	260 (2)
Substance T	82 ± 14 (50)	282 ± 33 (42)	292 ± 47 (14)	278 ± 59 (6)
Substance TRA	81 ± 16 (50)	280 ± 37 (42)	277 ± 34 (23)	247 ± 31 (7)

O = Vehicle only T = *p*-Toluenediamine formulation
TRA = *p*-Toluenediamine-resorcinol-*m*-diaminoanisole formulation

*Values given are means ($\bar{x}$) ± standard deviation ($\sqrt{[S(x^2)-\bar{x}S(x)]/(n-1)}$) for the numbers of animals (n) indicated in parentheses.

†Beginning of the experiment.

Haematology

All the haematological data recorded were within the normal ranges. No pathological methaemoglobin formation was observed even at 3 min or 4 hr after ip injection of a single highly toxic dose of 32 mg *p*-toluenediamine sulphate/kg. No Heinz bodies were found in animals treated with the experimental substances for 7 or 16 months or in those given a single sc injection of 1·5 mg *p*-toluenediamine sulphate/kg. A very small number of Heinz bodies was found 2–3 days after administration of several of these sc injections and also after the single sc injection of a comparable dose of TRA (1–2/1000 erythrocytes), but even in a dose of 12 or 24 mg/kg given ip, *p*-toluenediamine sulphate caused little or no increase in Heinz bodies. In a positive control experiment, phenylhydrazine dihydrochloride given ip as a 1% aqueous solution in a dose of 3 mg/kg body weight produced an incidence of Heinz bodies of 500–700/1000 erythrocytes.

Liver function

No impairment of liver function was detected in the experimental groups. Bromsulphthalein excretion by the liver was comparable in experimental and control groups and the serum transaminase values were not increased in the treated animals or in any of the random samples from animals with tumours.

Autopsy and histopathology

Autopsies revealed no pathological changes attributable to either of the long-term treatments. The main causes of death were pneumonia and lymphadenitis, the latter having the symptoms of a Klebsiella infection and being associated with abscess formation but not

Table 3. *Tumour incidence in control rats and rats treated with p-toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Treatment	Sex	No. of rats/group	Rats with tumours		Tumours		
			No.	% of group	Total no.	Type (no.)	Time of appearance* (months)
None (control)	M	50	4	8	4	Fibrolipoma	20
						Lymphosarcoma	23
						Hyperkeratotic papilloma	29
						Polymorphocellular sarcoma†	24
	F	50	12	24	15	Round-cell sarcoma†	11
						Round-cell sarcoma	19
						Cystadenomas (2)	22, 29
						Fibroadenomas (8)	16–29
						Adenomas (2)	17, 24
						Fibroma	17
Substance O	M	25	1	4	1	Round-cell sarcoma	18
	F	25	4	16	4	Round-cell sarcoma	24
						Carcinoma solidum	29
						Fibroadenoma	25
						Fibrosarcoma	12
Substance T	M	50	4	8	5	Fibromas (2)	18, 31
						Lipoma	24
						Round-cell sarcoma	21
						Lymphosarcoma	21
	F	50	10	20	11	Fibroadenomas (8)	15–26
						Carcinoma solidum	17
						Adenocarcinoma	23
						Adenoma	28
Substance TRA	M	50	3	6	4	Lymphosarcoma	29
						Adenoma	29
						Fibroma	18
						Adrenal tumour	25
	F	50	14	28	15	Cystadenoma	18
						Fibromas (2)	18, 25
						Fibroadenomas (6)	18–29
						Fusocellular sarcoma	15
						Lymphosarcomas (2)	28
						Hyperkeratotic papilloma	12
						Carcinoma solidum†	21
						Squamous cell carcinoma†	27

O = Vehicle only T = *p*-Toluenediamine formulation
TRA = *p*-Toluenediamine–resorcinol–*m*-diaminoanisole formulation
* Time at which tumour was detected, calculated from beginning of experiment.
† With metastases in the peritoneal cavity.

with any "non-specific irritation" (Huhn, 1966) of the lymph nodes in the form of lymphogenous tumour cell propagation and formation of metastases. Both of these diseases occur frequently in the Sprague-Dawley rats used, but were no more common in the experimental groups than in the controls. Histopathological studies similarly revealed no tissue changes that could be ascribed to treatment with the dyes.

Tumour incidence. Table 3 shows that tumours only became clinically detectable during the second half of the life of the rats. Of the 350 Sprague-Dawley rats used in the study, 52 (15%) developed tumours and six had more than one tumour, but the incidence, the proportion of males and females affected and the types of tumours were comparable in the experimental and control groups (Table 3). The 59 tumours found included 16 different types (Table 4), of which fibroadenomas were by far the most frequent, followed by fibromas, lymphosarcomas and round-cell sarcomas. The two skin tumours (hyperkeratotic papillomas) and ten sc tumours (six fibromas, one fibrolipoma, one fibrosarcoma, one lipoma and one round-cell sarcoma) were not found in the area of application of any of the substances.

Table 4. *Identity and location of tumours in control rats and rats treated with* p-*toluenediamine formulations by twice-weekly applications to the skin for 2 yr*

Type of tumour	Location of tumour	Sex	No. found in: Untreated controls M	Untreated controls F	Animals treated with substance: O M	O F	T M	T F	TRA M	TRA F
Adenocarcinoma (m)	E							1		
Adenoma (b)	E			2				1	1	
Carcinoma solidum (m)	E					1		1		1
Fibroadenoma (b)	E			8		1		8		6
Fibrolipoma (b)	Ms		1							
Fibroma (b)	Ms			1			2		1	2
Fibrosarcoma (m)	Ms					1				
Hyperkeratotic papilloma (b)	ES		1							1
Cystadenoma (b)	E			2						1
Lipoma (b)	Ms						1			
Lymphosarcoma (m)	M		1				1		1	2
Phaeochromocytoma (b)									1	
Squamous cell carcinoma (m)	E									1
Polymorphocellular sarcoma (m)	M		1							
Round-cell sarcoma (m)	M			2	1	1 (s)	1			
Fusocellular sarcoma (m)	M									1
Total number of tumours....			4	15	1	4	5	11	4	15

m = Malignant b = Benign E = Epithelial tissue M = Mesenchymal tissue
s = Subcutaneous tissue S = Skin

DISCUSSION

Although the amount of solution applied to the animal and the frequency of application both exceeded those likely to be encountered in practice, no evidence of carcinogenic or other toxic effects was detected in the experimental animals. The relatively high tumour incidence was to be expected in these Sprague–Dawley rats, which are somewhat susceptible

to spontaneous tumour formation and were examined or kept under observation for up to 2·5 yr. The tumour incidence and lifespan were comparable in all the experimental and control groups, however, and no tumours developed at the site of application. The other criteria studied (behaviour, feed consumption, body-weight changes, blood parameters, serum activity of glutamic-pyruvic transaminase and bromosulphthalein tests for liver function) were also comparable in experimental and control groups.

Although there is evidence, including the development of allergic reactions in hypersensitive subjects, that substances of the type examined penetrate through the epidermis and also into the hair shaft, no signs of skin irritation, such as the erythema, oedema or scar-tissue formation described by Carson, Weinberg & Goldhamer (1965), nor any increase in cell proliferation in the skin were observed. Kiese, Rachor & Rauscher (1968) showed that phenylenediamines were absorbed through the skin of dogs when applied in vehicles used in the dyeing of human hair and appeared unchanged in small amounts in the urine. Absorption was reduced when hydrogen peroxide was added to the mixture before application. Kiese & Rauscher (1968) demonstrated the absorption of *p*-toluenediamine through the human scalp during dyeing of the hair with a mixture of *p*-toluenediamine sulphate with resorcinol and hydrogen peroxide. The absorbed material was slowly excreted in the urine, principally as *N,N'*-diacetyl-*p*-toluenediamine. It was not known, however, whether the *p*-toluenediamine was absorbed as such, or was liberated in the body from some compound formed during the oxidation of the *p*-toluenediamine–resorcinol mixture.

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Etude sur le rat de la toxicité percutanée à long terme et des propriétés carcinogènes de teintures capillaires (colorants oxydants)

Résumé—On a étudié sur des rats la toxicité percutanée à long terme et le pouvoir carcinogène de la *p*-toluènediamine, du résorcinol et du *m*-diaminoanisol, produits qui sont d'importants composants de teintures pour les cheveux. On les a appliqués—la *p*-toluènediamine seule dans un porteur approprié ou chaque produit dans des mixtures de compositions similaires—deux fois par semaine, et ceci pendant 2 ans, à la peau rasée du dos des rats. Les doses étaient beaucoup plus fortes que celles utilisées dans la pratique. Un groupe d'animaux témoins n'a pas été traité et un second groupe n'a été traité qu'au seul porteur. Après les 2 années de traitement, on a prolongé de 6 mois l'observation des animaux survivants.

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LONG-TERM TOXICITY STUDIES ON OXIDATION HAIR DYES

C. BURNETT, B. LANMAN, R. GIOVACCHINI, G. WOLCOTT and R. SCALA
Cosmetic, Toiletry, Fragrance Association, Washington, D.C.

and

M. KEPLINGER
Industrial Bio-Test Laboratories, Chicago, Ill., USA

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Abstract—Three oxidation hair dye formulations, mixed with hydrogen peroxide as in use, were tested for long-term toxicity and carcinogenic activity by topical application to groups of 100 mice weekly or every alternate week for 18 months. Chemical intermediates present in the various formulations were *p*-phenylenediamine, 2,5-toluenediamine sulphate, resorcinol, *m*-phenylenediamine, 2,4-diaminoanisole sulphate and 2,4-toluenediamine. The latter compound has been shown to produce malignant hepatomas when fed to rats in a sub-optimal diet at a level of 0·1% for 35 wk. In this study none of the formulations produced evidence of systemic toxicity or carcinogenicity.

INTRODUCTION

The natural vegetable and mineral substances used for centuries to colour the hair have largely been replaced by "permanent" synthetic oxidation dyes. As a result of many technical improvements, the use of oxidation hair dyes has increased dramatically in the last 20 yr, accounting for 75% of the hair colouring products sold today.

The dyes are formed inside the hair by the oxidation of colourless intermediates, principally aromatic diamines and aminophenols, to imines which react very rapidly with colour modifiers or couplers to form indo dyes (Corbett & Menkart, 1973). Although these reactions occur rapidly, it has been shown (Kiese & Rauscher, 1968) that in small amounts (less than 0·2% of the applied dose) unreacted intermediates may penetrate the skin during the dyeing of human hair.

Heavy industrial exposure to certain aromatic amines has caused bladder cancer in the past (Clayson & Cooper, 1970). Hueper (1963) has shown that although these tumours arose from prolonged skin absorption, there was no evidence of any effect of these chemicals on the skin itself. The reason for this now seems obvious in view of studies showing that aromatic amines appear to require metabolic conversion to the *N*-hydroxy derivative to induce cancer (Miller & Miller, 1969). We have found no published data demonstrating that *N*-hydroxylation occurs when the aromatic diamines used in permanent hair dyes are oxidized and there is no evidence indicating an increase in malignancies in the likely target organs in women using these dyes regularly for many years (National Cancer Institute, 1971).

Evidence that the use of hair dyes is not a significant factor in the production of scalp malignancies has been provided by Dr. F. Urbach, Skin and Cancer Hospital of the Temple University School of Medicine (personal communication 1973). Over a period of 15 yr, Dr. Urbach found only 16 persons (eight men and eight women) with scalp tumours out of 1672 consecutive patients with biopsy-proven cancer of the skin. This incidence of primarily sunlight-induced basal-cell carcinomas of the haired scalp area has not changed since the condition was first reported in 1878 (MacDonald & Bubendorf, 1964).

The purpose of this study was to determine whether any toxicity is associated with long-term cutaneous exposure to important ingredients or reaction products in oxidation hair dyes. Included in the study was one compound, *m*-toluene diamine, which has been shown to produce hepatoma in rats when fed at a level of 0·1% in a sub-optimal diet for 35 wk (Ito, Hiasa, Konishi & Marugami, 1969). This compound, which is no longer used in hair dyeing in the United States, was used for a number of years at a concentration of less than 1% in oxidation dye formulations. The extent of its skin penetration and metabolism has not been determined. Oxidation dyes, which are applied to the hair as a reacting system, can only be meaningfully evaluated from the toxicological viewpoint by the same route of administration as occurs in use, namely skin application.

In hair dyeing, the user is exposed to unreacted *para* components (*p*-phenylenediamine, *p*-aminophenol) and couplers (*m*-phenylenediamine, 2,4-diaminoanisole, *m*-aminophenol, resorcinol, hydroquinone), as well as to reactive intermediates, particularly quinone imines and the various indo dyes. The oxidation of the *para* components by hydrogen peroxide is relatively slow and has been shown to be incomplete even after 24 hr. However, the coupling reactions are extremely fast and thus there is no significant build-up in the concentration of the intermediate quinone imines. Nevertheless, it is important that the testing protocol should involve exposure to these extremely reactive species. This cannot be done by testing with the intermediates themselves since, in aqueous media, they undergo rapid polymerization and or hydrolysis (depending on the pH) to give products that do not arise in the oxidation dyeing process. Similarly, if aged reaction mixtures were used,

there would be little or no exposure to the quinone imines since they are no longer formed once the hydrogen peroxide is depleted.

The feeding of aged reaction mixtures may, at first sight, appear to be an attractive compromise. However, under conditions prevailing in the stomach (a pH around 2 and a temperature of about 38°C), the indo dyes would undergo rapid hydrolysis to give a mixture of *para* components, hydroxybenzoquinones and reaction products thereof. The quinone products would not arise in other metabolic pathways and the ingestion route is thus unrepresentative of the metabolism of indo dyes. Furthermore, no intermediate quinone imines would be involved in this mode of testing. Thus, from the chemical standpoint, topical application of fresh reaction mixtures is the only protocol involving exposure to all the materials that are inherent in the application of oxidation hair dyes to human subjects. This can only be achieved by careful choice of composites not by the use of individual precursors in the presence of peroxide. On the basis of these considerations, the protocols and test compositions used in this study were selected.

EXPERIMENTAL

Materials. The hair-dye intermediates used in these studies were obtained from several suppliers and either met standards as purchased or were subjected to further purification. *p*-Phenylenediamine (lot 1143) and resorcinol (lot 312), purchased from Nyanza, Inc., Lawrence, Mass., were over 99% pure. *p*-Toluenediamine (lot K38671) from Naftone, Inc., New York, assayed at 98·5%. The 2,4-toluenediamine and *m*-phenylenediamine from Eastman Kodak, Kingston, Tenn., were recrystallized from ethanol and isopropanol to a final purity of over 98%. On a moisture-free basis, the 2,4-diaminoanisole sulphate (lot A56442) from Lowenstein Inc., Brooklyn, New York, assayed at 96–97%.

