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Epidermal Carcinogenicity of Bis[2,3-Epoxycyclopentyl]Ether, 2,2-Bis(*p*-Glycidyloxyphenyl)Propane, and *m*-Phenylenediamine in C3H and C57BL/6 Inbred Male and Female Mice

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY UNION CARBIDE CORPORATION · FOR THE DEPARTMENT OF ENERGY

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Errata concerning ORNL Technical Report No. 5375

A recent technical report by J. M. Holland, et al, issued by the Oak Ridge National Laboratory, contained two errors. To avoid propagating these errors and adding further to an already complex and confusing literature we would appreciate your attaching the following errata notice to all subsequent copies of the document entitled "Epidermal carcinogenicity of bis [2, 3-epoxycyclopentyl] ether, 2, 2-bis (pglycidyloxyphenyl) propane and m-phenylenediamine in C3H and C57BL/6 inbred male and female inbred mice."

J. F. Morehead

J. F. Morehead, Y-12, 9706-2, MS 002 (4-1591)

JFM/p

see page 40 for Errata

Becky Minturn 7/17/79

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BIOLOGY DIVISION

EPIDERMAL CARCINOGENICITY OF BIS[2,3-EPOXYCYCLOPENTYL]ETHER, 2,2-BIS(*p*-GLYCIDYLOXYPHENYL)PROPANE, AND *m*-PHENYLENEDIAMINE IN C3H AND C57BL/6 INBRED MALE AND FEMALE MICE

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EPIDERMAL CARCINOGENICITY OF BIS[2,3-EPOXYCYCLOPENTYL]ETHER, 2,2-BIS(*p*-GLYCIDYLOXYPHENYL)PROPANE, AND *m*-PHENYLENEDIAMINE IN C3H AND C57BL/6 INBRED MALE AND FEMALE MICE

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ABSTRACT

Application of bis[2,3-epoxycyclopentyl]ether (I), 2,2-bis(*p*-glycidyloxyphenyl)propane (II), or a 1:1 mixture of I and II three times weekly for 24 months induced malignant epidermal tumors in C3H and C57BL/6 mice. C57BL/6 mice were more sensitive to skin carcinogenesis as evidenced by a higher frequency of tumors occurring at earlier times. The proportion of mice responding at the highest doses were, for C3H: compound I, 5/80 (6%); II, 0/80; I + II, 51/80 (64%); and for C57BL/6: compound I, 5/39 (13%); II, 8/40 (20%); I + II, 32/40 (80%). The substantial difference in the potency of the materials applied as a 1:1 mixture as opposed to their effects when tested individually suggested synergistic interaction. Similar tests of the skin carcinogenicity of *m*-phenylenediamine were negative at all doses and in both strains. An increased incidence of lung tumors ($p < 0.004$) relative to vehicle control was detected in C3H mice treated at the highest dose of I.

INTRODUCTION

The purpose of this study was to determine the skin carcinogenicity of bis[2,3-epoxycyclopentyl]ether (I, CAS No. 2386-90-5), 2,2-bis(*p*-glycidyloxyphenyl)propane (II, CAS No. 1675-54-3), and *m*-phenylenediamine (III, CAS No. 108-45-2).* In addition, in order to detect possible interaction between these resin monomers, a 1:1 mixture of I and II was tested separately, hereafter referred to as the "mixture." All estimations

*CAS refers to Chemical Abstracts Registry.

of the quantitative relationship between dose, time, and tumor incidence are based on histologically confirmed epidermal skin tumors.

In order to detect whether any of the materials, after repeated application to the intact skin, were absorbed in quantities sufficient to evoke a systemic response, major organ systems were examined in mice dying or killed at the end of the study.

Since genetic susceptibility is known to vary substantially between inbred mouse strains, as well as between species, it was of interest to compare the responses of both sexes, using two divergent mouse strains, C3Hf/He (C3) and C57BL/6 (B6). Both strains are highly inbred with inbreeding coefficients approaching unity. They share a common resistance to spontaneous murine leukemia and for this reason are considered relatively long-lived strains. They differ considerably in spontaneous tumor incidence as well as in susceptibility to toxic agents.

In anticipation of future requirements for carcinogenesis bioassay of similar materials and mixtures, it was decided to run parallel tests of the compounds, using currently available, rapid, *in vitro* screening methods. If the results of the rapid and inexpensive methods agreed with the animal tests, considerable savings in both time and money could be realized in future studies of similar materials. Pertinent details concerning the nature of specific screening tests and results obtained on these materials are contained in Appendix A. Results of these tests, compared with animal carcinogenesis data, are considered in the discussion section of this report.

Appendix B contains information on methodological detail, results and conclusions concerning chemical identity, and purity of the compounds

tested. In addition, room air samples were obtained during animal exposure, both as a means of estimating exposure of technical personnel and for the purpose of indirectly estimating the respiratory exposure of the mice to the more volatile materials. As an additional measure of respiratory exposure, air samples were collected from mouse cages immediately after application of the materials.

Materials, Methods, and Experimental Design

Materials were obtained from commercial sources as indicated: I, II (lot No. 1742), and a 1:1 mixture of both (lot No. 1727) were all purchased from Union Carbide (Chemicals and Plastics Division, Hackensack, N.J.). Independent analysis of each material and the mixture (Appendix B) revealed that I contained approximately 10%, by weight, of two additional compounds. Infrared (IR) and nuclear magnetic resonance (NMR) analyses of fractions isolated by gel permeation chromatography and by vacuum distillation have identified these components as an epoxidized polyglycol (Mr > 500) and a small amount of phenyl glycidyl ether. No impurities were detected in II by NMR. NMR analysis of the 1:1 mixture of I and II revealed 50% of each plus a small amount of the components alluded to previously, showing the effect of dilution. Resin III (lot No. 060127) was obtained as a commercial-grade material from Aldrich Chemical and tested as supplied without further analysis. Postive-control benzo[a]pyrene was obtained from Sigma Chemical Company and was used as supplied without further purification. All materials were obtained in a single batch and kept in darkness at room temperature in well-stoppered amber glass containers. Dilutions of each material and the reference standard were

made biweekly in Spectro-grade acetone (Matheson-Coleman-Bell). Dilute solutions were kept in glass-stoppered Erlenmeyer flasks within an unlighted fume hood.

Inbred C3 and B6 mice were produced by the central animal facility of this laboratory and supplied free of enzootic murine viruses and endo- and ectoparasites. Mice were randomly entered into the experiment upon reaching 8 to 10 weeks of age. They were housed five per cage containing ground corncob bedding and given Purina 5010-C and hyperchlorinated-acidified (pH 2.5) water, ad lib. Animals were maintained for the duration of the study in a closed-colony limited-access conventional animal facility. The experimental design and dosage levels are given in Table 1.

Prior to commencing the long-term test, acute toxicity tests were run in order to establish the maximum tolerated dose. In the absence of overt toxicity, the compounds were applied at a concentration dictated by their physical characteristics. Toxicity tests consisted in exposing both sexes of mice daily, five days per week for two weeks, to various doses of each material dissolved in acetone. At the end of this period, survivors were weighed, killed, and examined histologically in order to detect evidence of systemic toxicity.

A 24-month cutoff was chosen for the long-term study because the spontaneous mortality rate increases rapidly after this time (Fig. 1A and B). If comparisons are possible, this duration of exposure would be equivalent, in terms of occupational risk, to a worker first encountering the materials as a young adult with exposure continuing well into retirement. It was also decided that, since skin tumor formation constituted the primary end point of the study, positive-control mice would be killed

Table 1. Experimental design

Mouse strain	Sex	No.	Concentration[a] (%)	Weekly dose[b] (mg)
		Bis[2,3-epoxycyclopentyl]ether		
C3	F	40	50 & 10 v/v	75 & 15
C3	M	40	50 & 10 v/v	75 & 15
B6	F	20	25 & 5 v/v	37.5 & 7.5
B6	M	20	25 & 5 v/v	37.5 & 7.5
		2,2-Bis(*p*-glycidyloxyphenyl)propane		
C3	F	40	50 & 10 v/v	75 & 15
C3	M	40	50 & 10 v/v	75 & 15
B6	F	20	50 & 10 v/v	75 & 15
B6	M	20	50 & 10 v/v	75 & 15
		1:1 mixture of I and II		
C3	F	40	50 & 10 v/v	75 & 15
C3	M	40	50 & 10 v/v	75 & 15
B6	F	20	50 & 10 v/v	75 & 15
B6	M	20	50 & 10 v/v	75 & 15
		m-Phenylenediamine		
C3	F	40	2 & 0.4 w/v	3 & 0.6
C3	M	40	2 & 0.4 w/v	3 & 0.6
B6	F	20	2 & 0.4 w/v	3 & 0.6
B6	M	20	2 & 0.4 w/v	3 & 0.6
		Benzo[*a*]pyrene		
C3	F	40	0.1 & 0.01 w/v	0.15 & 0.015
C3	M	40	0.1 & 0.01 w/v	0.15 & 0.015
B6	F	20	0.1 & 0.01 w/v	0.15 & 0.015
B6	M	20	0.1 & 0.01 w/v	0.15 & 0.015
		Acetone		
C3	F	40	100	0.15
C3	M	40	100	0.15
B6	F	20	100	0.15
B6	M	20	100	0.15

[a]Two dose levels for each sex and strain dissolved in acetone.

[b]Calculated on the basis of a 50-μl volume applied Monday, Wednesday, and Friday. All weights assume a density of 1.0.

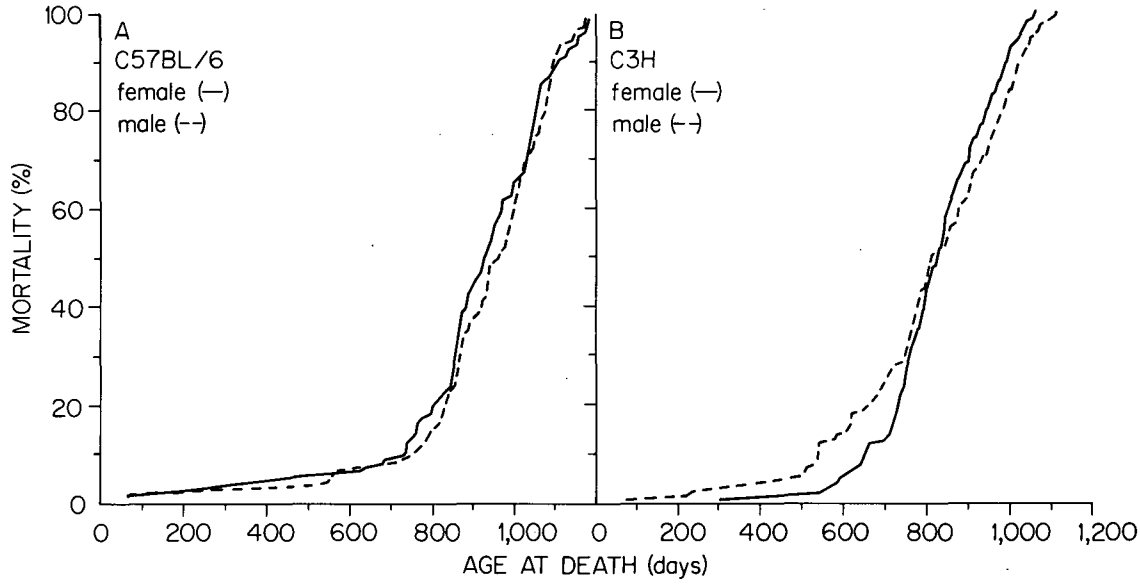


Fig. 1. Cummulative percent survival (ordinate) vs age in days (abscissa) in female (——) and male (---) (A) C57BL/6 mice; (B) C3H/He mice.

at a time determined by the frequency of tumor-bearing mice. The same rule also would have applied to any of the unknowns, had they proven to be sufficiently active as skin carcinogens to induce skin tumors in a number of animals adequate for estimation of tumor latency. Mice were shaved one week prior to commencing exposure and reshaved as required.