Animals and diet. Random-bred Swiss Webster mice of both sexes, 6–8 wk old, from Charles River Laboratories were housed individually and were given Ralston Purina Laboratory Chow and water *ad lib.*

Experimental design and procedure

The mice were randomly allotted to nine groups each of 100 mice and one group of 250 mice. There were equal numbers of each sex in each group. The experimental design is shown in Table 1. Three oxidation hair-dye formulations were tested (Table 2), each formulation being mixed with an equal volume of 6% hydrogen peroxide just prior to use. Once weekly or once every other week for 18 months, 0·05 ml of the mixture was applied to the skin of the mid-scapular area, which was kept free of hair by shaving with an electric clipper. The dose was spread just enough to prevent run-off. After each application all unused material was discarded. The vehicle-control group received the base with no added dye intermediate. The positive-control group was housed in a separate room and received weekly applications of 0·05 ml of a solution of 7,12-dimethylbenz[*a*]anthracene (DMBA) in acetone.

It is not always possible in a topical study to select the single dose of the positive-control carcinogen that will give the best indication of the sensitivity of the test animal. One may simply apply an overwhelming dose producing a high incidence of tumours in a short period of time, or it may be more meaningful in terms of the usual human exposure to apply small doses over longer periods and thus achieve the best estimate of the minimal tumorigenic dose. Poel (1956) has shown that 125 μg benzo[*a*]pyrene applied topically to mice as a single dose is not carcinogenic in the normal life span of the mouse, while 1 μg administered three times weekly for 40 wk (total 120 μg) produced tumours in nine of 42 mice by wk 40. Klein (1956) has shown that as little as 1·2 μg DMBA applied topically to Swiss mice over a 10-month period caused a 13% incidence of tumours. In the study reported here, a split-dose schedule was used, with mice receiving 50 ng DMBA/wk for 6 months, followed by 10 μg/wk for 4 months and finally 50 μg/wk for the remainder of the study.

The mice were observed daily for signs of toxicity. Each week they were weighed, the skin graded for irritation and papillomas and other gross lesions were noted. The progress of papillomas and other lesions

Table 1. *Experimental design of 18-month carcinogenicity study of hair-dye formulations, involving topical application to mice*

Group	Test material*	No. of mice/group†	Dose‡ (ml)	Dosing frequency
UC	None	250		—
PC	DMBA	100	0·05	W
A	PP-7588	100	0·05	W
B	PP-7586	100	0·05	W
C	PP-7585	100	0·05	W
D	PP-7588	100	0·05	F
E	PP-7586	100	0·05	F
F	PP-7585	100	0·05	F
G	Vehicle	100	0·05	W
H	Vehicle	100	0·05	F

UC = Untreated control PC = Positive control
DMBA = 7,12-dimethylbenz[*a*]anthracene
W = Weekly F = Fortnightly

*For identification of 'PP' samples, see Table 2.

†Each group consisted of equal numbers of males and females.

‡The applied dose of the hair-dye was 0·05 ml of a 1:1 mixture of the formulation and 6% hydrogen peroxide. The concentration of the positive control (DMBA in acetone) was adjusted so that 0·05 ml provided a weekly dosage of 50 ng DMBA for 6 months, 10 μg for 4 months and 50 μg for 8 months.

Table 2. *Experimental hair-dye formulations tested in an 18-month carcinogenicity study in mice*

Ingredient	Level (%) in experimental formulation		
	PP-7588	PP-7586	PP-7585
Oleic Acid	5·00	5·00	5·00
Isopropanol	3·00	3·00	3·00
Sodium sulphite	0·20	0·20	0·20
Ammonia (29%)	6·00	6·00	6·00
2,5-Toluenediamine sulphate	3·00	3·00	3·00
p-Phenylenediamine	1·50	1·50	1·50
Resorcinol	0·40	0·40	0·40
2,4-Toluenediamine base	0·20	—	—
2,4-Diaminoanisole sulphate	—	0·38	—
m-Phenylenediamine base	—	—	0·17
Deionized water	80·70	80·70	80·70

was charted monthly. Animals that died or that were killed because of general debility were autopsied and examined histopathologically when possible.

At the termination of the study all survivors were weighed and killed and a gross autopsy was performed. A sample of blood taken from a random selection of at least four mice of each sex in each group, except those groups treated only every other week, was analysed for haematocrit and haemoglobin, and mean corpuscular volume and mean corpuscular haemoglobin were calculated. A complete blood count, including differential, platelet and reticulocyte counts, was done. The livers of ten animals in each group were weighed and liver/body weight ratios were calulated. From each animal were taken sections of skin, thyroid, lung, gastro-intestinal tract (three sections), spleen, pancreas, liver, kidneys, adrenals, urinary bladder, ureter, mesenteric lymph nodes, sternal bone marrow and gonads. These tissues were fixed in 10% buffered formalin. Slides were prepared from haematoxylin/eosin-stained tissues of 13 male and 13 female mice in each test, vehicle-control and positive-control group and of 32 mice of each sex in the untreated control group and were examined microscopically for evidence of hair dye-induced toxicity. Also, every gross tumour-like lesion, regardless of location, was examined microscopically and classified.

RESULTS AND DISCUSSION

There were no overt signs of systemic toxicity in any of the dye-treated groups. The survival varied from 58 to 80%, except in the positive controls in which only 21% of the mice were alive after 18 months. Average body weights were comparable in all groups throughout the study. The absolute and relative liver weights were in the range of normal values for all groups and none of the differences were statistically significant by analysis of variance (Snedecor, 1966). All of the haematological data recorded were within normal limits. Table 3 shows mean terminal body weights, liver/body weight ratios and the percentage survival for each group.

Disease processes commonly seen in aged mice of this random-bred strain were observed in all groups with essentially the same frequency. These included chronic murine pneumonia, amyloidosis, chronic

Table 3. *Survival, mean terminal body weights and liver weight/body weight ratios in mice treated for 18 months with topically applied hair-dye formulations*

Group*	Survival of 18 months (%)	Terminal body weight (g)†		Liver weight/ body weight ratio†
		Males	Females	
UC	78	37·8	34·5	0·060
PC	21	40·6	34·5	0·058
A	62	36·0	31·3	0·066
B	65	34·8	31·1	0·067
C	66	37·1	33·0	0·060
D	68	37·7	32·7	0·056
E	69	37·4	35·0	0·053
F	80	36·2	32·5	0·062
G	59	37·4	30·8	0·064
H	62	36·8	33·1	0·062

*For identification of groups, see Table 1.

†Values for body weights are means for all survivors and those for liver weight expressed relative to body weight are means for ten mice in each group.

Table 4. *Incidence of alveologenic adenomas and carcinomas in mice treated topically with hair-dye formulations for 18 months*

Group*	No. of mice/group	Sex	No. of mice with lung tumours	
			Alveologenic adenoma	Alveologenic carcinoma
UC	125	M	23	4
	125	F	14	2
PC	50	M	2	1
	50	F	5	2
A	50	M	4	1
	50	F	8	1
B	50	M	6	0
	50	F	5	2
C	50	M	8	1
	50	F	7	1
D	50	M	6	1
	50	F	3	1
E	50	M	9	1
	50	F	13	0
F	50	M	18	2
	50	F	9	1
G	50	M	12	1
	50	F	8	1
H	50	M	11	2
	50	F	8	4

*For identification of groups, see Table 1.

focal nephritis, focal lymphoid infiltration in lungs, liver and kidneys and testicular atrophy. The incidence of alveologenic adenomas and adenocarcinomas (Table 4) was comparable among the various control and test groups and was within the range of control values for this strain of mice (Percy & Jonas, 1971). The type and distribution of all other tumours in the groups treated weekly and in the untreated, positive-control and vehicle-control groups are shown in Table 5.

For the first 5 months of the study, moderate alopecia occurred in about 50% of the animals of each sex in each of the three groups receiving the hair dyes once weekly. This was not seen in the groups treated every other week or in the vehicle or positive controls. The condition diminshed with time and after 11 months hair growth appeared normal. Microscopic examination of sections taken at the final autopsy showed that the skin and its appendages were normal in all mice in these groups. There were no papillomas or carcinomas of the skin except those in the positive control group. The other tumours of the skin, in whatever groups they occurred, were all outside the treated area.

The skins of the mice in the positive control group showed no response following the weekly application of 50 ng of the carcinogen for 6 months followed by 10 μg/wk for an additional 4 months. However, when the dose was increased to 50 μg/wk, there was an almost immediate response and tumours appeared on six animals. This dose proved to be irritating to the skin and mortality increased sharply from 18 to 46% in 3 months. Although the response was somewhat less than expected, it did serve to demonstrate the susceptibility of the animals to the action of the carcinogen.

No evidence of carcinogenic activity was seen in the hair dyes tested in this study. The results of a companion study which was carried out by the US Food and Drug Administration and which included groups of mice receiving topical applications of 3% *m*-toluenediamine in water/isopropanol, were also

Table 5. *Distribution of tumours other than alveologenic adenomas and carcinomas in untreated and positive controls and in mice treated once weekly with hair dyes*

		No. of mice affected and location of tumours in group*					
		UC	PC	A	B	C	G
Type of tumour	No. of mice/group...	250	100†	100	100	100	100
Lymphosarcoma		1 M. liver 1 M. kidney 1 F. lung	1 F. spleen 2 F. general				
Fibrosarcoma		1 M. dermis 1 F. dermis	1 M. dermis			1 F. dermis	
Fibroma		1 F. dermis 1 F. cervix 1 F. body wall		1 F. body wall		1. prostate 1 F. dermis	
Adenocarcinoma		2. mammary gland				2. mammary gland	3. mammary gland
Granulosa-cell tumour		1. ovary					
Leiomyosarcoma		1. uterus	1. uterus				
Reticular-cell sarcoma			1 M. kidney 1. prostate	1 F. liver, spleen, lung, lymph node 1 F. liver, lymph node			1 M. liver
Myxofibroma			1. uterus				
Squamous carcinoma			1 F. skin and lung				
Papilloma			2 M. skin				
Adenoacanthoma						1. mammary gland	
Osteogenic sarcoma							1 F. lung
Serosal fibroma							1 F. stomach

*For identification of groups, see Table 1.
†Two positive-control mice with skin papillomas in the treated area were lost as a result of death and autolysis.

negative for carcinogenicity and will soon be published (H. Blumenthal, personal communication 1974).

Included in these studies was a compound previously shown to be a potent hepatocarcinogen when fed to rats. This raises the question again of the relevance of testing topical products by oral administration and of the significance of studies showing the toxic effects of prolonged oral dosing with high levels of ingredients of products that have been shown to be safe when tested under reasonably exaggerated conditions of use. It is often argued that oral administration is the means of choice to achieve the exaggerated dosage levels necessary to detect, in small numbers of test animals, effects that may occur in a few exposed individuals in a large population. This oral dosing frequently assumes massive proportions bearing no reasonable relationship to normal exposure and is often limited only by the amount the animal can tolerate while remaining compensated and apparently normal. This kind of testing may give false and misleading information for a number of reasons: (1) Blood levels will undoubtedly occur that would never be seen in product use, and may result in abnormal metabolism and pathology; (2) due to the effects of pH and gut bacteria, there could be absorption of molecular species that would never occur with topical application; (3) metabolic conversion to a more (or less) toxic substance may occur, or may occur at a rate quite different from that following topical application; (4) chemical reaction with essential nutrients in the ingesta can produce toxicity as a result of deficiencies and imbalances that bear no relationship to any inherent toxicity of the compound tested; (5) the normal gut flora may be drastically altered causing additional nutritional problems; (6) the target organs and excretory patterns may not be representative of those occurring during normal exposure. As indicated in the Introduction, these considerations have particular relevance in the case of oxidative hair dyes. They are, however, valid also for other systems.

Conclusions

No evidence of toxicity, or carcinogenicity, was seen when several oxidative hair-dye formulations were tested in an 18-month topical study in mice. Included in the formulations were 2,5-toluenediamine sulphate, *p*-phenylenediamine, resorcinol, 2,4-diaminoanisole sulphate, *m*-phenylenediamine and 2,4-toluenediamine. The latter compound has produced liver cancer when fed to rats in a sub-optimal diet. Safety evaluation studies of products must involve actual conditions of use to produce meaningful results.

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DERMAL CARCINOGENICITY STUDY BY MOUSE-SKIN PAINTING WITH 2,4-TOLUENEDIAMINE ALONE OR IN REPRESENTATIVE HAIR DYE FORMULATIONS

Albert L. Giles, Jr., Choong W. Chung

Division of Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D.C.

Choudari Kommineni

Pathobiology Research Branch, Experimental Biology Laboratory, National Environmental Research Center, Environmental Protection Agency, Research Triangle Park, North Carolina

The chronic toxicity of 2,4-toluenediamine (2,4-TDA) alone or in combination with a hair dye complex (2,5-toluenediamine, p-phenylenediamine, and resorcinol) was studied in Swiss-Webster mice of both sexes by a skin-painting technique. The predominant neoplasms seen in these mice were primary pulmonary adenomas and adenocarcinomas. Skin neoplasms were seen in most groups of mice, including untreated control mice. Statistical analysis of the incidences of skin neoplasms among the various groups of mice did not show any significant differences. The 2,4-TDA alone or mixed with the hair dye complex did not produce any abnormal proliferation and maturation of the squamous epithelium of the skin. The 2,4-TDA under our experimental conditions was found to be nontoxic and noncarcinogenic to the skin of mice.

INTRODUCTION

Aromatic diamines are used as human hair dyes; one such dye is 2,4-toluenediamine (*m*-toluylenediamine; 2,4-TDA). Umeda (1955) reported that repeated subcutaneous injections of 2,4-TDA induced rhabdomyosarcomas in 100% of rats treated. Rats fed diets containing 2,4-TDA developed hepatocellular carcinomas (Ito et al., 1969).

To assess the carcinogenicity and toxicity of 2,4-TDA and other cosmetic ingredients of hair dyes when used topically, a 2-yr mouse-skin-painting study was conducted. Both use levels and exaggerated concentrations of 2,4-TDA were tested.

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Requests for reprints should be sent to Albert L. Giles, Jr., Division of Toxicology, Food and Drug Administration, Bureau of Foods HFF-154, 200 C Street, S.W., Washington, D.C. 20204.

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MATERIALS AND METHODS

Preparation of Chemicals

Formulations containing 0.2 or 0.6% 2,4-TDA in hair dye complex and hair dye base, hair dye base alone, and 6% peroxide were provided by the Cosmetic, Toiletry and Fragrance Association, Inc. (CTFA), Washington, D.C.; these samples were representative of formulations marketed in the United States. Compositions of the complex and base are given in Table 1. The concentration of 2,4-TDA in the formulations was determined monthly for 12 months by a gas-liquid chromatographic method (Goldstein et al., 1968) and remained essentially constant. For mouse-skin painting, 2,4-TDA formulations and base alone were diluted with an equal volume of 6% peroxide and were used within 10 min.

Standard 2,4-TDA[1] was prepared as a 6% solution by dissolving 400 mg in 6.5 ml of water-isopropanol[2] (75.1:11.1) in a 20-ml vial with continuous stirring for 2–4 hr over a magnetic stirrer. Aliquots were diluted with an equal volume of either distilled water or 6% peroxide for use in skin painting. Water-isopropanol mixed with an equal volume of 6% peroxide was used to paint the skin of a control group.

9,10-Dimethylbenzanthracene (DMBA),[3] the positive control compound, was prepared as 0.02 and 0.005% solutions in acetone.[4] These solutions were stored at 40°F and used as needed. Acetone from the same stock bottle was used to paint the skin of a control group.

[1] Supplied by the Clairol Co., New York, N.Y., which obtained it from the general market.
[2] Reagent grade, Fisher Scientific Co., Pittsburgh, Pa.
[3] ICN-K&K Laboratories, Inc., Plainview, N.Y.
[4] Reagent grade, J. T. Baker Chemical Co., Philadelphia, Pa.

TABLE 1. Composition of 2,4-Toluenediamine Hair Dye Formulations

Ingredient	Weight (%)
Dye complex	
2,5-Toluenediamine sulfate	3.00
p-Phenylenediamine	1.50
Resorcinol	0.40
2,4-Toluenediamine	0.20 or 0.60
Base	
Oleic acid	5.00
Isopropanol	3.00
Sodium sulfite	0.20
29% Ammonia	6.00
Deionized water	80.70 or 80.30
Total	100

TABLE 2. Treatment Protocol

Group no.	Treatment[a]	Dose (μg/wk) (2,4-TDA or DMBA)	Initial number of mice	
			Males	Females
1	0.2% 2,4-TDA in hair dye complex in base + P	50	28	28
2	0.6% 2,4-TDA in hair dye complex in base + P	150	28	28
3	Hair dye base + P	0	28	28
4	6.0% 2,4-TDA in WI + distilled water	1,500	28	28
5	6.0% 2,4-TDA in WI + P	1,500	28	28
6	WI + P	0	28	28
7	0.02% 9,10-DMBA in acetone	10	28	28
8A	Acetone	0	14	14
8B	0.005% 9,10-DMBA in acetone	2.5	28	28
9	Untreated controls (no hair clipped)	0	76	17

[a] Skin painting was done once a week. Abbreviations: 2,4-TDA = 2,4-toluenediamine; DMBA = dimethylbenzanthracene; P = 6% peroxide; WI = water-isopropanol (75.1:11.1).

Animals and Treatments

Swiss-Webster mice of both sexes, obtained from the National Institutes of Health mouse colony, were 4–7 wk old and weighed 15–20 g at the beginning of the experiment. The mice were allotted to nine groups consisting of 28 mice of each sex, except as noted in Table 2. Each mouse was housed individually in a plastic, shoe box type cage kept in a room maintained at 74 ± 2°F and was weighed weekly for the first year. Food[5] and water were available *ad libitum*.

The hair in the intrascapular region of each mouse was closely clipped with an electric clipper[6] each week, one day prior to painting. Each week, 0.05 ml of the appropriate test or control solution (Table 2) was applied to this region by using a tuberculin syringe without a needle. Each mouse was kept under a hair dryer[7] without heat to dry the applied solution before being returned to the cage. When signs of marked irritation, ulceration, or tumor formation were evident, the application of the chemical was discontinued until the skin looked "normal." To minimize variations in treatment, the order of painting was adjusted so that a mouse painted first in one week would be painted last the next week and vice versa. The hair of untreated controls was not clipped.

[5] Purina Laboratory Chow for Mice, Rats, and Hamsters, Ralston Purina Co., St. Louis, Mo.
[6] Model A2, size 40, Oster Corp., Milwaukee, Wis.
[7] Model 202, Oster Corp., Milwaukee, Wis.

Histopathology

Tissue specimens were taken from all major organ systems and tumors of mice found dead during the study (if not markedly autolyzed) or sacrificed when moribund or at 2 yr, the termination of the experiment. An overdose of ether was used to sacrifice the animals. These specimens were fixed in buffered 10% formaldehyde. Sections were cut 6 μm thick from the paraffin blocks and stained routinely with H&E before histopathology examination.

Complete histopathology examinations were performed on the following organs: skin, spleen, liver, bone marrow, lungs, myocardium, pancreas, kidney, bladder, stomach, large and small intestines, testis and seminal vesicle of males, ovary and uterus of females, pituitary, thyroid, adrenal, and brain.

The following special stains were used to resolve pathology diagnostic problems: phosphotungstic acid-hematoxylin (PTAH), Masson, Van Gieson, Alcian blue, periodic acid Schiff (PAS), methyl green pyronine, toluidine blue, and von Kossa's stain.

RESULTS

Body weight gains of mice in treated groups were not significantly different from those of mice in the untreated control groups. The survival rate of all mice was erratic and did not show sex dependency. From group 8A, which was treated with acetone only, tissues of five female mice were subjected to histopathology examinations. In the untreated control group, although there was a disproportionately large number of male mice (76) at start of the experiment, only 21 males were subjected to histopathology minations.

Two different concentrations of DMBA were used in this study. The 0.02% solution of DMBA produced marked irritation of the skin of mice; ulceration and 15% mortality were observed within 4 wk after the start of the experiment. The utilization of 0.005% DMBA solution obviated these problems, and the compound at this concentration proved to be a potent skin carcinogen. These observations confirm our earlier results (Giles and Byron, 1968).

The male and female mice in all groups developed both malignant and benign neoplasms (Table 3). There were no statistical differences between the sexes in the total number of neoplasms or in the incidence of neoplasms of a particular organ or type.

Primary lung neoplasms predominated in all groups except in the positive control groups 7 and 8B (Table 4) and were either adenomas or carcinomas. The incidences of skin neoplasms are given in Table 5; these neoplasms included squamous cell carcinomas, fibrosarcomas, myxosarcomas, myoepithelioma, liposarcoma, osteogenic sarcoma (untreated control group 9), and other types. Of the total number of neoplasms in the DMBA-treated

TABLE 3. Incidence of Malignant and Benign Neoplasms in Male and Female Swiss-Webster Mice (2,4-TDA Skin Carcinogenicity Study)

	Group no. [a]																			
	1		2		3		4		5		6		7		8A		8B		9	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No. of mice examined	19	13	19	15	13	17	21	9	11	14	16	14	17	13	14	5	11	13	21	12
Total no. of neoplasms	15	10	11	9	12	14	20	8	4	16	10	4	14	16	7	3	12	15	10	3
No. of mice with neoplasms	11	7	10	6	10	13	15	7	4	12	9	4	13	13	7	3	10	9	9	3
No. of mice with multiple neoplasms	3	3	1	2	2	1	5	1	0	3	1	0	1	3	0	0	2	5	1	0
No. of malignant neoplasms	10	7	7	5	8	8	12	4	4	10	5	1	12	15	6	1	10	12	7	3
No. of benign neoplasms	5	3	4	4	4	6	8	4	0	6	5	3	2	1	1	2	2	3	2	0

[a] See Table 2 for description of treatment.

TABLE 4. Incidence of Primary Lung Neoplasms in Male and Female Swiss-Webster Mice (2,4-TDA Skin Carcinogenicity Study)

	Group no.[a]																			
	1		2		3		4		5		6		7		8A		8B		9	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No. of mice examined	19	13	19	15	13	17	21	9	11	14	16	14	17	13	14	5	11	13	21	12
Total no. of lung neoplasms	10	3	8	5	9	10	17	6	4	8	7	2	2	4	5	2	4	3	6	3
No. of mice with lung neoplasms	10	3	8	4	9	10	15	6	4	8	7	2	2	4	5	2	4	3	6	3
No. of malignant lung neoplasms	7	2	6	2	7	4	11	3	4	5	4	0	1	3	4	0	2	3	6	3
No. of benign lung neoplasms	3	1	2	3	2	6	6	3	0	3	3	2	1	1	1	2	2	0	0	0

[a] See Table 2 for description of treatment.

TABLE 5. Incidence of Primary Skin Neoplasms in Male and Female Swiss-Webster Mice (2,4-TDA Skin Carcinogenicity Study)

	Group no.[a]																			
	1		2		3		4		5		6		7		8A		8B		9	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
No. of mice examined	19	13	19	15	13	17	21	9	11	14	16	14	17	13	14	5	11	13	21	12
Total no. of skin neoplasms	2	2	–	1	1	2	0	0	0	3	0	0	12	10	0	0	8	4	2[b]	0
No. of mice with skin neoplasms	2	2	–	1	1	2	0	0	0	3	0	0	12	10	0	0	8	3	2	0
No. of malignant skin neoplasms	2	2	–	1	1	2	0	0	0	3	0	0	12	10	0	0	8	4	1	0
No. of benign skin neoplasms	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0

[a] See Table 2 for description of treatment. Dashes indicate that mice were not examined.
[b] Neoplasm seen at the angle of the lower jaw was considered to be of dermal origin.

oups 7 and 8B, 73 and 44%, respectively, were skin neoplasms; statistical alysis of the incidence of skin neoplasms among all other groups did not show any significant differences. The adrenal glands, the reticuloendothelial system, and the female reproductive organs developed a few neoplasms.

DISCUSSION

Swiss-Webster mice manifest a high incidence of spontaneous lung neoplasms (Stoats, 1972; C. Kommineni, unpublished data, 1972). Statistical analysis of the incidence of lung neoplasms among the groups in this study did not show any significant differences; thus the 2,4-TDA and other hair dye ingredients did not augment the development of primary lung neoplasms. The incidence of skin neoplasms did not show statistically significant differences in any of the groups under test except for the positive control groups 7 and 8B (Table 5). Moreover, no deleterious effects, including signs of skin irritation, abnormal proliferation and maturation of squamous epithelium of the skin, or changes in the roots of the hair shafts, were seen in the skin sections examined from the painted mice exhibiting no skin neoplasms. The absence in these mice of the type of neoplasms that occur in rats treated with 2,4-TDA (Umeda, 1955; Ito et al., 1969) is of particular interest. The difference is probably due to (1) mode of treatment, (2) quality of diet, and (3) the species of animals used; the route of administration may be most important. 2,5-Toluenediamine and 2,4-diaminoanisole, which are similar hair dyes, did not cause any skin toxicity or carcinogenicity when given to rats by the percutaneous route (Kinkel and Holzmann, 1973). A chronic skin-painting study in mice with 2,4-TDA, -diaminoanisole, or 2,4-phenylenediamine in hair dye formulations failed to show evidence of toxicity or carcinogenicity in mice (CTFA, personal communication). Our present results in mice are in accord with these reports.

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Lack of Toxicity and Carcinogenicity of Some Commonly Used Cutaneous Agents[1,2]

FREJ STENBÄCK AND PHILIPPE SHUBIK

Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105

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Lack of Toxicity and Carcinogenicity of Some Commonly Used Cutaneous Agents. STENBÄCK, F. AND SHUBIK, P. (1974). *Toxicol. Appl. Pharmacol.* 30, 7–13. The potential carcinogenicity and toxicity of several commonly used cutaneous agents (benzophenone, propylene glycol, isopropyl myristate, resorcinol, 2-ethyl-1,3-hexanediol, *p*-aminobenzoic acid and pyrogallol) were studied in female Swiss mice by administering repeated applications of the chemicals on the skin for the life-span of the animals. Tumors seen in both control and treated animals were mainly lymphomas, hemangiomas of the liver and lung adenomas, as well as tumors of other organs. A statistically significant increase in tumor incidence caused by the chemical treatment was not seen. Skin lesions, slight inflammation and ulceration were seen, but no persistent cutaneous abnormalities occurred. A few skin tumors were seen in treated areas as well as in untreated areas and in control animals. Thus a carcinogenic or toxic potential which would affect the use of these agents in man was not detected.

Skin cancers are one of the largest groups of environmentally induced tumors; both radiation (sunlight) and certain chemicals are responsible (Hueper, 1942). These tumors may be reproduced readily in animal models as demonstrated with coal tar and petroleum oil constituents (Hartwell, 1951; Shubik and Hartwell, 1969).

Because regulations governing the testing of externally used chemicals are less extensive than those governing drugs, little is known about their toxicity and possible carcinogenicity. In this study, 7 compounds that may come into contact with human skin were selected because of their use as solvents, insect repellents, fixatives, sun-protecting agents, dispersing agents, as well as chemicals used in photographic developing and dye manufacture. These agents were applied to mouse skin, and local changes, systemic response and tumor occurrence were noted.

METHODS

Chemicals. Benzophenone, propylene glycol, resorcinol, 2-ethyl-1,3-hexanediol,[3] *p*-aminobenzoic acid, isopropyl myristate and pyrogallol[4] and 7,12-dimethylbenz-

[1] This work was supported by Public Health Service contract PH-43-68-959 from the National Cancer Institute.

[2] Part of this work was reported at the Twelfth Annual Meeting of the Society of Toxicology, New York City, March 18–22, 1973.

[3] Purchased from Aldrich Chemical Company, Milwaukee, Wisconsin.

[4] Obtained from Nutritional Biochemical Company, Cleveland, Ohio.

TABLE 1

NUMBER OF DIFFERENT TYPES OF HISTOLOGICALLY VERIFIED TUMORS IN SWISS MICE TREATED WITH DIFFERENT CHEMICALS

Treatment (50 mice concentration)	% Concentration (in acetone)	Total No. of tumor-bearing animals	% of tumor-bearing animals	Total No. of tumors	Lymphomas	Lung adenomas	Liver hemangiomas	Thymomas	Skin tumors	Other
Pyrogallol	50	22	44	28	11	8	3	—	—	6[a]
	25	18	36	23	12	6	3	—	—	2[b]
	5	25	50	30	20	4	1	—	—	5[c]
2-Ethyl-1,3-hexanediol	100	32	64	40	14	11	9	—	2	5[d]
	50	28	56	34	17	7	2	—	2	6[e]
	10	23	46	28	11	5	1	—	1	1[f]
Propylene glycol	100	20	40	23	9	7	4	1	—	2[g]
	50	26	52	31	13	13	2	2	—	1[h]
	10	26	52	30	15	7	1	1	—	6[i]
Isopropyl myristate	100	21	42	27	14	7	3	1	—	2[j]
	50	25	50	30	13	6	4	2	1	4[k]
	10	21	42	25	13	5	2	1	1	4[l]
p-Aminobenzoic acid	10	20	40	24	8	8	5	2	—	1[m]
	5	22	44	26	16	4	1	3	1	2[n]
	1	18	36	25	8	8	2	5	—	2[o]
Resorcinol	50	27	54	37	18	7	4	—	—	8[p]
	25	22	44	24	13	6	—	—	1	4[q]
	5	20	40	29	16	5	1	—	1	5[r]
Benzophenone	50	14	28	18	6	6	2	—	—	4[s]
	25	16	32	22	11	3	1	1	1	5[t]
	5	26	52	38	15	3	1	1	2	16[u]
Acetone	100	22	44	31	12	9	2	—	2	6[v]
DMBA	0.5	39	78	86	6	4	1	—	75	—
Untreated control (150 mice)	—	64	42	72	26	17	4	6	3	16[w]