Vital statistics recorded for each animal in the experiment included birth date, date exposure commenced, date the first skin tumor was observed, and death date. For purposes of risk assessment, exposure was calculated as the number of days from the initiation of treatment until the first skin tumor appeared or until death without tumor, whichever occurred first. At the time of death, mice were weighed and given complete gross necropsies that included examination of major organ

systems and the brain. Abnormalities were recorded on necropsy record sheets and tissues taken to confirm the gross diagnosis. Skin tumors and suspect skin lesions were routinely taken for histology.

Statistical methods used in the summary and interpretation include estimation of sample means, standard errors, and use of Student's t test to compare the average terminal body weights. More complex analyses have been applied to interpretation of skin tumor risk. Adjustments for intercurrent mortality were required (1) since removing a mouse prior to appearance of a skin tumor, termed censoring, precludes further observation of that mouse. That is, the proportion of mice without a skin tumor at a given number of days of exposure is estimated by adjusting the uncensored observations, for the censored observations. The unadjusted frequencies of lung and skin tumors and deaths for treated groups were compared with acetone control, using the Fisher exact test (2). Other techniques used have been developed for and applied to similar skin carcinogenesis bioassay data by the National Cancer Institute, and it was from them that we obtained computer programs and documentation (courtesy of Dr. Donald Thomas, NCI). Additional details concerning the use of these procedures and interpretation of various parameters used to measure the effect of a given treatment are cited with the specific data.

RESULTS AND ANALYSIS

Acute Toxicity Range-Finding Tests

On a weight basis, the most toxic of the unknown materials was *m*-phenylenediamine (III). At concentrations of 10% or greater in acetone,

signs of toxicity were observed in 10- to 12-week-old mice. After as few as two applications of 50 μl at the 10% concentration (∿10 mg), mice were observed to be depressed, dehydrated, and excreted a brown, discolored urine. Since the acetone solution of the diamine was also brown, it was likely that the material itself could have been excreted. To determine whether this was the case, sterile urine samples were collected by bladder puncture and subjected to column chromatography with UV monitoring (3). By comparing the urine of treated animals with untreated control, several new peaks were revealed in the urine of the treated mice. At least one peak was chromatographically identified as III; the other peaks were presumed to be catabolites. If more than two or three exposures at the 10% level were given, mortality was observed. In addition to urinary excretion, tissues and body fat were also brown, an indication that III dissolved in acetone readily penetrates the intact mouse skin. Other than discoloration, gross lesions were not observed. Microscopic lesions were confined to the liver and kidney, especially the latter, and consisted chiefly of toxic tubular nephrosis. Cause of death was presumed to be acute renal failure and uremic toxemia. Liver lesions were milder by comparison with diffuse degenerative changes characterized by swollen hepatocytes and mild periportal fatty change.

On the basis of these observations, III was tested at a 2% concentration without mortality, acute weight loss, or histologic evidence of systemic toxicity. Therefore, as is true for many chemicals, the line between a toxic and nontoxic dose was narrow; for example, 10 mg given at 5 mg/day for two days was toxic, while 10 mg given at 1 mg/day for ten days was not.

Acute toxicity was noted for only one other substance, bis[2,3-epoxycyclopentyl]ether, but only in one strain, B6. At a concentration of 50% (v/v) in acetone, signs of acute toxicity were observed. After one or two exposures, animals manifested a shock reaction, followed by prostration, coma, and death. Gross necropsy revealed a pale and friable liver and kidney. Microscopically, both tissues showed massive necrosis suggestive of a loss of blood supply. It was possible, although not proven, that this substance in this strain caused release or activation of pharmacologically active substances from storage sites within blood platelets and mast cells. Agents released from these cells could have caused constriction of the blood vessels supplying the liver and kidney, causing death by hepatorenal failure. That some sort of allergic reaction was responsible was suggested by the observation that in B6 animals surviving two or three applications at the 50% level, gradual recovery occurred in spite of continued application of the material. However, in order to minimize the risk of acute toxicity, a lower concentration of I was applied to B6 mice. Direct toxicity to II at 50% was not observed in either strain.

Nonneoplastic Epidermal Changes

Throughout the two-year exposure, allergic skin lesions were observed in mice exposed to I, II, or the mixture. No attempt was made to quantitate the lesions as they appeared to wax and wane. Seldom were more than a few animals in any given group affected. We had the impression that allergic dermatitis was more common early in the experiment and paradoxically more prevalent in the lower doses. Since treatment was

not interrupted in spite of these changes, the phenomenon of tolerance or "hardening," well known in the literature of acute epoxy effects, was frequently observed (4). What this may represent is an unplanned allergic desensitization, similar to that obtained when a sensitive individual is exposed to increasing doses of the allergen until response is diminished, a pattern very similar to that observed here.

Allergic reactions were initially characterized by a focal increase in skin thickness. The lesions eventually became exudative and were initially serous and later purulent. Associated with the exudative phase were erosions and ulceration. Bacteria frequently complicated the ulcerative lesions and no doubt contributed to their severity and persistence. However, in spite of these complications, healing usually occurred with only occasional scar formation.

Another nonneoplastic change induced by the compounds was accelerated hair growth. It was especially noted early in the experiment and occurred exclusively in the epoxy and benzo[*a*]pyrene groups. Stimulation of the hair follicles is interpreted as a manifestation of mild skin irritation with induced epidermal hyperplasia, which is a nonspecific biological response to a wide variety of irritants. It accompanies chemical as well as physical and mechanical irritation of the skin and may influence expression of skin tumors by stimulating cell division. Normal mouse skin is shown in Fig. 2A. The outermost layer is an acellular fibrillar layer of keratin beneath which are the living cells of the epidermis. These cells divide, differentiate, and are target cells for skin carcinogens. Epidermal cells rest upon a thin glycoprotein matrix called the basement membrane, which requires special staining procedures to demonstrate and which separates the outer epidermis from the deeper supporting

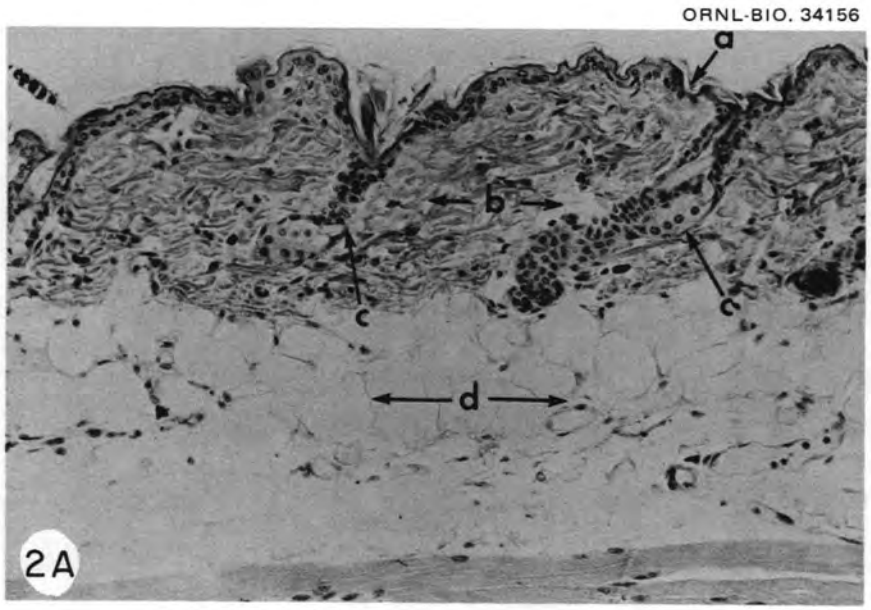
tissues of the dermis. The dermis consists principally of collagen and elastin fibers, blood and lymphatic vessels, nerves and cells of mesenchymal origin including fibrocytes, and emmigrant white cells from the circulation. Embedded within the dermis are hair follicles and associated sebaceous glands. Sweat glands are not present in the mouse.

One of the earliest changes recognizable at the clinical and light microscopic level is epidermal hyperplasia, in this instance associated with exposure to II. In comparison with normal mouse skin, the most notable difference is an increased number of cells in the epidermis as well as an increased mitotic index in cells adjacent to the basement membrane (Fig. 2B). It should be emphasied that these changes are nonspecific and occur in response to a wide variety of irritants. Clinically, epidermal hyperplasia is expressed as a thickened, dry, and scaly region of skin.

Twenty-Four-Month Study

Body weight is a useful index of general toxicity, especially in small mammals such as the mouse. To obtain a measure of whether mice were subject to toxicity, body weights were determined at the time of death for all groups. The average weight of mice treated with the highest dose of each test material was compared with acetone control using Dunnett's test (5). The results are given in Table 2 for C3 and in Table 3 for B6. These tests were run under the assumption of equal variance for all groups.

Significant weight loss indicative of subclinical toxicity was observed at the highest resin doses in both strains. This is interpreted



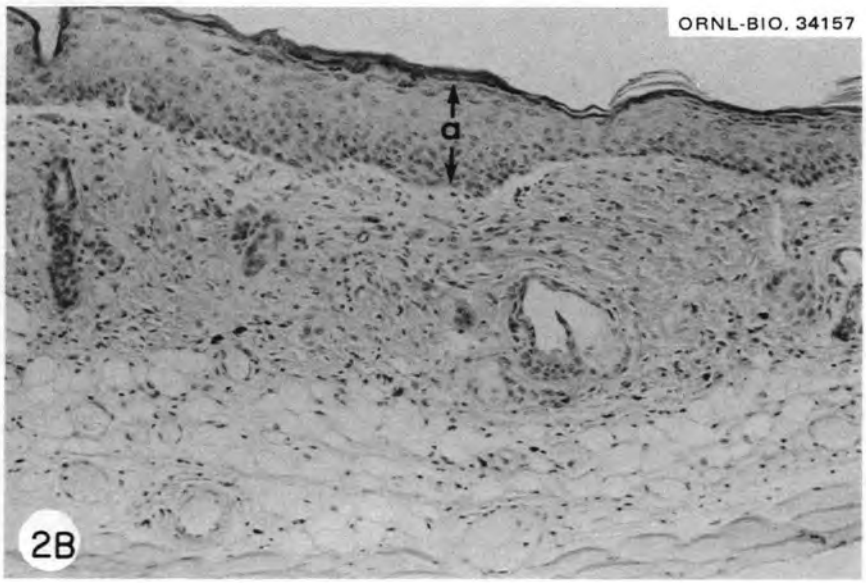


Fig. 2. (A) Normal mouse skin showing the epidermis (a), the collagenous dermis (b), hair follicles and attached sebaceous glands (c), and the subcutis (d), 160X; (B) hyperplasia of the epidermis associated with mild irritation. The number of cell layers in the epidermis is increased (a), associated with increased cell division rates in the basal layer, 160X.