[a] 2 mammary fibroadenomas, 2 mammary adenocarcinomas, 1 subcutaneou[illegible]angioma, 1 ovarian [illegible]
[b] 2 mammary adenocarcinomas.
[c] 1 ovarian hemangioma, 1 ovarian cystadenoma, 1 lung adenocarcinoma, 1 hemangioma of the ear, 1 ma[illegible]oadenoma.
[d] 2 forestomach papillomas, 1 subcutaneous fibroma, 2 subcutaneous fibrosarcomas.
[e] 2 mammary adenocarcinomas, 1 mammary fibroadenoma, 1 adnexal carcinoma of the eye, 1 subcutaneous [illegible]enoma, 1 spleen hemangioma.
[f] 1 forestomach papilloma.
[g] 2 mammary adenocarcinomas.
[h] 1 forestomach papilloma.
[i] 1 ovarian cystadenocarcinoma, 1 ovarian fibroma, 1 ovarian hemangioma, 2 subcutaneous fibromas, 1 forestomach papilloma.
[j] 1 subcutaneous fibroma, 1 adnexal adenocarcinoma of the eye.
[k] 1 forestomach papilloma, 1 mammary fibroadenoma, 2 ovarian hemangiomas.
[l] 2 ovarian hemangiomas, 1 mammary fibroadenoma, 1 forestomach papilloma.
[m] 1 forestomach papilloma.
[n] 1 subcutaneous fibromyxoma, 1 cecal adenocarcinoma.
[o] 1 ovarian hemangioma, 1 subcutaneous fibroma.
[p] 2 mammary fibroadenomas, 2 mammary adenocarcinomas, 1 uterine leiomyosarcoma, 1 ovarian fibroma, 1 uterine hemangioma, 1 subcutaneous fibroma.
[q] 1 forestomach papilloma, 1 subcutaneous fibrosarcoma, 1 mammary adenocarcinoma, 1 ovarian hemangioma.
[r] 1 ovarian hemangioma, 1 spleen hemangioma, 1 subcutaneous hemangioma, 1 ovarian fibroma, 1 mammary fibroadenoma.
[s] 2 spleen hemangiomas, 1 retroperitoneal fibromyxoma, 1 ovarian cystadenoma.
[t] 2 ovarian fibromas, 1 spleen hemangioma, 1 ovarian hemangioma, 1 subcutaneous fibrosarcoma.
[u] 3 spleen hemangiomas, 3 ovarian hemangiomas, 2 mammary adenocarcinomas, 1 mammary fibroadenoma, 1 subcutaneous hemangioma, 2 subcutaneous fibrosarcomas, 2 uterine adenomas, 1 uterine adenocarcinoma, 1 retroperitoneal fibromyxoma, 1 vaginal hemangioma.
[v] 1 mammary fibroadenoma, 1 mammary adenocarcinoma, 2 ovarian hemangiomas, 1 subcutaneous fibrosarcoma, 1 forestomach papilloma.
[w] 1 mammary fibroadenoma, 2 mammary adenocarcinomas, 1 subcutaneous fibroma, 3 subcutaneous fibrosarcomas, 1 subcutaneous hemangioma, 1 retroperitoneal fibromyxoma, 2 renal adenomas, 2 ovarian hemangiomas, 2 ovarian fibromas, 2 forestomach papillomas.

anthracene[5] (DMBA) were dissolved in acetone.[5] The concentrations are shown in Table 1. The chemicals were used as obtained from the manufacturer.

Animals. Seven-week-old female Swiss mice from the Eppley colony were used. The animals were randomized prior to the start of the experiments, and the littermates were separated. There were 50 animals per concentration group. Animals were housed in plastic cages with commercial bedding,[6] 10 per cage, and fed a commercial diet[7] and water ad libitum. One hundred thirty-five untreated animals served as controls, as well as 50 animals treated with the solvent alone and 50 animals treated with 7,12-dimethylbenzanthracene as a positive control.

Treatment. The chemicals (0.02 ml) were dropped on the dorsal skin between the flanks twice a week on a 1-inch square area which was shaved regularly. The animals were checked weekly, and all lesions, as well as tumors, were recorded. Animals were allowed to die spontaneously or were killed when moribund. Complete autopsies were performed on all animals. The skin from all animals, all grossly observed tumors and other lesions in the lungs, livers, kidneys, etc., from treated as well as control groups were studied histologically. Formalin-fixed paraffin-embedded specimens were cut and stained with hematoxylin-eosin and other stains when needed. The statistical significance of the results was evaluated using the methods presented by Armitage (1971).

RESULTS

Animals treated with pyrogallol showed no significant changes in the skin. Hyperplasia, ulceration or inflammation did not occur. The number of tumors produced was not significantly different from that in untreated controls (Table 1). Skin tumors were not found. The number and distribution of the tumors were similar to that in other groups. Lymphomas, lung adenomas and liver hemangiomas were the most common tumors. The survival rate was slightly lower than in the untreated control group, but comparable to other groups (Table 2).

Animals treated with 2-ethyl-1,3-hexanediol had a 46-64% tumor incidence which was higher than for untreated controls (42%). However, this incidence was not specifically related to tumor type (Table 1).

Isopropyl myristate led to 2 skin tumors, 1 on the abdomen and 1 on the eyelid Table 3).

Animals treated with resorcinol showed skin lesions with ulceration, inflammation and hyperplasia. Two skin tumors were seen, 1 on the back and 1 on the ear as well as 1 subcutaneous fibrosarcoma.

Animals treated with benzophenone had a small number of skin tumors. One animal receiving the 25% concentration had a tumor on the lip and 2 animals treated with the 5% concentration had tumors on the dorsal skin. In animals receiving repeated applications of DMBA a large number of skin tumors were seen--squamous cell papillomas, keratoacanthomas and squamous cell carcinomas (Table 2). The number of tumors of other organs was small owing to the short life-span of the animals (Table 3) as a result of the skin tumors.

[5] Fisher Scientific Company, Fairlawn, New Jersey.
[6] San-i-cel, Paxton Processing Company, Inc., Paxton, Illinois.
[7] Wayne, Allied Mills, Chicago, Illinois.

TABLE 2

SURVIVAL RATE OF SWISS MICE TREATED WITH DIFFERENT CHEMICALS

Treatment (50 mice concentration)	Concentration* (%)	Weeks												
		10	20	30	40	50	60	70	80	90	100	110	120	
Pyrogallol	50	50	49	49	46	45	27	24	21	14	2	0	—	
	25	50	49	48	44	42	35	27	17	14	5	0	—	
	5	48	47	46	45	41	31	26	19	11	6	0	—	
2-Ethyl-1,3-hexanediol	100	50	50	50	49	46	44	32	27	18	10	3	0	
	50	50	50	49	48	47	44	39	33	23	13	1	0	
	10	50	50	49	48	48	44	38	29	15	6	2	0	
Propylene glycol	100	50	50	50	49	46	41	39	28	16	10	2	0	
	50	50	50	50	49	45	42	33	29	20	8	1	0	
	10	50	49	49	48	45	37	30	18	13	6	1	0	
Isopropyl myristate	100	50	50	49	48	45	39	29	22	13	7	0	—	
	50	50	50	47	44	41	39	32	23	19	8	3	0	
	10	49	48	47	47	46	39	29	22	12	4	2	0	
p-Aminobenzoic acid	10	50	50	49	47	45	44	37	28	19	12	2	0	
	5	50	50	48	46	45	42	36	29	21	9	3	0	
	1	50	49	46	43	43	38	32	23	15	2	0	—	
Resorcinol	50	50	50	49	46	45	39	34	26	16	6	0	—	
	25	50	50	49	48	45	32	30	26	23	8	0	—	
	5	50	50	50	50	48	30	27	16	7	5	0	—	
Benzophenone	50	47	40	39	33	30	28	18	14	7	4	0	—	
	25	49	49	48	41	37	26	18	11	6	2	0	—	
	5	47	47	47	46	43	37	24	19	13	4	0	—	
Acetone	100	50	49	48	48	46	44	36	25	18	12	3	0	
Dimethylbenzanthracene	0.5	50	45	33	0									
Untreated control (150 animals)		150	148	146	142	138	123	111	82	62	35	9	3	0

* Dissolved in acetone.

TABLE 3

NUMBER AND LOCATION OF DIFFERENT TUMORS IN SWISS MICE IN RELATION TO TREATMENT

Treatment (50 mice/concentration)	Concentration[a] (%)	Location	Histopathology
2-Ethyl-1,3-hexanediol	100	1 on dorsal skin, 1 on tail	Squamous cell papillomas
	50	1 on dorsal skin, 1 on ear	Squamous cell carcinomas
	10	1 on eyelid	Squamous cell papilloma
Isopropyl myristate	50	1 on eyelid	Keratoacanthoma
	10	1 on abdominal skin	Squamous cell papilloma
p-Aminobenzoic acid	5	1 on ear	Squamous cell carcinoma
Resorcinol	25	1 on ear	Squamous cell carcinoma
	5	1 on dorsal skin	Squamous cell papilloma
Benzophenone	25	1 on lip	Squamous cell carcinoma
	5	2 on dorsal skin	Squamous cell papillomas
Acetone	100	1 on tail, 1 on ear	Squamous cell papillomas
Dimethylbenzanthracene	0.5	31 on dorsal skin	Squamous cell papillomas
	0.5	26 on dorsal skin	Squamous cell carcinomas
	0.5	11 on dorsal skin	Keratoacanthomas
Untreated control (150 animals)		1 on ear	Squamous cell carcinoma
		1 on dorsal skin, 1 on tail	Squamous cell papillomas

[a] Dissolved in acetone.

DISCUSSION

None of the chemicals studied produced a statistically significant increase in skin tumor incidence, when compared to untreated controls, and to those seen after repeated applications of DMBA. The skin tumor incidence seen in the treated animals (2-4%) was similar to that of the acetone control and untreated control animals. Also, a significant percentage of the tumors were on the tail, ear, eyelid, lip and abdomen, which did not have direct contact with the chemicals, this speaks against a causal relationship between tumor incidence and treatment. The possibility exists that the mice rub against each other, which might spread the chemical. However, in the test groups 72% were outside the treated area, in the positive control only 8%. Thus from our results it cannot be concluded that any of the chemicals studied are carcinogenic for skin. Swiss mice are very sensitive to skin tumors as seen in the positive control group, which shows a 78% skin tumor incidence after painting with a known chemical carcinogen such as DMBA 10 μg twice weekly.

The chemicals administered did not produce lesions in other organs directly related to the treatment. Resorcinol has been reported to be a goitrogenic agent in rats when injected subcutaneously (Doniach and Logothetopoulos, 1953), however, the reported goitrogenic effect related only to resorcinol-diacetate and resorcinol in peanut oil. The systemic or local effects of the other chemicals have not been studied extensively. Propylene glycol, a solvent used for pharmacologic studies, was originally suggested as a safe solvent by Seidenfeld and Hanzlik (1932). Davis and Jenner (1959) reported

propylene glycol to be less toxic than *N*,*N*-dimethylformamide and *N*,*N*-diacetylformamide. However, Zaroslinski *et al.* (1971) concluded that propylene glycol cannot be recommended as an inert cosolvent in pharmacologic studies. Gaunt *et al.* (1972) did not report any pathological findings or carcinogenic potential after feeding propylene glycol in concentrations up to 50,000 ppm to rats. Our findings are similar.

Fitzgerald *et al.* (1968) reported that isopropyl myristate had a local effect when applied on skin, causing erythema, followed by lichenization and fissure formation. Histologically, acanthosis, para- and hyperkeratosis, erosions and hemorrhages were seen, which regressed. Our results were similar in that initially cutaneous lesions were seen, but these regressed. Moderate dermatitis has also been reported in rabbits following 2-ethyl-1,3-hexanediol treatment (Draize *et al.*, 1948). In our studies no persistent changes occurred except for a slight inflammation and atrophy of the skin.

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TEH #95

TERATOLOGY AND PERCUTANEOUS TOXICITY STUDIES ON HAIR DYES

C. Burnett
1133 15th Street, N.W.
Washington, D.C. 20005
Cosmetic, Toiletry and Fragrance Assoc.

E. I. Goldenthal, S. B. Harris, F. X. Wazeter
Mattawan, Michigan 49071
International Research and Development Corp.

J. Strausburg, R. Kapp, R. Voelker
9200 Leesburg Tnpk
Vienna, VA 22180
Hazleton Laboratories America Inc.

Twelve hair dye formulations were tested for systemic toxicity by topical application twice weekly for 13 wk to groups of 12 New Zealand white rabbits and for teratologic effects following applications to groups of 20 pregnant Charles River CD rats on days 1, 4, 7, 10, 13, 16, and 19 of gestation. The three semipermanent formulations were applied as is, and the nine oxidation dyes were mixed 1:1 with 6% hydrogen peroxide just prior to application, as in normal use. The formulations included a broad spectrum of dyes and dye intermediates used or considered useful in oxidative and semipermanent hair color products.

In the teratology study no biologically significant soft tissue or skeletal changes were noted. Similarly, the mean numbers of corpora lutea, implantation sites, live fetuses, and resorptions per pregnancy, as well as numbers of liters with resorptions, were not significantly affected by the dye treatment. litters

In the percutaneous toxicity study there was no evidence of compound-induced systematic effects. Microscopic examination of 25 tissues from each animal gave no indication of histomorphologic evidence of toxicity. No dye discoloration of urine was seen at any time during the test or at necropsy. Some of the dye groups showed epidermal hyperplasia, which was probably a reflection of slight irritation due to the frequency of application of the oxidation formulations. systemic

INTRODUCTION

Questions of the safety of hair color products have been raised as a result of studies (Ames et al., 1975) showing mutagenic activity in

Requests for reprints should be sent to C. Burnett, Clairol Inc., 2 Blachley Road, Stamford, Connecticut 06902.

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Salmonella bacteria. On the basis of these results, Ames and co-workers speculate that hair dyes may be mutagenic to humans and probably carcinogenic. However, this extrapolation of the results of tests in bacteria to human safety has been questioned by the proponents of the Ames test and other screening procedures (de Serres, 1975). The regulatory agencies are taking a deliberate position in this matter and are considering the more relevant and weighty evidence emerging from animal studies in evaluating the safety of hair dyes (Anon., 1975).

The hair color industry has performed a number of chronic studies on major dye ingredients over the past 10 yr. Three such studies (Burnett et al., 1975; Kinkel and Holzman, 1973; Wernick et al., 1975) showed no evidence of carcinogenicity when a variety of dyes and dye intermediates were applied topically or were fed to rodents and dogs for up to 2 yr. Unpublished data (C. Burnett and co-workers) show that 11 hair dye chemicals found to be mutagenic (Ames et al., 1975) and to cause cytogenic abnormalities in some cases (Venitt et al., 1975) in *in vitro* tests had no effect on the germ cells of male rats treated with high doses of the dyes in a dominant lethal mutagenicity study.

The studies reported here are part of an ongoing hair dye industry safety testing program that includes a three-generation reproduction/carcinogenicity study.

TERATOLOGY STUDY IN RATS

Methods

Twelve hair dye composite formulations were tested, along with one positive and three negative control groups. The dyes were obtained from commercial suppliers and are either currently used or are considered potentially useful in hair color products today. The formulations (Tables 1-3) were applied topically at a dose of 2 ml/kg on days 1, 4, 7, 10, 13, 16, and 19 of gestation to groups of 20 mated Charles River CD female rats (presence of sperm in the vagina considered day 0 of gestation). Pilot studies had shown that the potential skin irritancy of most formulations would not permit more frequent application.