Table 2. Average terminal body weight in C3H mice

Weekly dose (mg)	Sex	No.	Mean weight (g)	Standard error	Student's t[a]
			Bis[2,3-epoxycyclopentyl]ether		
75	F	39	21.3	0.72	-4.14
75	M	39	23.8	0.85	-2.73
15	F	39	25.1	0.78	
15	M	40	26.6	0.66	
			2,2-Bis(*p*-glycidyloxyphenyl)propane		
75	F	39	23.5	0.81	-2.00
75	M	39	25.3	0.73	-1.49
15	F	36	26.1	0.61	
15	M	40	27.2	0.76	
			1:1 mixture of I and II		
75	F	38	24.7	0.65	-1.06
75	M	39	26.9	0.78	+0.96
15	F	40	26.2	0.93	
15	M	38	28.5	0.73	
			m-Phenylenediamine		
3	F	40	25.6	0.69	-0.19
3	M	39	27.2	0.74	+0.39
0.6	F	40	26.4	0.63	
0.6	M	39	27.6	0.69	
			Benzo[*a*]pyrene		
0.15	F	40	26.3	0.31	+0.46
0.15	M	40	29.8	0.33	+3.93
0.015	F	40	27.4	0.48	
0.015	M	40	32.3	0.34	
			Acetone		
0.15	F	40	25.8	0.82	
0.15	M	40	26.8	0.69	

[a]Dunnett's method for multiple comparisons, two-sided test comparing unknowns and acetone control, with values of Dunnett's t of 2.61 and 3.18 for probability levels of 0.05 and 0.01 respectively. Sign (+ or -) indicated direction of the difference.

Table 3. Average terminal body weight in C57BL/6 mice

Weekly dose (mg)	Sex	No.	Average weight (g)	Standard error	Student's t[a]
			Bis[2,3-epoxycyclopentyl]ether		
37.5	F	20	23.1	0.57	-2.26
37.5	M	19	25.1	1.21	-3.18
7.5	F	20	25.8	0.83	
7.5	M	20	31.1	0.57	
			2,2-Bis(*p*-glycidyloxyphenyl)propane		
75	F	19	23.9	0.83	-1.16
75	M	20	25.4	1.26	-2.84
15	F	20	23.5	1.08	
15	M	20	28.4	0.98	
			1:1 mixture of I and II		
75	F	18	18.2	1.05	-5.59
75	M	20	18.5	1.22	-8.23
15	F	20	23.5	0.66	
15	M	18	26.1	1.11	
			m-Phenylenediamine		
3	F	19	23.8	0.40	-1.63
3	M	20	28.0	0.61	-1.58
0.6	F	20	23.3	0.88	
0.6	M	20	28.5	0.55	
			Benzo[*a*]pyrene		
0.15	F	18	22.9	0.28	-3.10
0.15	M	20	29.7	0.49	+0.75
0.015	F	20	24.4	0.37	
0.015	M	20	29.8	0.41	
			Acetone		
0.15	F	16	24.9	0.62	
0.15	M	20	29.2	0.46	

[a]Values of Dunnett's t of 2.67 and 3.29 for probability levels of 0.05 and 0.01 respectively.

as positive evidence that animals were challenged at a nearly maximum resin dose. In other words, dose levels higher than 50% would probably have been too toxic to permit evaluation of chronic effects. The apparent significant weight gain shown for benzo[a]pyrene positive controls is presumed to be an artifact. Since benzo[a]pyrene mice were killed on the basis of the frequency of skin tumors, they were much younger, as a group, than were the acetone controls. Body weight, after reaching a maximum in mature mice, gradually declines with the onset of natural senescence. We think it likely that declining body weight of the vehicle controls was responsible for the positive effect of the benzo[a]pyrene treatment on body weight.

The odds ratio technique was applied to determine whether mortality rate for treated animals differed significantly from acetone control. The technique compares the ratio of odds for survival in treated to the odds for survival in control for successive intervals over the duration of the study. Using the maximum-likelihood procedure, the ratio of difference in survival rate, comparing treated mice and acetone control, was calculated along with a one-sided P for the significance of difference. The results of these calculations are given in Table 4 for C3 and Table 5 for B6. The procedure and additional examples of its application are described in greater detail elsewhere (6). Significantly increased mortality ($p < 0.05$) was noted in C3 mice only in females at the highest dose of I. In B6 mice, males showed significantly greater mortality from I at both doses and from II and III at the highest dose only. Significantly greater mortality was noted in both sexes of B6 exposed to the mixture at high dose and in males also at low dose.

Table 4. Cumulative percent mortality for the duration of the experiment in C3H mice

Weekly dose (mg)	Sex	Interval (days) 56–500	501–600	601–700	701–800	P[a]
		Bis[2,3-epoxycyclopentyl]ether				
75	F	2	5	25	62	0.05
75	M	8	10	18	45	0.56
15	F	8	15	25	55	0.31
15	M	2	5	10	38	0.35
		2,2-Bis(*p*-glycidyloxyphenyl)propane				
75	F	2	12	32	48	0.26
75	M	10	15	35	55	0.16
15	F	5	10	30	48	0.28
15	M	0	2.5	12	42	0.40
		1:1 mixture of I and II				
75	F	0	5	15	52	0.28
75	M	2	8	25	42	0.54
15	F	0	5	18	42	0.55
15	M	5	18	30	50	0.30
		m-Phenylenediamine				
3	F	0	5	22	45	0.43
3	M	2	10	15	42	0.46
0.6	F	0	2	18	50	0.34
0.6	M	5	12	25	48	0.42
		Benzo[*a*]pyrene				
0.15	F	0				
0.15	M	0				
0.015	F	2				
0.015	M	0				
		Acetone				
0.15	F	0	5	15	42	
0.15	M	2	8	20	45	

[a]One-sided probability comparing each group with the corresponding acetone control. Calculations were based on 40 animals of each sex per compound.

Table 5. Cumulative percent mortality for the duration of the experiment in C57BL/6 mice

Weekly dose (mg)	Sex	Interval (days) 56–500	501–600	601–700	701–800	P[a]
		Bis[2,3-epoxycyclopentyl]ether				
37.5	F	0	5	10	21	0.48
37.5	M	0	0	15	35	<0.01
7.5	F	0	5	15	15	0.32
7.5	M	0	5	15	20	0.05
		2,2-Bis(*p*-glycidyloxyphenyl)propane				
75	F	5	10	15	35	0.41
75	M	0	5	20	25	0.02
15	F	0	0	25	25	0.55
15	M	0	5	5	15	0.12
		1:1 mixture of I and II				
75	F	5	10	30	80	<0.01
75	M	0	5	35	80	<0.01
15	F	0	5	10	30	0.56
15	M	0	5	20	25	0.02
		m-Phenylenediamine				
3	F	5	5	10	10	0.20
3	M	5	10	10	35	<0.01
0.6	F	0	20	25	35	0.39
0.6	M	0	0	5	10	0.25
		Benzo[*a*]pyrene				
0.15	F	10				
0.15	M	5				
0.015	F	0				
0.015	M	0				
		Acetone				
0.15	F	5	20	20	25	
0.15	M	0	0	0	0	

[a]As in Table 4, with the exception that there were 20 animals of each sex used per compound.

Thus, weight loss and overt mortality reinforce one another as integrative measures of general toxicity. Sex differences were apparent, as were differences attributable to strain. The basis for these differences is unknown.

Assessment of tumor incidence in tissues other than skin was a secondary objective of this study. That is, while skin carcinogenesis was the primary focus of this study, we were also interested in whether the unknown materials penetrated the skin of a mouse in amounts sufficient to alter nonepidermal tumor risk relative to the acetone control. The reason we considered this a secondary end point was that the dose absorbed was unknown, and the experimental protocol was sufficient to detect only those tumors visible to the naked eye. The incidence of histologically confirmed tumors observed at death is given for C3 (Table 6) and B6 (Table 7). Tumor incidence fluctuated greatly and either was uncorrelated with dose or comparable to the acetone control with one possible exception, lung tumors in C3 mice. Using Fisher's exact test (2), the frequency of lung tumors in C3 (sexes pooled) was increased following I at 75 mg/week ($p = 0.004$). All other comparisons were not significant ($p > 0.05$). Lung tumor risk was not significantly increased in any of the B6 groups ($p > 0.05$).

If the increased lung tumor frequency in C3H mice is real, an estimate of vapor-phase exposure becomes important. These calculations are based on air samples obtained from mouse cages after application of I at both the 50 and 10% level and following III at the 2% level. Details concerning the test procedures can be obtained from Appendix B. The vapor-phase concentration of II was not monitored because it was determined

Table 6. Frequency of C3H mice[a] with neoplastic disease in tissues other than skin

Compound	Weekly dose (mg)	Sex	Frequency of tumor-bearing animals by organ or histologic type										
			Lung	Liver	Kidney	Bladder	Reticulum cell sarcoma and lymphoma	Ovary	Other endocrine	Breast	Connective tissue	Other epithelial	Other Mesenchymal
I	75	F	9	14	0	0	6	13	0	3	5	0	0
	75	M	5	16	1	0	3		1		0	1	0
	15	F	3	3	0	0	3	14	1	6	8	2	0
	15	M	4	28	1	0	2		1		0	0	0
II	75	F	6	3	0	0	3	15	2	4	9	0	1
	75	M	3	22	0	0	3		1		1	0	0
	15	F	1	5	0	0	5	18	0	10	9	0	5
	15	M	1	21	2	0	11		0		0	1	2
I + II	75	F	1	8	0	0	5	15	2	8	10	0	2
	75	M	3	14	1	0	5		0		1	2	0
	15	F	2	5	0	0	6	14	0	6	6	3	3
	15	M	6	19	0	0	6		0		4	0	0
III	3	F	5	3	0	0	7	19	0	3	11	0	4
	3	M	4	19	0	0	4		0		3	0	1
	0.6	F	5	4	1	0	11	13	1	6	6	2	3
	0.6	M	3	21	0	0	13		0		0	0	2
Benzo[*a*]pyrene	0.15	F	0	0	0	0	0	0	0	0	3	0	0
	0.15	M	0	0	0	0	0		0		1	0	0
	0.015	F	0	0	0	0	0	0	0	0	0	0	0
	0.015	M	0	5	0	0	0		0		0	0	0
Acetone	0.15	F	1	6	0	0	6	17	0	11	8	1	0
	0.15	M	2	20	0	0	4		0		1	0	1

[a]Forty animals of each sex were used for each material and dose.

Table 7. Frequency of C57BL/6 mice[a] with neoplastic disease in tissues other than skin

Compound	Weekly dose (mg)	Sex	Frequency of tumor-bearing animals by organ or histologic type										
			Lung	Liver	Kidney	Bladder	Reticulum cell and lymphoma	Ovary	Other endocrine	Breast	Connective tissue	Other epithelial	Other Mesenchymal
I	37.5	F	1	0	0	0	7	0	0	0	0	1	1
	37.5	M	1	1	0	0	3		0		4	0	0
	7.5	F	0	0	0	0	5	0	0	0	0	0	0
	7.5	M	1	3	0	0	4		0		1	0	1
II	75	F	0	2	0	0	10	1	0	1	0	0	0
	75	M	1	0	0	0	8		0		0	0	0
	15	F	0	1	0	0	7	0	1	0	0	1	1
	15	M	1	5	0	0	7		0		0	0	0
I + II	75	F	1	1	0	0	2	0	0	0	0	1	0
	75	M	0	1	0	0	1		0		0	0	0
	15	F	0	1	0	0	12	1	1	0	0	0	0
	15	M	4	0	0	0	5		0		0	1	0
III	3	F	0	0	0	0	3	0	0	0	0	0	0
	3	M	0	0	0	0	5		0		0	0	2
	0.6	F	1	1	0	0	7	0	1	0	0	1	2
	0.6	M	0	2	0	0	8		0		2	0	1
Benzo[*a*]pyrene	0.15	F	0	0	0	0	0	0	0	0	1	0	1
	0.15	M	0	0	0	0	0		0		0	0	0
	0.015	F	0	0	0	0	0	0	0	0	0	0	0
	0.015	M	0	0	0	0	0		0		0	0	0
Acetone	0.15	F	0	3	0	0	6	2	0	0	0	2	1
	0.15	M	1	1	0	0	3		0		0	0	0

[a]Twenty animals of each sex were used for each material and dose.

that this substance does not volatilize appreciably at mouse skin temperature. Following routine application of the materials to five mice at 50 μl each, silica-gel tubes were inserted into the mouse cage to a level extending to the breathing zone. Cage air was pulled through the filters at a rate of 1 liter/min for 110 min. Compounds trapped in the tubes were then analyzed and the quantities expressed as micrograms per liter of air. Sampling within a cage treated with III gave values of 0.02 μg/liter for III and 0.10 and 0.04 μg/liter for the solid and liquid isomer of I respectively (lower limit of sensitivity, 1 μg/ml in the assay solvent for either isomer). On this basis, some crossover of the more volatile I did occur. Sampling the 50% I cage failed to detect III in amounts in excess of 0.01 μg/liter, while concentration of the solid isomer of I was 1.26 and of the liquid isomer was 0.49 μg per liter of air (1.75 μg total). Sampling cages treated at the 10% I level also failed to reveal crossover of III and gave a concentration for the solid isomer of I of 1.09 μg/liter and for the liquid isomer 0.46 μg/liter. Similar concentrations of I at both the 50 and 10% levels suggested that even at the 10% level there was sufficient material to equilibrate with cage air for the entire sampling period. However, it is likely that vapor exposure would be more prolonged at the higher concentrations and that the sampling period (maximum 110 min) was not long enough to reflect this difference.

In order to obtain a first-order approximation of the amount of substance I that mice could have inhaled, we have made the following assumptions: (1) the sampling procedure itself did not alter the air exchange rate within a cage; (2) an arbitrary 4-hr period during which

time the materials continue to volatilize; (3) a mouse respiratory minute volume of 24 ml/min (ref. 7); (4) all of substance I trapped by silica-gel tubes reaches the lungs; (5) substance I reaches the lungs by no route other than inhalation; (6) potential crossover of III to I cages did not influence the response.

Based on these assumptions, if, in a 4-hr period, a mouse inhaled 5.76 liters of air containing 1.75 μg/liter of compound I, the inhaled dose would be 10 μg per exposure, or 30 μg per week. If vapors persist longer than 4 hr or if sampling at 1 liter/min in a 9-liter cage reduced the instantaneous maximum concentration, then the dose estimate would be low. Alternatively, if inhaled vapors either absorb to the upper airways or remain in the air stream, then lung dose would be less than "silica-gel" dose. Since either or both of these possibilities are likely, we express no certainty in our estimate of lung dose.

Another explanatory note, applicable to both strains, concerned the apparently decreased frequency of nonepidermal neoplastic disease in mice treated with benzo[*a*]pyrene. This is because these mice were killed at an age determined by the presence of a skin tumor. Since skin tumors developed rapidly, mice in this group were much younger as a group than were other mice in the experiment and thus were not at risk for diseases developing late in life.

For the purpose of establishing the carcinogenicity of the various materials, the most important end point was skin tumorigenesis. Overall frequencies for various tumor types are given in Table 8 for C3 and in Table 9 for B6. Tumor frequencies alone are not adequate to define potency; however, they do give an overall impression of the difference

Table 8. Skin tumor frequency by histologic type in C3H mice[a]

Weekly dose (mg)	Sex	Papilloma	Carcinoma		Total
			Localized	Metastatic	
		Bis[2,3-epoxycyclopentyl]ether			
75	F	1	2	0	3
75	M	1	1	0	2
15	F	0	0	0	0
15	M	0	0	0	0
		2,2-Bis(*p*-glycidyloxyphenyl)propane			
75	F	0	0	0	0
75	M	0	0	0	0
15	F	0	0	0	0
15	M	0	0	0	0
		1:1 mixture of I and II			
75	F	12	6	1	19
75	M	13	19	0	32
15	F	3	1	1	5
15	M	4	8	2	14
		m-Phenylenediamine			
3	F	0	0	0	0
3	M	0	0	0	0
0.6	F	0	0	0	0
0.6	M	0	0	0	0
		Benzo[*a*]pyrene[b]			
0.15	F	1	39	0	40
0.15	M	2	38	0	40
0.015	F	6	25	1	32
0.015	M	11	28	1	40
		Acetone			
0.15	F	1	0	0	1
0.15	M	0	0	0	0

[a]Forty animals per sex, dose, and compound.

[b]Benzo[*a*]pyrene was applied for 220 days at the 0.15 level and for 375 days at the 0.015 level.

Table 9. Skin tumor frequency by histologic type in C57BL/6 mice[a]

Weekly dose (mg)	Sex	Papilloma	Carcinoma		Total
			Localized	Metastatic	
		Bis[2,3-epoxycyclopentyl]ether			
37.5	F	0	1	0	1
37.5	M	1	3	0	4
7.5	F	0	0	0	0
7.5	M	0	0	0	0
		2,2-Bis(*p*-glycidyloxyphenyl)propane			
75	F	1	1	0	2
75	M	0	6	0	6
15	F	0	0	0	0
15	M	1	0	0	1
		1:1 mixture of I and II			
75	F	2	13	0	15
75	M	0	11	6	17
15	F	1	0	1	2
15	M	0	0	1	1
		m-Phenylenediamine			
3	F	0	0	0	0
3	M	0	0	0	0
0.6	F	0	0	0	0
0.6	M	0	0	0	0
		Benzo[*a*]pyrene[b]			
0.15	F	2	17	1	20
0.15	M	1	19	0	20
0.015	F	6	11	1	18
0.015	M	5	14	1	20
		Acetone			
0.15	F	0	0	0	0
0.15	M	0	0	0	0

[a]Twenty animals per sex, dose, and compound.

[b]Benzo[*a*]pyrene was applied for 220 days at the 0.15 level and for 310 days at the 0.015 level.

between compounds. Considering first the C3 strain, it was apparent that I at the highest dose was weakly carcinogenic. In this strain neither III nor II, at the doses tested, elicited skin tumors. An unusual aspect of this experiment was the striking potentiation of carcinogenesis when the two resins were combined. Based on crude frequencies, the effect resulted in approximately a tenfold difference in tumor yield. A reminder that all tissues possess an intrinsic background tumor incidence was the detection of a benign tumor in a single female treated with acetone only. The statistical procedures applied to these data take account of this observation in the estimation of tumor risk relative to acetone control in this strain.

The same general pattern was observed in B6 (Table 9), plus the demonstration of this inbred strain's greater genetic sensitivity to skin carcinogens. It is probably this sensitivity that accounts for the detection of weak carcinogenicity due to II. As was the observation in C3, substance III also failed to induce skin tumors in B6. Synergistic carcinogenesis was noted for the mixture, with tumor frequencies approaching 100% in males.

Gross, microscopic, and behavioral features of these tumors were varied, but a few patterns did emerge. The tumors themselves varied in size and gross appearance from 1- to 2-mm focal lesions to complex growths often involving the entire back. Since, with the exception of the benzo[*a*]pyrene control group, tumor-bearing mice were not killed until the end of the experiment, many of the more malignant lesions were metastatic to both regional lymph nodes (Fig. 3A) and lung (Fig. 3B). The probability of metastasis appeared to be proportional to tumor

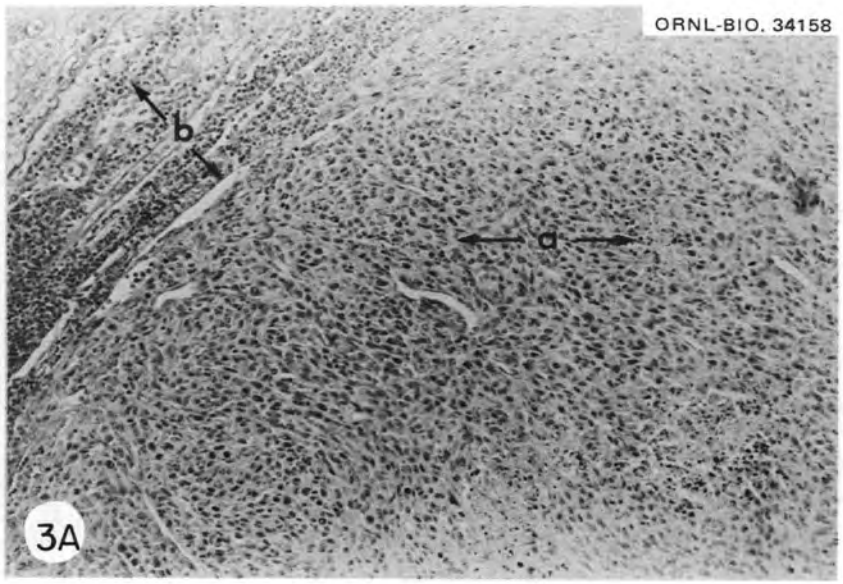


Fig. 3. (A) Squamous cell carcinoma metastatic to a regional lymph node. The nodule of tumor cells (a) has displaced the normal lymphatic tissue (b), causing gross enlargement and distortion of the lymph node, 160X. (B) Lung showing widespread metastasis of epidermal squamous carcinoma. Tumor cells infiltrate and obliterate lung tissue surrounding small bronchioles (a) and blood vessels (b), 30X.

residence time. This explains the greater frequency of metastasis in mice treated with unknowns as well as the greater frequency of metastasis at the lower doses of benzo[*a*]pyrene.

Tumor malignancy was determined histologically, on the basis of cellular characteristics as well as infiltrative local extension. In instances where tumors were multiple, the animal's diagnosis reflected the most advanced tumor stage. For example, if a mouse had several papillomas, a few locally infiltrative carcinomas, and a metastatic solitary carcinoma, for purposes of the data table, this animal would have been diagnosed as have a metastatic carcinoma and would not be counted in either of the other two categories. In the case of the unknown materials, tumor multiplicity was not considered an interpretable parameter because long residence times as well as malignancy of many tumors contributed to confluency.