The 12 formulations tested represented the two major classes of hair color products used. Three formulations (P-22, P-23, and P-24) were of the semipermanent type that consist of preformed pigments that are deposited on the hair shaft from solvent-detergent bases. The nine remaining formulations were of the type that result in the oxidation of colorless intermediates, which are deposited as colored polymers in the hair shaft. These oxidation formulas have an ammoniacal soap base and

TABLE 1. Composition of Formulations 7401-7406, P-21, P-22, P-25, and P-26

Compounds tested	Formulation[a]									
	7401	7402	7403	7404	7405	7406	P-21	P-22	P-25	P-26
p-Phenylenediamine	3.0	2.0				4.0	1.0			
p-Toluenediamine sulfate		3.0	6.0					0.25	0.4	0.6
Resorcinol	1.7	1.7		1.0	2.0				0.25	0.2
2,4-Diaminoanisole sulfate	2.0	4.0								0.02
2,5-Diaminoanisole sulfate					6.0					
m-Aminophenol			0.7			0.7			0.09	0.02
o-Aminophenol					0.3					
p-Aminophenol			1.0						0.2	0.04
1-Naphthol			0.5							
m-Phenylenediamine				1.5						
o-Phenylenediamine				1.0						
2-Nitro-*p*-phenylenediamine	1.1									
4-Nitro-*o*-phenylenediamine			0.25							
2-Amino-4-nitrophenol					0.4					
2-Amino-5-nitrophenol						0.5				
4-Chlororesorcinol						2.0				
p-Aminodiphenylamine hydrochloride				2.0						
N-methyl-*p*-aminophenol sulfate				1.0						0.05
2,4-Diaminophenol							0.2			
2-Methylresorcinol							1.0			
4-Amino-2-nitrophenol							0.3			
Hydroquinone							0.2			
Pyrogallol							0.4			
t-Butyl hydroquinone							0.3			
Sodium picramate							0.1			
Toluenetriol (2,4,5)								0.5		
Benzenetriol (1,2,4)								0.5		
N,N-bis(2-hydroxyethyl)-*p* phenylenediamine sulfate							1.0	0.5		
2,5-Diamino-4-methylanisole di-HCl									0.6	
6-Hydroxybenzomorpholine									1.1	
2-Methyl-5-(carbamylmethyl)aminophenol										2.4
3-Carbamylmethylaminophenol										0.7
Oleic acid	5.0	5.0	15.0	5.0	5.0	5.0	15.0		8.0	8.0
Ispropanol	3.0	3.0	10.0	3.0	3.0	15.0	10.0		3.0	3.0
Sodium sulfite	0.2	0.2	0.2	0.2	0.2	0.2	0.1	1.6		
29% Ammonia	6.0	6.0	9.0	6.0	6.0	6.0	9.0		6.0	6.0
Glycerine			4.5							
Propylene glycol			9.0				5.0			
40% Diethylenetriaminepentacetic acid (Na salt)									2.4	2.4
35% Sodium bisulfite									1.3	1.3
Ascorbic acid							0.2			
Lauric diethanolamide							2.0			
Sodium lauryl sulfate							2.95			
Sulfonated castor oil							4.0			
Carbitol							5.0			
Ethoxylated octyl phenol							6.0			
Ethoxylated nonyl phenol							3.0			
Ethoxylated cholesterol							1.0			
Perfume							0.4			
Vinyl pyrrolidone copolymer quaternary								2.0		
Polyoxylated ethylated fatty alcohol								0.533		
Polyoxyethylene (23) lauryl ether								0.04		
Perfume								0.0127		
Stearyl dimethyl benzyl ammonium chloride								0.0667		
Silicone								0.533		
SDA 40 alcohol								25.0		
Ethyl acetate								1.992		
Triethnolamine								0.10-0.15		

[a] All formulations Water Q.S. 100.

are mixed with an equal volume of 6% hydrogen peroxide just prior to use.

The hair at the site of application on the dorso-scapular area was shaved closely the day before the solutions were applied. The negative control animals were untreated but were shaved. Three separate and discrete negative control groups were maintained in order to determine the degree of variability among small groups of control animals and to utilize this information in assessing treatment effects.

TABLE 2. Composition of Formulation P-23

Chemical or trade name	Percent
Basic violet 3	0.002
HC black 1	0.003
Solvent black 5 (nig. SS)	0.200
Acid black 2 (nig. WS blue)	0.200
Acid black 2 (nig. WS jet)	0.200
Direct red 23	0.200
Solvent black 5 (nig. WS jet)	0.150
Solvent blue 16	0.150
Brill. red safranine	0.005
Methylene blue	0.100
HC brown No. 1	0.200
AC red 259	0.150
Solvent black 5 (nig. SSJ)	0.200
Solvent yellow 63	0.050
Acid orange 89	0.070
Acid black 107	0.200
Acid blue 168	0.100
Solvent brown 43	0.080
Acid red 213	0.030
Acid blue 188	0.060
HC blue No. 3	0.200
Solvent yellow 90	0.100
Acid yellow 127	0.200
Acid orange 88	0.200
4-Amino-1-dihydroxy-5-nitroanthraquinone	0.200
4-Amino-4'-nitroazobenzene	0.200
2,4-Dinitro-6-chloro-2'-acetamido-4-bis-(2-hydroxyethyl)amino-5'-methoxyazobenzene	0.200
2,5-Diamino-6-nitrobenzthiazole	0.200
4-[(5-methylsulfonylbenzthiazole)-2-azo]-*N*-(2-cyanoethyl)-*N*-2-chloro-2-hydroxypropyl)aniline	0.200
Carbitol	5.000
Triethanolamine	1.500
Hydroxyethylcellulose	2.400
Citric acid	0.300
Partial sodium salt of *N*-lauryl β-iminodipropionate	1.500
Nonionic surfactant (dextrol XL-15)	0.480
1,3-Dimethylol-5,5-dimethyl hydantoin	0.100
Methyl paraben	0.050
Water	84.620

skeletal anomalies. Fetal examinations were performed in a random order with treatment group identity unknown to the examiner.

All statistical analyses compared the treatment groups with the control groups. The number of females exhibiting resorption sites, number of females exhibiting two or more resorptions, number of dead or resorbed fetuses, and the number of fetuses with soft-tissue or skeletal anomalies and accessory ribs was compared using chi-square test criterion with Yates' correction on 2 X 2 contingency tables as described by Steel and Torrie (1960) or Fisher's exact probability test (Siegel, 1956) as appropriate to judge the significance of difference.

The mean number of corpora lutea, implantation sites, live fetuses, and resorption sites was compared by analysis of variance (one-way classification) as described by Steel and Torrie (1960) using Dunnett's (1964) multiple comparison tables to judge the significance of differences.

The live fetal weights were compared by analysis of variance (hierarchal classification) as described by Steel and Torrie (1960) using Dunnett's (1964) multiple comparison tables to judge the significance of differences.

Statistically significant differences between groups were judged valid only when there were significant differences between any one of the dye treated groups and each of the three untreated control groups.

Results and Discussion

No signs of toxicity were seen throughout the study. Except for the changes in the color of the skin and hair at the site of dye application, no irritation or other changes in appearance were seen.

Changes in female body weights were similar for rats in the untreated controls and all dye-treated groups. A marked reduction in maternal weight gain through gestation was observed in the rats receiving acetylsalicylic acid as compared with either the untreated control rats or dye-treated rats.

Mean food consumption for all groups throughout gestation was similar except for rats in the acetylsalicylic acid group. These rats showed a moderate decrease in food consumption from days 7 to 13 of gestation. This decrease was not seen from days 13 to 20 of gestation.

The dye formulations produced no significant differences in the mean number of corpora lutea, implantation sites and live fetuses, and the sex ratio when compared with the untreated control groups. No differences between groups were seen regarding the number of females exhibiting resorption sites or mean resorptions per pregnancy. No significant changes were observed regarding soft-tissue anomalies between the dye-treated groups and the untreated control groups. Normally occurring skeletal variations were present in all groups; the most frequent variation noted was accessory ribs.

A statistically significant increase in skeletal anomalies appeared in the group receiving formulation 7402. Nine fetuses of the 169 (described in Tables 7 and 8) examined revealed anomalies that are regarded as minor skeletal changes. Seven of the nine fetuses displayed notched ribs and two displayed short ribs. These minor skeletal changes were seen in only 3 of 20 litters examined and are not regarded as biologically significant. The data are shown in Tables 4-6.

The results observed in the positive control group, namely an increase in embryotoxicity (e.g., increase in teratogenicity, increase in embryo death, and decrease in fetal weight), are in accord with the literature concerning the effects of aspirin upon fetal rat development (Kimmel and Wilson, 1973; Kimmel et al., 1971; McColl et al., 1965).

Conclusion

From this investigation, the administration of formulations 7401, 7402, 7403, 7404, 7405, 7406, P-21, P-22, P-23, P-24, P-25, and P-26 every third day of the gestation period produces no embryotoxic or teratogenic effects. The dyes and dye intermediates tested in these formulations include most of those used in oxidative and semipermanent hair color products today in the United States.

PERCUTANEOUS TOXICITY IN RABBITS

Methods

The 12 hair dye formulations shown in Tables 1-3 were applied topically twice weekly for 13 wk to groups of 12 adult New Zealand white rabbits (six of each sex). Except for P-22, P-23, and P-24, all formulations were mixed with an equal volume of 6% hydrogen peroxide prior to application. The sites of application on the dorsolateral aspects of the thoracic-lumbar area (one on each side of the midline) were alternated to minimize skin irritation. The dose was 1 ml/kg of the 1:1 oxidation mixtures and of those formulations used as is. This was the maximum dose that could be applied on the side of the rabbits without runoff. The hair at the site of application on the back and sides of each rabbit was clipped short throughout the study. The application sites on three animals of each sex in each group were abraded on the first treatment day of each week. The rabbits were restrained in holding stocks for 1 hr following each application and were then shampooed, rinsed, dried, and returned to their cages.

The rabbits in the three independent control groups of 12 rabbits each were treated identically except that no dyes were applied. The animals were weighed weekly during the study. Hematologic and clinical chemistry determinations and examination of urine was performed on all animals at 0, 3, 7, and 13 wk. These studies included determination of complete

TABLE 4. Summary of Teratology Study in Rats Receiving 7401, 7402, 7403, 7404, 7405, and 7406

Observations	Control I untreated	Control II untreated	Control III untreated	Acetylsalicylic acid (250 mg/kg-day)	7401 (2 ml/kg)	7402 (2 ml/kg)	7403 (2 ml/kg)	7404 (2 ml/kg)	7405 (2 ml/kg)	7406 (2 ml/kg)
Maternal parameters										
Total no. females	20	20	20	20	20	20	20	20	20	20
Mean no. corpora lutea	15.35	13.55	15.25	16.15[a]	14.05	14.05	12.85[b,c]	15.15	15.55	14.55
Mean no. implantation sites	12.40	12.10[c]	13.90	13.25	13.40	13.30	12.60	13.60	14.30[a,b]	13.00
No. females exhibiting resorption sites	13	14	12	15	10	13	14	11	11	14
No. females exhibiting 2 or more resorptions	9	7	6	15[d]	6	6	7	6	7	8
No. females aborting	0	0	0	0	0	0	0	0	0	0
Fetal parameters										
Mean no. live fetuses/group	10.65	10.85	12.50	8.70[e]	12.40	12.25	11.15	12.40	12.95	11.15
Mean live fetal weight (g)	3.38	3.58	3.62	2.89[f]	3.60	3.70	3.82[g]	3.63	3.79[g]	3.68
No. dead or resorbed fetuses (%)	35 (14.11)	25 (10.33)	28 (10.07)	91 (34.34)[f]	20 (7.46)[h]	21 (7.89)[h]	29 (11.51)	24 (8.82)	27 (9.44)	37 (14.23)
Mean no. resorptions/pregnancy	1.75	1.25	1.40	4.55[a,i]	1.00	1.10	1.45	1.20	1.35	1.80
Sex ratio, M:F	106:107	102:115	119:131	100:75	116:132	118:127	121:102	137:111	117:142	100:124
No. fetuses with soft-tissue anomalies (%)	4 (6.35)	4 (6.06)	6 (7.79)	21 (36.84)[f]	4 (5.19)	3 (3.95)	4 (6.06)	8 (10.81)	4 (5.00)	3 (4.54)
No. fetuses with skeletal anomalies (%)	0 (0.00)	1 (0.67)	2 (1.16)	40 (34.19)[f]	2 (1.17)	9 (5.32)[d,g]	0 (0.00)	1 (0.57)	1 (0.56)	1 (0.63)
No. fetuses with accessory ribs only (%)	75 (50.00)	56 (37.09)	72 (41.62)	32 (27.35)[c,f]	86 (50.29)[j]	57 (33.73)[g]	64 (40.76)	35 (20.11)[f]	72 (40.22)	78 (49.37)[j]

[a] Significantly different from control II at $p < 0.01$.
[b] Significantly different from control I at $p < 0.05$.
[c] Significantly different from control III at $p < 0.05$.
[d] Significantly different from controls II, III at $p < 0.05$.
[e] Significantly different from control III at $p < 0.01$.
[f] Significantly different from controls I, II, III at $p < 0.01$.
[g] Significantly different from control I at $p <$ 0.05. 0.01
[h] Significantly different from control I at $p < 0.05$.
[i] Significantly different from controls I, II at $p < 0.05$.
[j] Significantly different from control II at $p < 0.05$.

TABLE 5. Summary of Teratology Study in Rats Receiving P-21, P-22, P-23, an

Observations	Control I untreated	Control II untreated	Control III untreated
Maternal parameters			
Total no. females gravid	20	20	20
Mean no. corpora lutea	15.35	13.55	15.25
Mean no. implantation sites	12.40	12.10[c]	13.90
No. females exhibiting resorption sites	13	14	12
No. females exhibiting 2 or more resorption sites	9	7	6
No. females aborting	0	0	0
Fetal parameters			
Mean no. live fetuses/group	10.65	10.85	12.50
Mean live fetal weight (g)	3.38	3.58	3.62
No. dead or resorbed fetuses (%)	35 (14.11)	25 (10.33)	28 (10.07)
Mean no. resorptions/pregnancy	1.75	1.25	1.40
Sex ratio, M:F	106:107	102:115	119:131
No. fetuses with soft-tissue anomalies (%)	4 (6.35)	4 (6.06)	6 (7.79)
No. fetuses with skeletal anomalies (%)	0 (0.00)	1 (0.67)	2 (1.16)
No. fetuses with accessory ribs only (%)	75 (50.00)	56 (37.09)	72 (41.62)

[a] Significantly different from control II at $p < 0.05$.
[b] Significantly different from control III at $p < 0.05$.
[c] Significantly different from control III at $p < 0.01$.
[d] Significantly different from controls II, III at $p < 0.05$.
[e] Significantly different from controls I, II, III at $p < 0.01$.

d P-24

Acetylsalicylic acid (250 mg/kg-day)	P-21 (2 ml/kg)	P-22 (2 ml/kg)	P-23 (2 ml/kg)	P-24 (2 ml/kg)
20	20	20	20	20
16.15[a]	13.45	14.30	14.80	14.35
13.25	11.65[b]	13.80[a]	13.50	13.50
15	8	11	12	13
15[d]	4	5	6	7
0	0	0	0	0
8.70[c]	11.00	12.50	12.15	12.35
2.89[e]	3.79[f]	3.75[g]	3.96[d,f]	3.88[f]
91 (34.34)[e]	13 (5.58)[f]	26 (9.42)	27 (10.00)	23 (8.52
4.55[h,i]	0.65[g]	1.25	1.35	1.10
100 : 75	116 : 104	133 : 118	123 : 120	129 : 118
21 (36.84)[e]	7 (10.29)	5 (6.33)	9 (12.16)	3 (4.05)
40 (34.19)[e]	1 (0.65)	2 (1.16)	3 (1.78)	1 (0.06)
32 (27.35)	83 (54.60)[b,h]	57 (33.14)[f]	66 (39.05)	66 (38.15)

[f] Significantly different from control I at $p < 0.01$.
[g] Significantly different from control I at $p < 0.05$.
[h] Significantly different from control II at $p < 0.01$.
[i] Significantly different from controls I, III at $p < 0.05$.