Diagnostic criteria are best illustrated by giving specific examples of various tumor types. Lesions were enough alike in the two strains to make the criteria of one equally applicable to the other. More advanced focal epidermal hyperplasia was referred to as an acanthotic or hyperkeratotic plaque (Fig. 4). These were discrete lesions and were not true neoplasms, but were considered to represent an immediate precursor to the benign papilloma (Fig. 5). Features distinguishing the papilloma from the preneoplastic acanthoma were changes more of degree than of kind. In the papilloma, fronds of hypercellular epidermis projected deeply into the epidermis, and in the mature tumor often penetrated to the subcutis. The external surface of these tumors was highly convoluted. Among features that helped distinguish the papilloma from the more

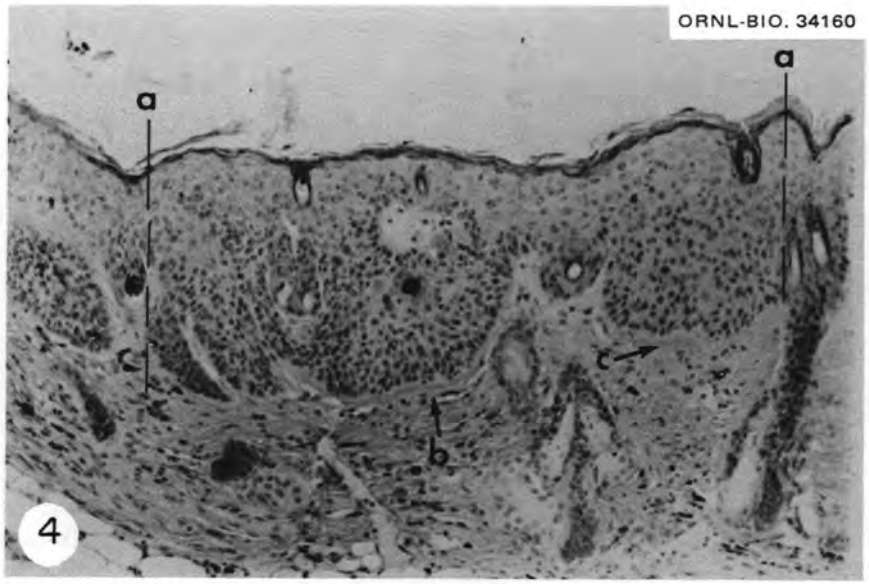


Fig. 4. Focus of proliferating epidermal cells referred to as an acanthotic plaque (a). The cells are beginning to encroach upon the underlying dermis (b) but still remain aligned with respect to the basement membrane (c), 160X.

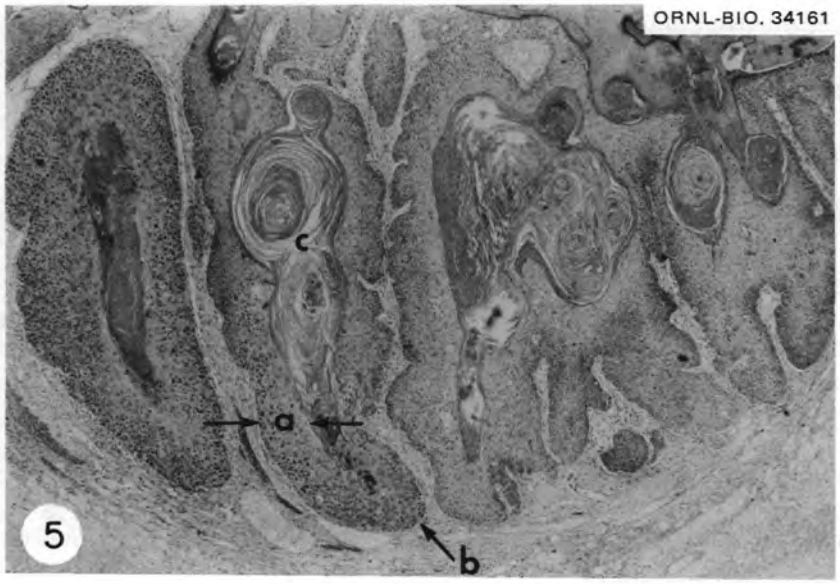


Fig. 5. Mature squamous papilloma distinguished by prominent, thickened bands of epidermis (a) extending deep into and through the dermis with epidermal cells in contact with subcutaneous adipose tissue (b). Onion-skin lamellae of keratin produced by differentiating tumor cells (c) are scattered throughout the section, 30X.

aggressive (malignant) carcinoma was preservation of normal epithelial growth polarity in the former. In papillomas, the neoplastic epithelial cells pass through a more or less ordered progression from the undifferentiated dividing state to the end stage of differentiation, the formation of keratin. Another criterion for determination of malignancy was the interface between epithelium and underlying dermis, the basement membrane. Benign tumors respected this physiologic and biochemical barrier, and thus there was a sharp line of demarkation between epithelium and underlying dermis. Malignant cells do not appear to form basement membranes, and thus structural relationships are not preserved. Lastly, the appearance of the cells themselves provides additional evidence for malignancy. The cells of a benign tumor are typically epithelial, while malignant tumors were comprised of cells that often could not be classified. In addition, the cells of benign tumors usually differ only slightly from one another, while cellular characteristics within malignant tumors differ greatly. It should be emphasized that the line between benign and malignant is often not sharply defined, that individual tumors vary considerably from area to area, and that a decision of malignancy is always based on a composite analysis of all the facets mentioned. An illustration of an early (Fig. 6), advanced (Fig. 7), and anaplastic (Fig. 8) squamous cell carcinoma is included as a means of illustrating the features described above.

Each treated group was compared with the respective acetone control, using a modification of Cochran's method of combining contingency tables (8,9). For C3 mice, skin tumor incidence in both sexes of mice at the high dose of I was 5/80. This increase was significant, with a

Fig. 6. Early squamous cell carcinoma exhibiting a less well ordered growth pattern. Clusters of neoplastic epidermal cells (a) are scattered throughout the dermis that also shows reactive fibroplasia (b), 30X.

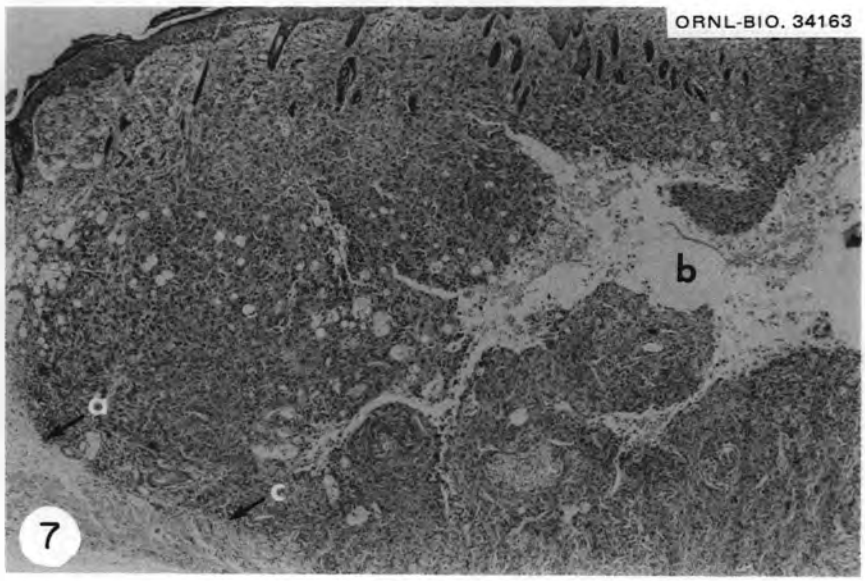


Fig. 7. More advanced squamous cell carcinoma showing lateral extension beneath the epidermis by invasion and infiltration of subcutaneous fat (a). The central area of the tumor has undergone necrosis and is cystic (b). Near the bottom of the photograph, tumor cells are invading and destroying the cutaneous musculature (c), 30X.

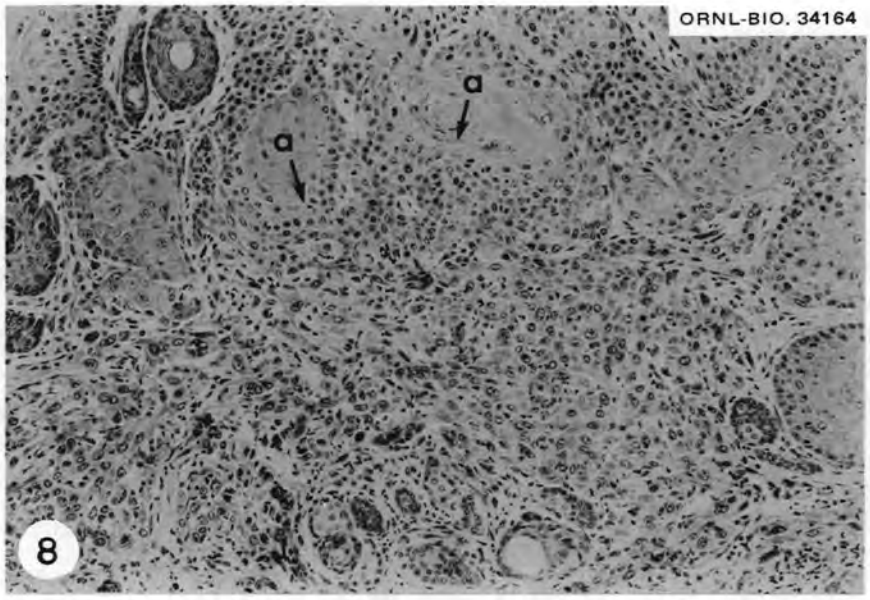


Fig. 8. Closer view of a malignant squamous carcinoma showing extreme variability in cell size and shape as well as loss of normal alignment with the basement membrane (a), 160X.

probability of 0.043. All other comparisons of C3 were either obviously significant (mixture and benzo[*a*]pyrene) or obviously nonsignificant (II or III). In B6 mice there were four borderline results: at the 37.5-mg dose of I, 5/41; at the 75-mg dose of II, 8/40; at the 15-mg dose of II, 1/40; and the 15-mg dose of the mixture, 3/40. The difference between unknown and acetone control was significant for I at 37.5-mg ($P = 0.044$) and for II at 75 mg ($P = 0.014$). Differences were nonsignificant for II at 15 mg and for the mixture at 15 mg ($P > 0.05$). In all other instances the results were obvious.

Another parameter useful for comparison of the various materials and dose levels is relative time to appearance of tumor after adjustment for differences in mortality rate between groups. Using the Kaplan-Meier procedure (1) for adjusting the data for intercurrent mortality, we have calculated the expected duration of exposure necessary to achieve

a given probability of skin tumor in mice exposed at each dose level when sufficient tumor data were available. The calculations were based on the distribution of times to appearance of the first skin tumor on each mouse, adjusted for deaths of mice without observable skin tumors. The data for C3 mice, both sexes pooled, are given in Table 10 and for B6 mice in Table 11. The absence of an entry, at a given level of probability, means that tumor risk in the unknown group did not exceed acetone control over the 720-day duration of the experiment.

Many methods have been advocated for estimation of relative risk or hazard based on experimental animal data. The assumptions necessary for these estimations require ample caution in extrapolating the conclusions. Nevertheless, a hazard index does give an overall impression of relative potency, and for this reason we have calculated this index for the mixture of I and II relative to benzo[a]pyrene. One approach is to fit the data to a function that interrelates the susceptibility of the animal and the dependent (time to tumor) and independent (dose rate) experimental variables. The Weibull distribution has been found to be useful for making comparisons between compounds when animals are exposed to constant, repetitive doses and the measured response variable is time to tumor (10).

In the three-parameter Weibull model the rate of appearance of new tumor-bearing animals is $bk(t - w)^{k-1}$, where t is the number of days from initiation of treatment until a skin tumor is detected, and w and k are constants. The constant w is the length of the minimum latent period and may be a characteristic of the animal species used for the test. The constant k determines the shape of the distribution and may

Table 10. Duration of exposure necessary to achieve a specified probability of skin tumor in surviving C3H mice, sexes pooled[a]

Compound	Weekly dose (mg)	Tumor positive	Probability of tumor (Kaplan-Meier)			
			0.1	0.25	0.50	0.75
I	75	5	724[b] (689, *c*)	*c*	*c*	*c*
I + II	75	45	524 (495, 595)	620 (570, 658)	696 (661, 731)	742 (730, *c*)
I + II	15	20	588 (536, 685)	690 (618, 737)	*c*	*c*
Benzo[*a*]pyrene	0.15	80	108 (103, 112)	113 (111, 115)	124 (117, 128)	131 (129, 134)
Benzo[*a*]pyrene	0.015	69	229 (219, 241)	252 (240, 268)	275 (270, 279)	296 (282, 307)

[a]Eighty animals were used per compound and dose.

[b]Elapsed time in days from initial exposure to given probability of tumor. Numbers in parentheses are approximate 95% confidence limits.

[c]Estimated duration of exposure is beyond the end of the experiment.

Table 11. Duration of exposure necessary to achieve a specified probability of tumor in C57BL/6 mice, sexes pooled[a]

Compound	Weekly dose (mg)	Tumor positive	Probability of tumor (Kaplan-Meier)			
			0.1	0.25	0.50	0.75
I	37.5	5	710[b] (595, *c*)	*c*	*c*	*c*
II	75	8	605 (582, 744)	724 (651, *c*)	*c*	*c*
I + II	75	32	390 (354, 410)	416 (395, 437)	461 (430, 517)	559 (477, 636)
Benzo[*a*]pyrene	0.15	40	78 (72, 86)	90 (81, 91)	93 (90, 100)	104 (98, 105)
Benzo[*a*]pyrene	0.015	39	145 (126, 173)	172 (154, 180)	193 (178, 199)	209 (196, 221)

[a]Forty animals were used per compound and dose.

[b]Same as in Table 10.

[c]Same as in Table 10.

be related to the number of discrete steps or stages necessary for expression of a skin tumor (11). The proportionality factor, b, is a scale parameter directly related to the chemical dose.

The model is fitted to the actual proportions of tumor-bearing animals, corrected for intercurrent mortality (1), by simultaneously determining the factor b for each group, while a common value of k, and likewise of w, is estimated. The function is nonlinear, and a computer program furnished by the National Cancer Institute was used to estimate the parameters by the method of maximum likelihood (12). For proper interpretation of the parameter b, a good fit to the data for each group and constancy of k and w among groups are necessary. A comparison of the fitted lines (dashed) to the actual data (solid) for C3 (Fig. 9) and B6 (Fig. 10) are given for high and low doses of both benzo[a]pyrene and the mixture of I and II. Common values of k and w estimated from all strains, doses, and compounds were 6.2 and 48.5 respectively.

Tests of the assumption of constant k and w appear valid (Pr = 0.05) for all except the B6 group at the highest dose of I + II. Including these data forced statistical rejection of the hypothesis of constant k and w (Pr < 0.001). However, for purposes of risk assessment, we have elected to assume k and w constant on the strength of the other seven data groups. Table 12 gives the parameter b for each group. By assuming a relationship between b and dose we can estimate the tumorigenic efficiency of I + II and benzo[a]pyrene in relation to dose. An estimate of the potency at any dose can be obtained by assuming that the logarithms of the parameter b and the logarithms of doses are linearly related. This assumption could not be tested, since only two nonzero dose levels were used for each compound.

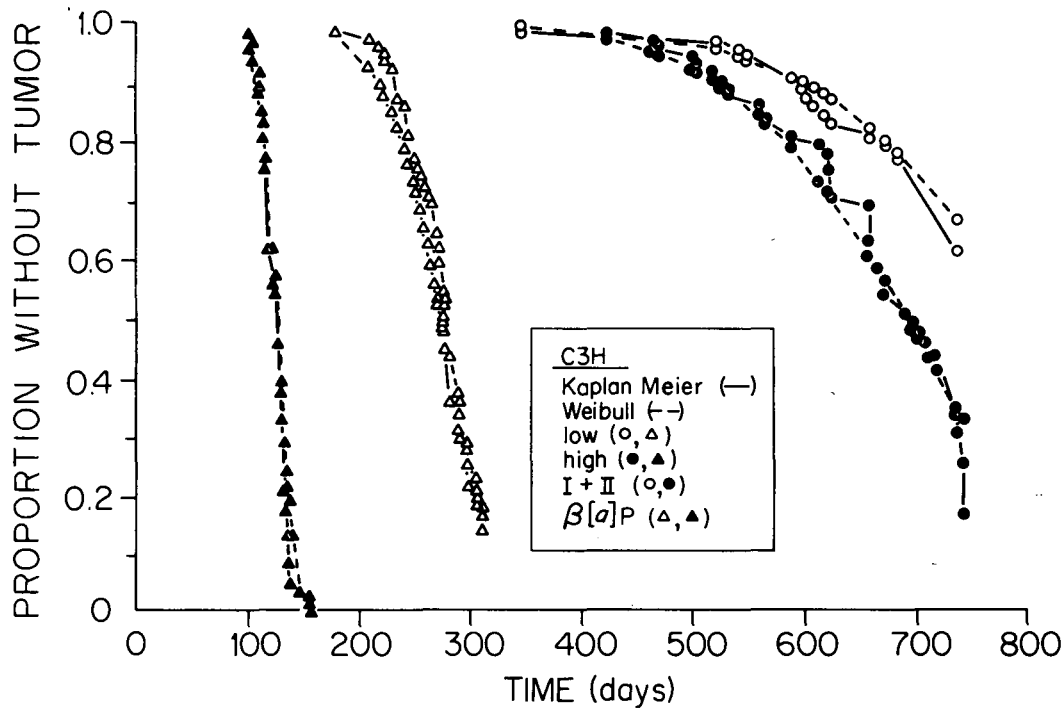


Fig. 9. Weibull distribution lines (---) superimposed on the age-specific tumor incidence rate (——) for C3H/He mice, both sexes pooled. Proportion of animals surviving without tumor (ordinate) vs duration of exposure (abscissa) in days. Symbols correspond to benzo[*a*]pyrene at 0.15 (▲) or 0.015 (Δ) mg/week or the 1:1 mixture of compounds I and II at 75 (●) or 15 (○) mg/week.

Relative potency is estimated by the ratio of doses which induce an equivalent response. When the dose-response lines are parallel, the relative potency is constant. A test of the assumption of parallelism between the dose-response lines for the mixture and benzo[*a*]pyrene could not be rejected ($P > 0.3$). On this basis, parallel lines were fit to the data for each strain at the high and low doses of I + II and benzo[*a*]pyrene respectively (Fig. 11). The distance between the two lines, in units of log dose at any arbitrary level of b, is a measure

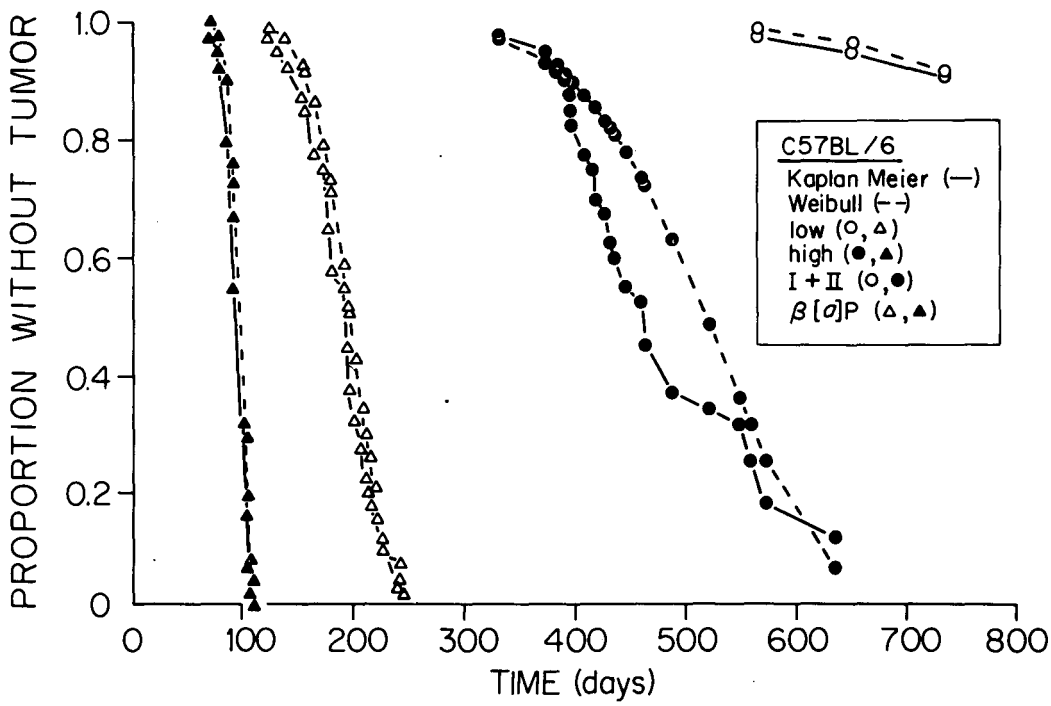


Fig. 10. Weibull distribution lines (---) superimposed on the age-specific tumor incidence rate (——) for C57BL/6 mice, both sexes pooled. Proportion of animals surviving without tumor (ordinate) vs duration of exposure (abscissa) in days. Symbols correspond to benzo[*a*]pyrene at 0.15 (▲) or 0.015 (Δ) mg/week or the 1:1 mixture of compounds I and II at 75 (●) or 15 (○) mg/week.

of difference in potency between the two materials. The calculated difference is 4.84, as indicated in Fig. 11 by the horizontal dashed line. The antilog of 4.84 is 69,000; thus the potency of benzo[*a*]pyrene was approximately 69,000 times that of the mixture. Because dose response data for I + II, tested as individual compounds, were limited, we did not attempt to estimate their individual potencies. However, comparison of the observed tumor yield, at equivalent exposure intensities, indicated that they were substantially weaker skin carcinogens than the mixture.

Table 12. Estimated parameter, b, for Weibull fit to skin carcinogenesis data, assuming constant k and w[a]

Material	Strain	Weekly dose (mg)	b
I + II	C3	75	3.59×10^{-18}
I + II	B6	75	2.54×10^{-17}
I + II	C3	15	1.38×10^{-18}
I + II	B6	15	3.31×10^{-19}
Benzo[*a*]pyrene	C3	0.15	1.83×10^{-12}
Benzo[*a*]pyrene	B6	0.15	3.03×10^{-11}
Benzo[*a*]pyrene	C3	0.015	2.35×10^{-15}
Benzo[*a*]pyrene	B6	0.015	3.14×10^{-14}

[a] k = 6.2; w = 48.5, with standard errors of 0.41 and 6.03 respectively.