TABLE 6. Summary of Teratology Study in Rats Receiving P-25 and P-26

Observations	Control I untreated	Control II untreated	Control III untreated	Acetylsalicylic acid (250 mg/kg-day)	P-25 (2 ml/kg)	P-26 (2 ml/kg)
Maternal parameters						
Total no. females	20	20	20	20	20	20
Mean no. corpora lutea	15.35	13.55	15.25	16.15[a]	14.20	14.75
Mean no. implantation sites	12.40	12.10	13.90[b]	13.25	12.70	14.05[b]
No. females exhibiting resorption sites	13	14	12	15	16	12
No. females exhibiting 2 or more resorptions	9	7	6	15[c]	10	8
No. females aborting	0	0	0	0	0	0
Fetal parameters						
Mean no. live fetuses/group	10.65	10.85	12.50	8.70[d]	10.85	12.50
Mean live fetal weight (g)	3.38	3.58	3.62	2.89[e]	3.91[b,f]	3.86[f]
No. dead or resorbed fetuses (%)	35 (14.11)	25 (10.33)	28 (10.07)	91 (34.34)[e]	37 (14.57)	31 (11.03)
Mean no. resorptions/pregnancy	1.75	1.25	1.40	4.55[a,g]	1.85	1.55
Sex ratio, M:F	106:107	102:115	119:131	100:75	106:111	127:123
No. fetuses with soft-tissue anomalies (%)	4 (6.35)	4 (6.06)	6 (7.79)	21 (36.84)[e]	2 (3.08)	3 (3.90)
No. fetuses with skeletal anomalies (%)	0 (0.00)	1 (0.67)	2 (1.16)	40 (34.19)[e]	1 (0.66)	0 (0.00)
No. fetuses with accessory ribs only (%)	75 (50.00)	56 (37.09)	72 (41.62)	32 (27.35)[f,h]	63 (41.44)	76 (43.93)

[a] Significantly different from control II at $p < 0.01$.
[b] Significantly different from control II at $p < 0.05$.
[c] Significantly different from controls II, III at $p < 0.05$.
[d] Significantly different from control III at $p < 0.01$.
[e] Significantly different from controls I, II, III at $p < 0.01$.
[f] Significantly different from control I at $p < 0.01$.
[g] Significantly different from controls I, III at $p < 0.05$.
[h] Significantly different from control III at $p < 0.05$.

blood count, methemoglobin, fasting blood sugar, blood urea nitrogen, alkaline phosphatase, and serum glutamic oxaloacetic transaminase. Urine was examined for color, pH, albumin, glucose, occult blood, and microscopic elements.

The clinical chemistry determinations were performed on blood obtained by cardiac puncture by SMA-12 analysis using the Technicon auto analyzer. Hematology studies utilized the automated electronic Coulter F counter and reference methods (Schalm, 1975). Ames Labstix were used for the urinalysis.

All survivors were sacrificed after 13 wk and examined for gross abnormalities. Organ-body weight ratios were determined for liver, kidneys, adrenals, heart, thyroid, spleen, and brain. Twenty-five tissues [skin from treated and untreated areas, lymph nodes (mesanteric, axillary, and cervical), spleen, stomach, duodenum, colon, liver, gall bladder, adrenals, nerve with adjacent muscle, eyes, pancreas, kidneys, urinary bladder, ovaries, testes, bone, bone marrow, heart, lung, thyroid, brain, and skeletal muscle under the site of application and elsewhere (thigh)] from each animal were stained with hematoxylin and eosin and examined microscopically. Statistical analysis of the data on body weight gains, hematology, clinical chemistries, and absolute and relative organ weights was performed using the analysis of variance F test and Student's t test. When variances differed significantly, Student's t test was modified (t') and Cochran's approximation was utilized (Snedecor and Cochran, 1967).

Results and Discussion

No evidence of compound-induced toxicity was seen. Body weight gain of all test groups was at least equal to that of the controls. Five control and five test animals died during the study due to complications resulting from cardiac puncture while collecting blood. There were scattered statistically significant differences in the clinical chemistry and hematologic values between test and control groups at the various sampling intervals (Tables 7-10). However, these differences were not considered to be of toxicologic significance because of either the direction or continuity of the differences or the fact that they fell within the range of historical control values.

There were a few instances when there were statistically significant differences in relative organ weights between a test group and the combined controls where the differences were not significant when the group was compared with each control group separately. In no instance were any of these differences accompanied by histomorphologic evidence of toxicity. The results of the urinalyses were unremarkable. No dye discoloration of the urine was seen at any time during the test. The treated skin showed slight thickening in some groups, particularly groups 7403 and P-21. This was not unexpected, due to the frequency of dye application.

TABLE 7. Mean Blood Chemistry Values for Female New Zealand Rabbits Subjected to 13-wk Repeated Dermal Applications of Hair Dyes with Standard Deviations

Group no.	Glucose (mg%)	BUN (mg%)	Alkaline phosphatase (K-A units)	SGOT (K units)
C-1,2,3[a]	124.69	22.19	9.26	27.94
±	22.31	3.94	3.49	11.85
P-22	134.20	24.20	9.62	24.60
±	9.01	4.38	4.16	11.99
P-23	142.67 S+[b]	24.83	8.85	26.67
±	5.92	4.12	1.46	9.16
P-24	143.17	26.33 S+	7.98	23.17
±	25.22	3.72	2.07	10.78
P-21	122.67	23.50	7.07 S−[c]	22.67
±	8.98	3.45	1.12	8.26
P-25	127.00	25.50	11.42	18.00
±	6.87	5.28	5.09	10.68
P-26	122.20	28.80 S+	8.62	19.60
±	6.91	5.40	3.15	11.13
7401	121.33	20.00	8.83	27.83
±	4.89	1.41	3.65	12.21
7402	125.33	21.83	6.52	20.50
±	6.92	2.71	1.75	7.87
7403	127.83	20.83	9.28	22.33
±	8.86	4.31	4.57	7.71
7404	130.17	20.17	9.70	26.67
±	10.91	1.60	2.68	12.31
7405	141.00	23.83	8.68	26.00
±	15.05	3.49	2.79	16.53
7406	132.67	21.83	7.47	28.50
±	14.88	2.48	2.42	15.32

[a] Combined controls.

[b] S+, statistically significantly higher than control ($p < 0.05$).

[c] S−, statistically significantly lower than control ($p < 0.05$).

No

TABLE 8. Mean Blood Chemistry Values of 13-wk Repeated Dermal Applications of Hair Dyes on Male New Zealand Rabbits with Standard Deviation

Group no.	Glucose (mg%)	BUN (mg%)	Alkaline phosphatase (K-A units)	SGOT (K units)
C-1,2,3[a]	130.29	17.00	11.13	30.06
±	21.44	3.27	4.25	13.93
P-22	144.17	20.50 S+[b]	8.70	23.83
±	11.64	1.97	1.54	7.31
P-23	138.33	21.67 S+	9.48	21.50
±	19.74	3.33	5.91	12.18
P-24	128.67	23.33 S+	10.73	26.67
±	8.36	3.67	2.92	20.50
P-21	130.50	20.33	7.32 S−[c]	33.67
±	7.94	6.22	1.75	12.37
P-25	135.50	17.17	9.25	43.33
±	14.31	0.98	3.34	29.88
P-26	121.50	16.83	7.75 S−	22.33
±	3.45	1.83	1.77	9.61
7401	131.50	17.83	7.97	26.17
±	12.36	3.71	2.57	14.99
7402	128.00	18.00	14.53	34.50
±	16.73	1.79	15.41	13.72
7403	132.83	18.33	8.98	28.67
±	3.43	4.41	2.90	10.29
7404	134.20	18.40	7.28	28.40
±	13.88	1.52	2.51	13.72
7405	137.33	16.17	11.43	18.50
±	5.65	2.56	5.35	11.64
7406	140.67	16.00	8.02	23.00
±	9.16	1.67	1.86	2.10

[a] Combined controls.
[b] S+, statistically significantly higher than control ($p < 0.05$).
[c] S−, statistically significantly lower than control ($p < 0.05$).

TABLE 9. Mean Hematological Values of 13-wk Repeated Dermal Applications of Hair Dyes on Female New Zealand Rabbits with Standard Deviations

Group no.	HCT (%)	Hb (g%)	RBC ($10^6/m^3$)	WBC ($10^3/m^3$)	Met-Hb (%)
C-1,2,3[a]	37.31	12.54	5.75	6.51	1.46
±	1.67	0.68	0.29	1.52	0.85
P-22	37.80	12.66	5.79	7.86	0.54 S−[c]
±	1.30	0.52	0.30	0.97	0.39
P-23	36.25	12.57	6.30	6.80	0.88
±	2.84	0.68	1.14	1.18	0.41
P-24	38.00	12.77	6.17	7.33	0.77
±	0.89	0.37	0.61	0.96	0.43
P-21	40.00	13.30 S+[b]	6.05	6.48	0.83
±	2.97	0.91	0.27	1.12	0.46
P-25	35.67	11.87 S−	5.69	7.37	0.95
±	1.63	0.59	0.35	2.54	0.86
P-26	34.60 S−	11.56 S−	5.37	5.78	1.34
±	1.14	0.45	0.55	1.42	1.10
7401	35.50 S−	12.67	5.77	6.42	0.90
±	1.64	0.84	0.46	1.33	0.75
7402	37.33	12.52	5.77	6.30	1.47
±	2.07	0.82	0.46	1.27	0.59
7403	38.42	11.47 S−	6.10 S+	7.53	0.83
±	2.42	0.72	0.35	3.46	0.94
7404	36.83	11.95	5.55	5.40	1.42
±	1.83	0.88	0.29	1.23	1.04
7405	39.50 S+	13.00	6.03	7.10	1.33
±	2.59	0.69	0.26	1.57	0.88
7406	39.92	13.12	6.05 S+	7.33	0.17 S−
±	3.69	1.11	0.29	3.17	0.45

[a] Combined controls.
[b] S+, statistically significantly higher than control ($p < 0.05$).
[c] S−, statistically significantly lower than control ($p < 0.05$).

TABLE 10.- Mean Hea

TABLE 10. Mean Hematological Values of 13-wk Repeated Dermal Applications of Hair Dyes on Male New Zealand Rabbits with Standard Deviations

Group no.	HCT (%)	Hb (g%)	RBC ($10^6/m^3$)	WBC ($10^3/m^3$)	Met-Hb (%)
C-1,2,3[a]	38.74	13.35	6.33	6.54	1.42
±	2.29	0.79	0.53	1.37	1.11
P-22	38.00	12.53 S−[c]	5.90	8.02 S+[b]	0.50
±	2.10	0.50	0.29	0.70	0.45
P-23	38.42	13.17	5.98	8.18 S+	0.87
±	1.56	0.56	0.39	2.17	0.45
P-24	40.00	13.27	6.19	8.57 S+	0.23 S−
±	2.10	0.73	0.12	1.22	0.26
P-21	38.50	13.17	6.09	7.32	1.00
±	1.22	0.56	0.32	1.34	0.43
P-25	39.17	13.23	6.19	6.52	1.40
±	2.40	0.87	0.28	2.17	1.04
P-26	38.00	12.93	5.98	6.72	0.87
±	2.83	0.88	0.19	1.36	0.77
7401	39.08	13.35	6.30	6.53	0.52
±	3.61	2.12	0.55	0.85	0.57
7402	40.25	13.60	6.28	5.30	0.48 S−
±	1.33	0.75	0.20	1.20	0.51
7403	38.17	11.98 S−	5.99	6.73	0.78
±	1.17	0.77	0.31	1.95	0.65
7404	38.80	12.66	5.82	7.30	2.86 S+
±	3.35	0.81	0.63	0.58	0.94
7405	40.83	13.80	6.19	6.95	1.63
±	2.93	0.91	0.28	1.30	1.19
7406	39.83	13.60	6.26	6.45	0.27 S−
±	3.13	1.12	0.47	1.49	0.45

No gross abnormalities were seen at necropsy, and no microscopic lesions were seen that were judged to be due to the administration of the hair dye formulations. The incidence and severity of disease processes common to laboratory rabbits was not affected by the experimental treatments.

Conclusion

Hair dye formulations, representing oxidative and semipermanent hair color products widely used in the United States today, were applied to rabbits twice weekly for 13 wk. This represents a considerable exaggeration of once-monthly hair dyeing in normal product usage. There was no evidence of systemic toxicity.

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Cocarcinogenic and Tumor-Promoting Agents in Tobacco Carcinogenesis [1, 2]

B. L. Van Duuren *and* **B. M. Goldschmidt** [3, 4]

MARY—A series of 21 tobacco smoke components and ted compounds were tested for cocarcinogenic activity on mouse skin. The compounds were applied to mouse skin (50 female ICR/Ha Swiss mice/group) three times weekly with a low dose (5 μg/application) of benzo[a]pyrene (B[a]P). The test compounds were of five classes: aliphatic hydrocarbons, aromatic hydrocarbons, phenols, and long-chain acids and alcohols. The following compounds enhanced remarkably the carcinogenicity of B[a]P: catechol, pyrogallol, decane, undecane, pyrene, benzo[e]pyrene, and fluoranthene. The following compounds inhibited B[a]P carcinogenicity completely: esculin, quercetin, squalene, and oleic acid. Phenol, eugenol, resorcinol, hydroquinone, hexadecane, and limonene partially inhibited B[a]P carcinogenicity. Six of the 21 compounds were also tested as tumor promoters in two-stage carcinogenesis. No direct correlation existed between tumor-promoting activity and cocarcinogenic activity. The cocarcinogens pyrogallol and catechol did not show tumor-promoting activity. Decane, tetradecane, anthralin, and phorbol myristate acetate showed both types of activity. Structure-activity relationships and possible modes of action were described.—J Natl Cancer Inst 56: 1237–1242, 1976.

From carcinogenicity tests of cigarette-smoke condensate (CSC) and its known content of aromatic hydrocarbon carcinogens on mouse skin, it was concluded in 1958 that these carcinogens do not account for the observed carcinogenicity of CSC (*1–3*). Subsequent studies by oth- s (*4*) led to the same conclusion, and several investiga- (*5–8*) undertook studies on the nature of tumor-pro- ting agents in CSC. However, none of the compounds or tobacco tar fractions tested as tumor promoters in two-stage carcinogenesis showed convincing tumor-promoting activity. Some reports described cocarcinogenic activity on mouse skin by aromatic hydrocarbons (*9*) and long-chain aliphatic hydrocarbons (*10–12*). In (*9–12*) and our study, cocarcinogenesis, in the restricted sense, is a regimen in which the cocarcinogen, applied repeatedly on mouse skin with low doses of a carcinogen such as benzo[*a*]pyrene(B[*a*]P), enhances considerably the carcinogenic activity of the carcinogen.

This type of experiment, i.e., cocarcinogenesis, was only recently applied to the problem of tobacco carcinogenesis. In preliminary reports, the cocarcinogenic activity of several known CSC components such as benzo[*e*]-pyrene (B[*e*]P), pyrene, and catechol was described (*13, 14*); B[*a*]P was the carcinogen. Since cocarcinogenesis testing is more relevant to animal or human exposure to CSC or cigarette smoke, respectively, compared with tumor promotion, we made a more extensive study of the cocarcinogenic activity of known and suspected components of CSC.

Included in these cocarcinogenesis experiments were eight phenolic compounds, four aromatic hydrocarbons, six nonaromatic hydrocarbons (aliphatic, olefinic, and alicyclic), oleic acid, stearic acid, and lauryl alcohol. We also tested six of these compounds for promoting activity in two-stage carcinogenesis tests on mouse skin with]P as the initiating agent. The results of these new eriments, as well as the final results of several incomplete experiments published in (*14*), are described here.

MATERIALS AND METHODS

Chemicals.—The 21 test compounds as well as B[*a*]P, 1,8-dihydroxy-9-anthrone (anthralin), and phorbol myristate acetate (PMA) were prepared and/or purified as summarized in table 1. The criteria of purity are also given in table 1.

Preparation and storage of test solutions.—B[*a*]P and the compounds to be tested were made up biweekly in amber containers and stored at 4° C. The B[*a*]P and each test or control compound were made up in one solution so that they were applied simultaneously on the same area of mouse skin.

Animals.—Female ICR/Ha Swiss mice (ARS/Sprague-Dawley Inc., Madison, Wis.) were vaccinated against ectromelia and tested beginning at age 6–8 weeks. They were housed on sterile hardwood chips (Iso-Dri; Fisher & Son, Bound Brook, N.J.) in stainless-steel cages (10/cage), fed Purina Laboratory Chow and water ad libitum, and weighed regularly. The animal rooms were maintained at 22–24° C.

Bioassay procedure.—The backs of the mice (50 mice/experimental group) were clipped 2 days before the initial treatment and thereafter as needed for the duration of the experiment. The solutions were applied by micropipette in acetone or in dimethyl sulfoxide (DMSO) by calibrated paintbrush, three times weekly.

Animals were observed regularly; tumors were recorded, and those greater than 1 mm in diameter were counted and charted regularly. Only tumors persisting for 30 days or more were counted in the cumulative totals; the data presented are based on these chartings. Animals bearing tumors that appeared grossly to be carcinomas were killed approximately 2 months after the tumors were clinically classified as malignant or when animals were moribund. All animals were autopsied. Specimens from tumors and any abnormal tissues were excised, fixed in unbuffered 10% formalin, blocked in paraffin, stained with hematoxylin and eosin, and confirmed histologically. Included in the experimental protocol were control groups receiving carcinogen or test compound only, solvent only, and a group given no treatment.

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[3] Laboratory of Organic Chemistry and Carcinogenesis, Institute of Environmental Medicine, New York University Medical Center, New York, N.Y. 10016.

[4] This work was carried out in collaboration with Mrs. C. Katz, Mrs. S. Melchionne, and Miss A. Smith. Histopathologic diagnoses were done by Dr. I. Seidman, Department of Pathology, and Dr. D. Roth, Department of Environmental Medicine, New York University Medical Center.

TABLE 1.—*Purification of test compounds*

Compound	Source [a]	Method of purification and properties [b]
Phenols		
Phenol	A	Distillation; bp: 182° C/760 mm
Esculin (6-glycosyl-7-hydroxycoumarin)	A	Recrystallization; water; mp: 196–201° C
Eugenol (4-allyl-2-methoxyphenol)	A	Distillation; bp: 78–81° C/0.08 mm
Quercetin (3,3',4',5,7-pentahydroxyflavone)	B	Recrystallization; ethanol/water; mp: 313–315° C
Catechol (1,2-dihydroxybenzene)	A	Recrystallization; toluene; mp: 102–105° C
Resorcinol (1,3-dihydroxybenzene)	A	Recrystallization; toluene; mp: 106–109° C
Hydroquinone (1,4-dihydroxybenzene)	A	Recrystallization; water; mp: 169–171° C
Pyrogallol (1,2,3-trihydroxybenzene)	B	Recrystallization; ethyl acetate/toluene; mp: 129–132° C
Anthralin (1,8-dihydroxy-9-anthrone)	K	Recrystallization; hexane/benzene; mp: 178–179° C (*15*)
Hydrocarbons (nonaromatic) [c]		
Squalene	E	n_D^{26} 1.4892
Limonene	E	Distillation; bp: 42–45° C/2.5 mm
Decane	A	n_D^{26} 1.4050
Undecane	A	n_D^{26} 1.4110
Tetradecane	B	n_D^{26} 1.4221
Hexadecane	A	n_D^{26} 1.4281
Aromatic hydrocarbons [d]		
Pyrene	A	Recrystallization; ethanol; mp: 149–150° C
Benzo[*ghi*]perylene	A	Recrystallization; xylene; mp: 274–275° C
B[*e*]P	C	Recrystallization; benzene; mp: 179–180° C
Fluoranthene	A	Zone refined; mp: 107–109° C
B[*a*]P	A	Recrystallization; benzene/methanol (1:3); mp: 179° C
Long-chain alcohols and acids		
Oleic acid	S, B	Fractional distillation; bp: 164° C/0.1 mm
Stearic acid	E	Vacuum dried; mp: 67–68° C
Lauryl alcohol	E	Distillation; bp: 99–100° C/2.5 mm
PMA	Croton oil	Solvent fractionation and column chromatography (*16*)

[a] A = Aldrich Chemical Co., Milwaukee, Wis.; B = J. T. Baker Laboratory, New York, N.Y.; K = K & K Laboratories, Plainview, N.Y.; C = Consolidated Midland Corp., Brewster, N.Y.; E = Eastman Organic Chemicals, Rochester, N.Y.; S = Sigma Chemical Co., St. Louis, Mo.

[b] Nuclear magnetic resonance, IR and UV spectra, and thin-layer chromatography, with the use of at least two solvent systems, were obtained, where applicable, for the purified samples; these analyses confirmed the assigned structures and purity of the test compounds. bp = boiling point; mp = melting point.

[c] n_D^{26} = refractive index, each 26° C [sodium (D) line].

[d] In addition to the other analyses (footnote *b*), fluorescence and mass spectra also were obtained for these compounds.

Twenty-one compounds were tested for cocarcinogenic activity with B[*a*]P. Each compound, at the dosages indicated under "Results," was tested by simultaneous application with 5 μg B[*a*]P/0.1 ml solvent. Anthralin and PMA were used as positive controls in the cocarcinogenesis experiments, since they are potent cocarcinogens (*13, 14*).

Six of the 21 compounds were also tested as tumor-promoting agents, i.e., by repeated application of the test compound on mouse skin (50 mice/group) after an initiating dose of 150 μg B[*a*]P. Fourteen days after the primary treatment, animals were given applications of the compound three times weekly for the duration of the tests. Duration of tests, dosages, and vehicles are shown in the tables under "Results." PMA and anthralin were positive controls for these experiments also.

The dosages of test compounds used were based on preliminary short-term (2–4 wk) evaluations and were the highest dosages at which skin-damaging effects were minimal.

RESULTS

Findings from the two series of cocarcinogenesis experiments are in table 2. The first series was terminated at 368 days; the second, at 440 days. The rates of tumor appearance for a few of the test compounds are illustrated in text-figure 1. Pyrene, benzo[*ghi*]perylene, and B[*e*]P were each tested at two or three dosages. For all three compounds the increased dosages resulted in notably higher tumor incidences. Except the aromatic hydrocarbons, the test compounds were all applied at high dosages (2–25 mg/application). The dosages of aromatic hydrocarbons ranged from 4–40 μg per application. The importance of dosage in the determination of tumor induction is discussed later. It is instructive first to compare the effects of the whole range of chemicals and dosages used on B[*a*]P carcinogenesis.

The following compounds more than doubled the numbers of mice with papillomas and carcinomas (our indexes of cocarcinogenic activity) compared with the numbers of such mice among the B[*a*]P–acetone controls: catechol, pyrogallol, decane, undecane, pyrene (40-μg dose), B[*e*]P (15-μg dose), and fluoranthene. In the B[*a*]P control group (440-day expt), the total number of papillomas among 50 mice was 26. The seven compounds listed above, when tested as cocarcinogens, increased this number by a low of 66 for pyrene to a high of 126 for fluoranthene; i.e., these cocarcinogens also largely increased tumor multiplicity compared with the B[*a*]P control. Except fluoranthene, these cocarcinogens did not notably decrease the days to the appearance of the first tumor compared with findings for the B[*a*]P controls.

Benzo[*ghi*]perylene, tetradecane, and lauryl alcohol gave results that suggested weak to moderate cocarcino-

TABLE 2.—*Cocarcinogenesis: Bioassay in mouse skin* [a]

Carcinogen [b]	Cocarcinogen (dose)	Days on test	Days to first papilloma	Mice with papillomas/ total papillomas [c]
Phenols				
B[*a*]P	Phenol (3 mg)	368	267	7/9 (3)
None	Phenol (3 mg)	368	355	1/1 (0)
B[*a*]P	Esculin (25 mg)[d]	368	—	0
None	Esculin (25 mg)[d]	368	—	0
B[*a*]P	Eugenol (10 mg)	368	299	4/4 (2)
None	Eugenol (10 mg)	368	—	0
B[*a*]P	Quercetin (25 mg)[d]	368	—	0
None	Quercetin (25 mg)[d]	368	—	0
B[*a*]P	Catechol (2 mg)	368	299	36/90 (31)
None	Catechol (2 mg)	368	292	1/1 (1)
B[*a*]P	Resorcinol (10 mg)	368	249	5/6 (2)
None	Resorcinol (10 mg)	368	—	0
B[*a*]P	Hydroquinone (5 mg)	368	254	7/11 (3)
None	Hydroquinone (5 mg)	368	—	0
B[*a*]P	Pyrogallol (5 mg)	440	253	40/95 (33)
None	Pyrogallol (5 mg)	440	—	0
Hydrocarbons (nonaromatic)				
B[*a*]P	Squalene (25 mg)	440	400	1/1 (0)
None	Squalene (25 mg)	440	—	0
B[*a*]P	Limonene (10 mg)	440	295	13/13 (4)
None	Limonene (10 mg)	440	—	0
B[*a*]P	Decane (25 mg)	440	230	38/73 (34)
None	Decane (25 mg)	440	406	1/1 (0)
B[*a*]P	Undecane (25 mg)	440	195	44/105 (41)
None	Undecane (25 mg)	440	—	0
B[*a*]P	Tetradecane (25 mg)	440	120	32/58 (19)
None	Tetradecane (25 mg)	440	—	0
B[*a*]P	Hexadecane (25 mg)	440	314	5/8 (2)
None	Hexadecane (25 mg)	440	—	0
Aromatic hydrocarbons				
B[*a*]P	Pyrene (4 μg)	368	250	12/14 (6)
B[*a*]P	Pyrene (12 μg)	368	186	26/42 (20)
B[*a*]P	Pyrene (40 μg)	440	229	35/66 (26)
None	Pyrene (12 μg)	368	—	0
None	Pyrene (40 μg)	440	—	0
B[*a*]P	Benzo[*ghi*]perylene (7 μg)	368	238	19/31 (10)
B[*a*]P	Benzo[*ghi*]perylene (21 μg)	368	222	20/39 (18)
None	Benzo[*ghi*]perylene (21 μg)	368	—	0
B[*a*]P	B[*e*]P (5 μg)	368	249	24/33 (9)
B[*a*]P	B[*e*]P (15 μg)	368	246	33/79 (27)
None	B[*e*]P (15 μg)	368	—	0
B[*a*]P	Fluoranthene (40 μg)	440	99	39/126 (37)
None	Fluoranthene (40 μg)	440	—	0
Long-chain alcohols and acids				
B[*a*]P	Oleic acid (25 mg)	440	—	0
None	Oleic acid (25 mg)	440	—	0
B[*a*]P	Stearic acid (4 mg)[e]	440	195	25/38 (7)
None	Stearic acid (4 mg)[e]	440	—	0
B[*a*]P	Lauryl alcohol (10 mg)	440	226	21/27 (13)
None	Lauryl alcohol (10 mg)	440	—	0
Control groups				
B[*a*]P	Acetone	368	251	14/16 (10)
B[*a*]P	Acetone	440	210	16/26 (12)
B[*a*]P	DMSO	368	252	13/16 (6)
B[*a*]P	PMA (2.5 μg)	368	60	45/260 (37)
None	PMA (2.5 μg)	368	174	5/5 (0)
B[*a*]P	Anthralin (80 μg)	368	159	25/58 (15)
None	Anthralin (80 μg)	368	307	2/2 (0)
None	Acetone	440	—	0
None	None[f]	368	—	0
None	None[f]	440	—	0

[a] We used 50 female ICR/Ha Swiss mice per group. Two sets of experiments are presented here. The first experiment lasted for 368 days; the second, for 440. Median survival times were greater than the days of testing, except for those experiments in which the carcinoma incidences were high. The high incidences of carcinoma occurred with the potent cocarcinogens: pyrogallol, undecane, tetradecane, pyrene (at 12 and 40 μg/application), fluoranthene, lauryl alcohol, and PMA, when applied simultaneously with B[*a*]P.

[b] B[*a*]P was applied in the same solution as the cocarcinogen (5 μg/0.1 ml acetone or DMSO) three times weekly to the dorsal skin. Acetone and DMSO solutions were applied by micropipette and a calibrated brush, respectively.

[c] *Numbers in parentheses* are numbers of mice with squamous carcinoma.

[d] DMSO was the solvent; solvent for all others was acetone.

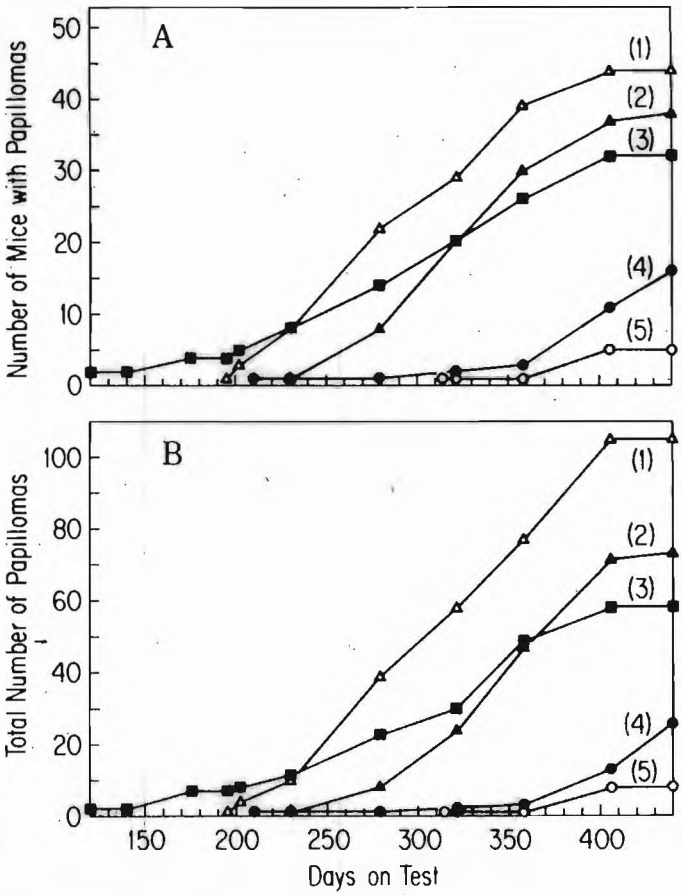
[e] Applied in 0.2 ml acetone due to limited solubility.