Fieller (13) has suggested a means for obtaining an exact lower confidence limit on the estimate of relative potency. Using his procedure we obtain a lower 95% confidence limit of 13,400. Therefore, based on the data obtained in this experiment, benzo[*a*]pyrene is estimated to be 69,000 times more potent a skin carcinogen than the mixture of I and II, with a lower 95% confidence limit of 13,400. Our purpose in providing these estimates is to give an impression of what is meant by "strong" and "weak" carcinogenic effect. The ratios should not be interpreted to indicate that a given individual will be more likely to develop lung cancer from smoking than from exposure to any of these agents. This sort of direct extrapolation between risks, compounds, and species is simply not possible. Bioassay data only provide criteria of relative hazard for the particular materials and in the particular combinations and forms tested.

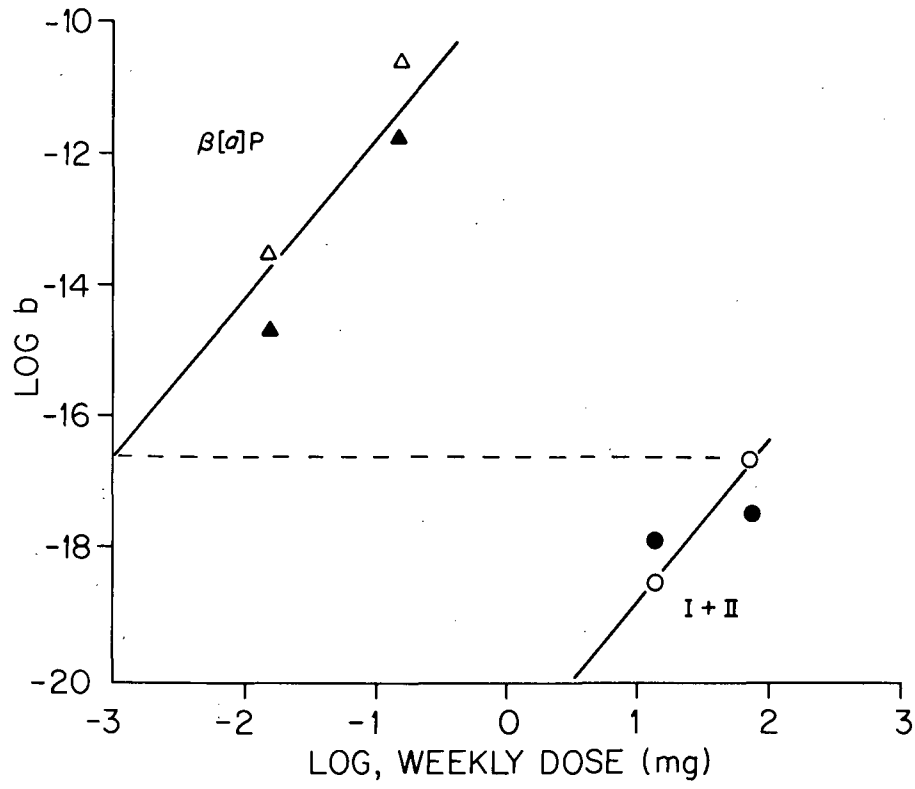


Fig. 11. Relative potency of benzo[*a*]pyrene and the mixture of I and II. The logarithm of the Weibull proportionality parameter, b (ordinate), is plotted vs the logarithm of the weekly dose rate (abscissa). Symbols correspond to either benzo[*a*]pyrene in both C3H (▲) and C57BL/6 (Δ) or the 1:1 mixutre of I and II in both C3H (●) and C57BL/6 (○). The horizontal distance between parallel lines fitted to the data for each material is the difference in log weekly dose rate necessary to elicit a common probability of response.

DISCUSSION

Weil *et al.* (14) conducted lifetime skin carcinogenesis tests in male mice with both I and II tested separately. In two experiments with II they observed a single skin tumor after 16 months of exposure to the undiluted material. Skin tumors were not observed in mice exposed to I

at a 30% concentration in acetone. Both of these experiments with II and I were complicated by poor survival after 16 months of exposure that significantly reduced the number of animals at risk in the latter third of the experiment. Hine *et al.* (15) also conducted life-span carcinogenicity tests on an unspecified Shell bis-phenol-based resin similar to II. Groups of 30 male C3 mice were painted three times weekly with 0.2 μl of a 5% acetone solution of this material. After 24 months, 4 of 13 surviving animals had developed malignant skin tumors, three carcinomas and one fibrosarcoma.

On the basis of these published observations, we conclude that there is consistent evidence that material II is a weak carcinogen, a finding confirmed in the current study. The only published account of tests with I failed to demonstrate activity; however, for the reasons mentioned, the negative results of this test are unconvincing. Our data show clearly that not only is I an active skin carcinogen but may be more potent than II.

A result of the present study that may be without precedent in the epoxy resin literature was the observation of synergistic interaction between I and II, when applied to mouse skin as a 1:1 mixture. Skin tumors occurred in greater numbers of mice and with shorter latencies than would be expected on the basis of the response to the resins separately. Among several possible explanations for this effect we first considered that an impurity or reaction product may be present in the mixture that did not occur in either substance alone. To test this possibility, each substance and the mixture of them were subjected to gel permeation chromatography and fractional distillation, and the

resultant fractions were characterized by IR and NMR spectroscopy. These techniques detected no impurities or constituents in the mixture that were not also found in the materials as supplied; on this basis, we conclude that an impurity is unlikely to explain the result. The next possibility we considered was that, if I was more efficient (potent) as a skin carcinogen than II, and since I is more volatile, it could matter in what form I was applied to the skin. Resin II is a viscous, essentially nonvolatile material that persists for several hours after acetone has evaporated from the mouse's skin. Resin I, on the other hand, is considerably less viscous and more volatile and does not persist on mouse skin. On this basis we postulate that an equal-parts mixture of the two may enhance skin penetration of either or both. Whether or not penetration increases, it is likely that the persistence of I increases as a result of decreased volatility and increased viscosity. An increased penetration rate, persistence, or both could result in an increase in absolute dose to critical target cells and thus explain enhanced potency of the mixture.

Another set of possibilities concerns potential biological interaction between the two resin components. If we postulate that I is a weak tumor initiator and that II is a tumor promoter, then the combination would be expected to show synergistic interaction. However, we consider this an unlikely possibility for the reason that if either resin were a tumor initiator, then, according to theory, it should also prove to be an efficient complete carcinogen when applied repetitively. Finally, it is possible that one or both of the resins must be metabolically activated after gaining entry to the target cell. If one resin

enhances the metabolism of the other, or if metabolism is mutually stimulated, this also could be a factor contributing to the greater effect obtained with the mixture. Thus we are left with several alternative explanations, none being mutually exclusive, plus additional alternatives that we have not considered.

The increased sensitivity of B6 mice to both toxic as well as carcinogenic effects of the chemicals was striking as well as a clear demonstration of the need to exercise caution in drawing two general conclusions from test results based upon a single strain or species. This is especially true when effects are minimal, as was the case with II. If the experiment had been done exclusively on C3 mice, then we would have concluded that II, at the doses tested, was noncarcinogenic; but this was not the case, as was clearly demonstrated in B6 mice.

Previous data concerning the toxicity and carcinogenicity of III in animals reveal this compound to be toxic as well as allergenic (16-18). Long-term animal tests to determine the carcinogenicity of III have been limited, and the data are conflicting. Saruta *et al.* (19) described an injection-site sarcoma induced in one of five Wistar-King rats after 11 months of injection at 9 mg/kg every other day. In the same experiment, III given by the same method failed to induce any tumors at 18 mg/kg. Burnett *et al.* (20) applied hair dyes containing an unspecified amount of III once weekly to groups of 100 mice for 18 months. Under these conditions, none of the materials produced evidence of systemic toxicity or carcinogenicity.

Our data indicate that at a dose approaching the maximum tolerated level, III neither induces skin tumors nor substantially increases the

incidence of tumors in other tissues in spite of evidence that skin penetration occurred readily with this material, thus supporting the observations of Burnett.

Microbial mutagenesis tests revealed III to be a potent mutagen requiring metabolic activation (Appendix A). This observation is consistent with a report by Ames *et al.* (21) concerning the microbial mutagenicity of oxidation-type hair dyes that contain III. Both I and II and the 1:1 mixture of them were determined to be low-level mutagens requiring metabolic activation. Based on the frequency of mutations per unit dose, the mixture showed no evidence of synergism in the Ames microbial test.

Cultured human leukocytes were evaluated for the frequency of induced chromosomal aberrations following treatment with various doses of each material. All compounds were cytotoxic, and various subtoxic doses failed to increase the frequency of aberrations. However, there was a suggested increase in breaks and gaps noted with the mixture. Another mutational bioassay using cultured Chinese hamster ovary cells was run in parallel with the microbial tests (Appendix A). This test also revealed the materials to be cytotoxic, and all were characterized as weak mutagens, including III and the mixture.

In summary, all the screening methods were equally capable of detecting biological activity in the form of cytotoxicity. No quantitative correlation was observed between mutagenesis, whether in bacteria or hamster cells, and mammalian carcinogenicity. There appears to be little correlation between mutagenesis tests as evidenced by the mutagenicity of III in salmonella but not in hamster cells. For I and II

the microbial tests were consistent and agreed favorably with the animal bioassay in that the materials separately were weak carcinogens. The exception noted for the mixture may be additional evidence favoring the dosimetric hypothesis advanced previously. That is, it may be unreasonable to expect salmonella to reflect differences in skin penetration. The lack of agreement between *in vitro* tests as well as between mutagenicity and carcinogenicity for III should serve to provide clear warning of the risks involved in oversimplified interpretation of *in vitro* data. No *in vitro* test yet proposed can simulate actual exposure conditions that determine the response of critical target cells in the intact animal. The occurrence of false positives noted for III in this experiment is a further indication that while the correlation between proven carcinogenicity and mutagenicity is quite good, the converse is most certainly not true. Many, if not most, carcinogens are also efficient mutagens, but many mutagens are poor carcinogens, if carcinogenic at all. For this reason we feel it unwise to extrapolate *in vitro* mutagenesis data to an assessment of carcinogenic risk in animals. Mutagenesis tests in their present form are useful as rapid screening methods for vast numbers of compounds, but as replacements for carcinogenicity tests in whole animals they may yield results that have little to do with actual expression of neoplastic disease.

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Appendix A

GENETIC TOXICOLOGY

J. L. Epler A. W. Hsie

Biology Division, Oak Ridge National Laboratory

Tier Assays — J. L. Epler

The objectives of this study were (1) to assess the genetic activity of the test materials in microbial assays (the Ames salmonella system) with and without the addition of mammalian enzyme preparations, and (2) to assess the activity of the test materials in the chromosomal breakage assay, utilizing human leukocytes.