[f] 100 animals.

genic activity. The following four compounds completely inhibited B[*a*]P carcinogenesis on mouse skin at the dosages used: esculin, quercetin, squalene, and oleic acid. The compounds phenol, eugenol, resorcinol, and hexadecane reduced the tumorigenicity of B[*a*]P by 50% or more in all three tumor indexes given in table 2. Hydroquinone and limonene showed weak inhibitory activity. Stearic acid was the only compound that gave inconclusive results. The number of animals with tumors and total papillomas was higher than that in the control groups, whereas the number of animals with carcinomas was not.

Of the 21 compounds tested for carcinogenic activity, only catechol induced a skin carcinoma when applied alone, i.e., without B[*a*]P. Phenol and decane, when applied without B[*a*]P, each induced a papilloma in 1 of 50 mice. PMA, a known potent tumor promoter and cocarcinogen (*13, 14*), was as active as, or slightly more active than, the seven most potent cocarcinogens in this study. However, PMA was used at a dose several orders of magnitude lower than that used for the other compounds and caused a markedly higher tumor multiplicity and shorter latent period compared with the other active test compounds. Anthralin, also a known tumor promoter and cocarcinogen, showed the expected cocarcinogenicity (*14*) but was considerably less active than the seven most potent cocarcinogens of the 21 test compounds described here. No tumors existed in the solvent-treated and untreated control groups.

Concerning the dosages used, as dictated by short-term toxicity evaluations, the following observations are noteworthy: 1) The four compounds that completely inhibited B[*a*]P carcinogenesis were all given at 25 mg per application. However, 2) the long-chain aliphatic hydrocarbons were all used at the same high dose; decane, undecane, and tetradecane showed cocarcinogenic activity, whereas hexadecane caused marked inhibition. 3) Of the five simple phenols tested, all in the range of 2–10



TEXT-FIGURE 1.—A: Number of mice with papillomas; B: total number of papillomas. Curves 1: B[*a*]P + undecane; curves 2: B[*a*]P + decane; curves 3: B[*a*]P + tetradecane; curves 4: B[*a*]P alone; curves 5: B[*a*]P + hexadecane. Dosages and other data are given in "Materials and Methods" and table 2.

TABLE 3.—*Two-stage carcinogenesis: Tumor-promoting activity of cocarcinogens and inactive analogues* [a]

Secondary [b] treatment (dose)	Days of testing	Median survival time, days	Days to first papilloma	Mice with papillomas/ total papillomas [c]
Pyrene (40 μg)	448	>448	414	1/1 (1)
Fluoranthene (40 μg)	448	>448	401	1/1 (1)
Catechol (2 mg)	448	>448	—	0
Resorcinol (10 mg)	449	>449	—	0
Hydroquinone (5 mg)	409	>409	—	0
Pyrogallol (5 mg)	449	>449	328	1/1 (0)
PMA (2.5 μg)	449	357	54	43/155 (18)
Anthralin (80 μg)	434	>434	85	9/14 (2)
Acetone	450	>450	—	0
No treatment [d]	443	>427	—	0

[a] We used 50 female ICR/Ha Swiss mice per group, except for the anthralin experiment in which 20 mice were used.

[b] 150 μg B[a]P/0.1 ml acetone applied to dorsal skin once by micropipette. For the anthralin experiment, the initiating dose was 100 μg B[a]P. For the duration of the test, the promoters were applied to the dorsal skin 3 times weekly in 0.1 ml acetone beginning 14 days after initiator. For data on the application of promoting compounds, *see* table 2.

[c] *Numbers in parentheses* are numbers of mice with squamous carcinoma.

[d] 100 mice.

mg per application, catechol and pyrogallol were active and phenol, resorcinol, and hydroquinone inhibited B[*a*]P carcinogenesis.

Six of the 21 compounds tested as cocarcinogens were also tested as tumor promoters in two-stage carcinogenesis on mouse skin, with B[*a*]P as the initiating agent (table 3). Anthralin and PMA were positive controls and showed the expected tumor response (*15, 16*). The results of control experiments for these test compounds, i.e., promoter without B[*a*]P pretreatment, are in table 2. Of the six compounds, only fluoranthene and pyrene each caused a skin carcinoma in 1 of 50 mice. Pyrogallol caused a papilloma in 1 of 50 mice. The other three phenols did not show any tumor-promoting activity.

DISCUSSION

Cocarcinogenic Activity

The results obtained in this work are noteworthy, not only with respect to tobacco carcinogenesis, but also with respect to possible modes of biologic action of a variety of chemical structural types.

Using incidences of skin papillomas and carcinomas and papilloma multiplicity as indexes of biologic activity, one can differentiate four categories within 20 of the 21 test compounds (table 4). Because inconclusive results were obtained with stearic acid it is not included in this table.

A few of the compounds listed in table 4 have been reported in earlier studies. Phenol was tested earlier with a different protocol (*17*): B[*a*]P was the carcinogen, applied three times weekly, with twice weekly applications of a 5 or 10% solution of phenol on alternate days. A weak cocarcinogenic response was observed. Our earlier work (*13*), in which we used the same protocol as that used in the present studies, showed partial inhibition of B[*a*]P carcinogenesis by phenol, which agrees with the present findings.

Fluoranthene and pyrene were tested earlier (*9*) as cocarcinogens by application with B[*a*]P. Although details of these experiments were not reported, the data presented indicated significant cocarcinogenic activity for both compounds, which agrees with our present findings. Hoffmann and Wynder (*9*) observed tumor inhibitory effects when phenanthrene and benz[*a*]anthracene were applied with B[*a*]P. Horton and associates (*10–12*) tested a variety of long-chain hydrocarbons, including some of those listed in table 4 as "accelerators" in carcinogenesis on mouse skin, and reported on the cocarcinogenic activity of some of these materials. In another study (*18*), tumor inhibitory effects were observed for several noncarcinogenic aromatic hydrocarbons administered simultaneously with 3-methylcholanthrene to mice by sc injection. To our knowledge the other compounds listed in table 4 have not been tested for cocarcinogenic activity.

Structure-Activity Relationships

Although all four aromatic hydrocarbons tested showed weak to potent cocarcinogenic activity, the same was not

TABLE 4.—*Cocarcinogenesis experiments: Summary*

Cocarcinogenic activity		Inhibitory activity	
Potent	Weak or moderate	Partial	Complete
Catechol	Benzo(*ghi*)perylene	Phenol	Esculin
Pyrogallol	Lauryl alcohol	Eugenol	Quercetin
Decane	Tetradecane	Resorcinol	Squalene
Undecane		Hexadecane	Oleic acid
Pyrene		Hydroquinone	
B[e]P		Limonene	
Fluoranthene			

true for the long-chain hydrocarbons and phenols. Therefore, though decane, undecane, and tetradecane showed cocarcinogenic activity, hexadecane, tested by the same procedures and at the same dosage, partially inhibited B[*a*]P carcinogenesis. Horton and co-workers (*10–12*) commented on the effect of chain length on cocarcinogenic ctivity. In the case of the phenols, both catechol and pyrogallol contain vicinal phenolic functions and are potent cocarcinogens, whereas all the other phenols tested partially or completely inhibited B[*a*]P carcinogenesis. None of these compounds except quercetin contains a vicinal phenolic function in its structure. This suggests that hydrogen bonding and/or metal chelation have some role in cocarcinogenic activity. PMA and anthralin, which are potent cocarcinogens, also contain functional groups that will facilitate hydrogen bonding and metal chelation.

Effect of Dosage

The dose effect has been alluded to under "Results." In addition, the relative dosages of B[*a*]P and test compounds used did not necessarily correspond to their respective levels in CSC. Except those of the aromatic hydocarbons, the concentrations of test compounds are known only in terms of order of magnitude. Additional bioassays will be needed to clarify the matter of relative concentrations of B[*a*]P and cocarcinogens.

Comparison of Tumor-Promoting and Cocarcinogenic Activity

There is a clear-cut difference in the experimental protocols used in cocarcinogenesis and tumor-promotion (two-stage carcinogenesis) experiments on mouse skin. Since both procedures were used in this study, it is relevant to compare the biologic activities, in the two types of experiments, of the 21 compounds that were examined. The comparison (table 5) includes results from the present work and from earlier reports.

The results of some of these experiments were inconclusive; nevertheless, some observations can be made. Catechol and pyrogallol, both potent cocarcinogens, are devoid of promoting activity. Phenol, a weak promoter (*5, 19, 23*) is not a cocarcinogen and, in fact, partially inhibits B[*a*]P carcinogenicity. Decane, tetradecane, anthralin, and PMA show both types of activity; anthralin and PMA are the most potent tumor-promoting agents known. The pattern that emerges from this short list of compounds indicates that the terms "cocarcinogen" and "tumor promoter" as defined by us, cannot be used interchangeably.

Mode of Action of Cocarcinogens and Inhibitors

Because of the wide differences in chemical structures, reactivity and physical properties of the cocarcinogens and tumor inhibitors described in this report, it is expected that they exert their biologic activities in different ways. There are several possible effects. The test compounds may simply alter the rate of absorption, and hence disappearance, of the carcinogen B[*a*]P from mouse skin. This type of action will depend largely on the lipophilic and/or hydrophilic nature of the test compound.

A second possible effect of the cocarcinogen or inhibitor is to alter the metabolic pathway of B[*a*]P by activation or deactivation of various enzymes, e.g., epoxidizing or hydroxylating enzymes. In this respect, all four aromatic hydrocarbons tested showed cocarcinogenic activity in our present work. Since aromatic hydrocarbons can induce epoxidizing enzymes, this possible mode of action was examined in preliminary studies (*24*). With standard procedures (*25*), all four aromatic hydrocarbons induced aryl hydroxylating enzymes in mouse skin. However, the level of activation was low and did not correlate directly with cocarcinogenic activity. The phenolic cocarcinogens and inhibitors decreased the aryl hydroxylating enzyme activities in mouse skin. It can be concluded that, even though alteration of metabolic pathways of B[*a*]P involving aryl hydroxylating enzymes is a possible mode of action for the aromatic hydrocarbons, the same probably does not apply for phenolic compounds and for the long-chain aliphatic compounds. This conclusion is strengthened by our observation that chain length is important in the determination of cocarcinogenic or inhibitory activity for the aliphatic hydrocarbons and that the positions of hydroxyl functions are important in the determination of the same biologic activities for the phenolic compounds tested. It is also noticeable that the unsaturated aliphatic compounds squalene and oleic acid completely inhibit B[*a*]P carcinogenicity. The preferential oxidation of these materials possibly interferes with the conversion of B[*a*]P to its activated carcinogenic intermediate.

TABLE 5.—*Comparison of cocarcinogenic and tumor-producing activities*

Compound	Cocarcinogenic activity [a]	Tumor-promoting activity [a]
Phenols		
Phenol	+(*17*), −(*13*), −(TR)	+(*5, 17, 19*)
Esculin	−(TR)	−(*20*)
Eugenol	−(TR)	±(*20*)
Quercetin	−(TR)	−(*5*)
Catechol	+(TR)	−(*19*), −(TR)
Resorcinol	−(TR)	±(*19*), −(TR)
Hydroquinone	−(TR)	−(*19*), −(TR)
Pyrogallol	+(TR)	−(*19*), −(TR)
Anthralin	+(TR)	+(*15*), +(TR)
Hydrocarbons (nonaromatic)		
Squalene	−(TR)	−(*17*)
Limonene	−(TR)	NT
Decane	+(TR)	+(*21*)
Undecane	+(TR)	NT
Tetradecane	+(TR)	+(*21*)
Hexadecane	−(TR)	±(*21*)
Aromatic hydrocarbons		
Pyrene	+(*9*), +(TR)	±(TR)
Benzo[*ghi*]perylene	+(TR)	NT
B[*e*]P	+(TR)	NT
Fluoranthene	+(*9*), +(TR)	±(TR)
Long-chain alcohols and acids		
Oleic acid	−(TR)	+(*22*)
Stearic acid	±(TR)	−(*22*)
Lauryl alcohol	±(TR)	NT
PMA	+(*13*), +(TR)	+(*5*), +(TR)

[a] + = positive; − = negative; ± = inconclusive. TR = this report; NT = not tested

Precursors

Of the 21 compounds tested for cocarcinogenic activity, 18 reportedly are components of CSC (*26, 27*). Pyrogallol, quercetin, and esculin have not been found, though it

has been reported that pyrogallol dimethyl ether occurs in CSC (*28*).

Catechol and pyrogallol are two of the most potent cocarcinogens uncovered in this study. Catechol does not occur in tobacco leaf, yet it is the most abundant phenol in CSC. The yield of catechol is 0.4–0.5 mg per cigarette from 85-mm nonfilter cigarettes and 0.2–0.3 mg from filter cigarettes (*29*). It is believed that catechol is formed in the process of tobacco combustion from tobacco flavanoids such as rutin, quercetin, and chlorogenic acid (*29*). Other precursors are probably also responsible for catechol in smoke. Another study (*30*) showed an inverse relationship between the catechol content of CSC and the nitrate content of the tobacco leaf used for the preparation of the CSC. Pyrogallol may be in fresh CSC but rapidly reacts with other smoke components; in addition, pyrogallol is rapidly oxidized by air. Most fractionation procedures used in the past to prepare subfractions of tobacco tar are expected to completely degrade pyrogallol, since alkali has been used extensively in the fractionation of tobacco tars; pyrogallol is rapidly degraded in air in the presence of alkali. Gallic acid (3,4,5-trihydroxybenzoic acid), a likely precursor of pyrogallol, has not been reported in tobacco leaf, (*26, 27*) but it occurs widely in the plant kingdom (*31*). The other compounds, i.e., aliphatic and olefinic hydrocarbons and long-chain acids and alcohols, occur in both tobacco leaf and smoke (*26, 27*).

Conclusion

The results in this report demonstrate the important function of cocarcinogenic agents in tobacco carcinogenesis. Not only are the active agents potent but also phenols, such as catechol, and the long-chain aliphatic hydrocarbons constitute a part of whole CSC. Moreover, the results suggest that other compounds in these categories contribute to cocarcinogenic activity.

The nature of other cocarcinogenic compounds, the mode of action of cocarcinogens, and further studies on tumor inhibitory agents are all areas that deserve further study in the development of a less hazardous cigarette.

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