Materials and Methods

Indicator strains. The salmonella typhimurium strains used in the various assays are listed below. All strains were obtained through the courtesy of Dr. Bruce Ames, Berkeley, California.

TA 1535 hisG46, uvrB, rfa (missense)

TA 100 hisG46, uvrB, rfa (missense plus R factor)

TA 1537 hisC3076, uvrB, rfa (frameshift)

TA 1538 hisD3052, uvrB, rfa (frameshift)

TA 98 hisD3052, uvrB, rfa (frameshift plus R factor)

Materials. Materials tested were identical to those used in the animal studies, and the same abbreviations are used to describe them as were used in the main report.

Microbial protocol. In the screening of test materials the four strains TA98, TA1535, TA1537, and TA100 were generally employed. Standard experimental procedures have been given by Ames, McCann, and Yamasaki (Mutat. Res. 31: 347-64, 1975). Briefly, the strain to be treated with the potential mutagen(s) is added to soft agar containing a low level of histidine and biotin along with varying amounts of the test substance. The suspension containing approximately 2×10^8 bacteria is overlaid on minimal agar plates. The bacteria undergo several divisions with the reduced level of histidine, thus forming a light lawn of background growth on the plate and allowing the mutagen to act. Revertants to the wild-type state appear as obvious large colonies on the plate. The assay can be quantitated with respect to dose (added amount) of mutagen and modified to include "on-the-plate" treatment with the liver homogenate required to metabolically activate many compounds.

Materials and/or control compounds to be tested were suspended in dimethylsulfoxide (DMSO, supplied sterile, spectrophotometric grade from Schwarz-Mann) to concentrations in the range of 10 to 20 mg of solids. The potential mutagen was in some cases assayed for general toxicity (bacterial survival) with strain TA1537. Generally, the fraction was tested with the plate assay over at least a 1000-fold concentration range with the tester strains. Revertant colonies were counted after 48 hr of incubation. Data were recorded and plotted vs added concentration, and the approximate slope of the induction curve was determined. It is assumed that the slope of the linear dose-response range reflects the mutagenic activity. Positive or questionable results were clarified using a narrower range of concentrations. All studies were carried out

with parallel series of plates plus and minus the liver enzyme preparation for metabolic activation. The background lawn of bacterial growth was routinely examined so that any effects attributed to massive cell death and subsequent growth of the few surviving bacteria (availability of more histidine) could be differentiated from mutation induction. Routine controls demonstrating the sterility of samples, enzyme or S-9 preparations, and reagents were performed. Positive controls with known mutagens were carried out in order to recheck strain response and enzyme preparations.

Leukocyte protocol. Chromosomal preparations of leukocytes cultured from human peripheral blood (Moorhead, Nowell, Wellman, Battips, and Hungerford, 1960) were used to score induced aberrations and compared with untreated controls. Blood was drawn with 2% heparin (1000 units/ml) in a sterile syringe and transferred to a sterile centrifuge tube. After centrifugation at 1000 rpm for 10 min, the buffy coat was drawn off and inoculated into RPMI medium containing 27% human serum and 3% phytohemagglutinin at pH 7.0. The culture was incubated at 37°C until mutagen treatment. After treatment the cells were rinsed twice with Hanks' balanced salt solution, and new medium was added. Chromosome preparations were made in the usual manner. The cells are collected after 52 to 54 hr of incubation, including a pretreatment of 2×10^{-7} *M* colchicine for 2.5 hr. The cells are centrifuged for 5 min, the medium decanted, and the cells resuspended with 4 ml of Hanks' solution. The resuspended cells are centrifuged for 5 min and the Hanks' solution decanted down to a total volume of 1 ml. The cells are resuspended with 3 ml of warm water (37°C) and incubated at 37°C for 15 min with constant agitation.

The suspension is again centrifuged and the supernatant removed. The cells are fixed with 1 part glacial acetic acid and 3 parts methyl alcohol for 20 min with two changes of fixative. Air-dried preparations are made and are stained with 2.5% giemsa water solution, mounted with Euparal, and covered.

Results. Table A.1 summarizes the response, measured as revertants per plate, of the salmonella strains after treatment with varying doses of the test materials. Table A.2 lists positive control data obtained from other materials. All materials were tested in duplicate in at least two separate experiments. Rechecks of the dose response curves were run when necessary over a narrow range. In summary, the preliminary findings with the Ames test are as follows:

1. Resin III is a potent mutagen affecting, specifically, the frame shift strain TA98. Metabolic activation with mammalian extracts (rat liver) is necessary. Both phenobarbital and Aroclor 1254 induce the enzymes necessary to activate the compound with Aroclor showing the best activity.
2. Resin II is a low-level mutagen affecting the missense strains TA1535 and TA100. Metabolic activation is required.
3. Resin I is a low-level mutagen affecting only the highly sensitive missense strain, TA100. Metabolic activation is not required.
4. The mixture of I + II reflects only the expected (approximate) combined effect of the constituents. No synergistic effect is seen.

Table A.1. Dose response relationships for mutation induction in salmonella typhimurium

Compound	Conc. (μg)	Revertants per plate[a]											
		TA1535			TA1537			TA98			TA100		
		None	ϕBarb	Aroclor	None	ϕBarb	Aroclor	None	ϕBarb	Aroclor	None	ϕBarb	Aroclor
m-Phenylenediamine	0	12	11	13	7	11	9	32	41	36	158	171	147
	25	7	17	14	6	21	8	25	206	271	183	197	160
	50	9	12	11	14	27	19	26	494	820	158	228	220
	125	12	9	11	7	24	38	28	826	1500	157	291	275
	250	12	6	13	11	43	50	44	1420	2500	126	192	293
	500	8	8	12	14	48	85	49	1250	4000	128	211	383
2,2-Bis(*p*-glycidyloxyphenyl)propane	0	12	11	12	8	9	9	32	30	36	147	191	158
	100	11	24	11	8	6	11	27	20	47	100	279	312
	250	18	21	23	10	10	8	46	30	31	125	271	338
	500	18	36	35	8	4	13	22	32	36	131	275	357
	750	26	52	68	5	8	11	25	15	46	150	273	393
	1000	25	93	110	2	2	6	24	31	37	119	144	274
Bis[2,3-epoxycyclopentyl]ether	0	8	11	11	12	13	10	46	30	41	155	179	150
	100	10	14	13	12	12	7	41	20	45	231	153	197
	250	12	17	9	11	12	8	57	30	53	239	192	137
	500	11	10	9	9	13	11	48	32	48	256	167	162
	750	15	9	11	12	14	7	27	15	34	240	181	132
	1000	4	14	8	10	14	4	35	31	34	137	122	126
1:1 mixture of I and II	0	8	11	11	12	10	10	45	30	41	159	191	150
	100	9	13	12	19	13	7	35	31	41	201	130	103
	250	12	18	14	13	4	8	24	23	36	331	116	98
	500	10	54	23	3	3	11	26	22	36	373	74	94
	750	5	48	41	4	8	7	19	24	25	424	111	146
	1000	9	104	38	3	9	4	15	28	37	422	117	132

[a]Wild-type revertants per plate with 2×10^8 bacteria originally applied; none = without activation; ϕBarb = activated with enzyme prep from rat livers induced with 0.1% sodium phenobarbital in the drinking water; Aroclor = activated with enzyme prep from rat livers induced with Aroclor 1254 (2 ccg/kg).

Table A.2. Control data obtained from pure reference materials

Compound	Dose (μg)	Revertants per plate			
		TA1535	TA1537	TA98	TA100
8-Aminoquinoline	20[a]	22	1614	132	424
4-Nitroquinoline-N-oxide	1	120	35	550	720
Ethyl methane sulfonate	0.5[b]	10,000	6	37	250
Benzo[*a*]pyrene	15[c]	22	293	763	420
Acetoaminofluorene	2.5[a]	13	64	2000	810
None	0	17	9	38	235

[a]Activated with phenobarbital-induced rat liver prep.

[b]Measured as microliters.

[c]Activated with Aroclor 1254 rat liver prep.

Table A.3 represents the summary of our findings with the human leukocyte system. None of the test materials gives a significant increase over control levels of chromatid aberrations. However, an abnormal occurrence of "gaps" and breaks was seen with the mixture. Both I and II and especially II were cytotoxic at the higher concentrations used in cell treatments.

Discussion. Resin III is obviously a potential hazard, and the attention of users should be quickly shifted to whole-animal testing for carcinogenicity. The question of genetic hazard of the compound is, in another context, not just a problem for the industrial world but the population in general, for example, hair dye use of the same compound.

Both I and II, regretfully, fall into the category that a whole array of compounds will fall into with respect to short-term testing; they are "slightly positive" and only at "high concentrations" (judged by the effective concentration range of known mutagens-carcinogens).

Table A.3. Induction of chromosomal aberrations in cultured human leukocytes

Conc. (μg/ml, final)	Cells scored	Gaps	Chromatid breaks	Isochromatid breaks	Chromatid interchange
			m-Phenylenediamine		
200	100	4	1	1	
50	100	2	0	0	
5	100	2	1	1	
			2,2-Bis(*p*-glycidyloxyphenyl)propane		
100	only a few mitotic cells were scored probably cytotoxic				
50	100	2	1	1	
5	100	3	1	0	
			Bis[2,3-epoxycyclopentyl]ether		
200	100	0	2	0	
100	100	0	0	0	
20	100	1	0	0	
			1:1 mixture of I and II		
200	150	0	7	2	
100	100	6	1	1	
20	100	3	0	1	
			Control		
	300	5	3	0	1

The borderline results with the leukocyte system are enough to question their action. Further testing in other comparative short-term tests would be necessary, and certainly attention should be paid even to low-level effects in the whole-animal carcinogen screen.

Cytotoxicity and Mutagenesis in Mammalian Cell Culture — A. W. Hsie

The purpose of this study was to evaluate the cytotoxicity and mutagenicity of four industrial materials in the Chinese hamster ovary (CHO) cell system. Cytotoxicity was determined by the colony-forming ability of single cells. Mutagenicity was measured by induction of 6-thioguanine (TG) resistance. These mutations are known to affect a specific gene on the X-chromosome specifying hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity.

Materials and methods

Cell culture. These studies were performed with subclones of the CHO cell line designated CHO-K_1-BH_4, isolated following selection in aminopterin medium. Cells were routinely cultured in our regular medium, which is Ham's F12 (Pacific Biological Co.) containing 5% (F12FCM5) or 10% (F12FCM10) heat-inactivated (56°C, 30 min), extensively dialyzed fetal calf serum (Pacific Biological Co.) in plastic tissue culture dishes (Falcon or Corning Glass Works) under standard conditions of 5% CO_2 in air at 37°C in a 100% humidified incubator. These cells grow in aminopterin medium as well as in regular medium with 5 or 10% dialyzed fetal calf serum with a population doubling time of 12 to 13 hr. Cells were removed with 0.05% trypsin for subculture. Cell number was determined with a Coulter counter (model B, Coulter Electronics).

Determination of cytotoxicity and mutagenicity. CHO cells were plated at 5×10^5 cells/100-mm dish in medium F12FCM10. After a 16- to 24-hr growth period (cell number = $\sim$1.0 to 1.5×10^6 cells/plate), the

epoxy resin compounds were dissolved in DMSO (Schwarz-Mann), added to the medium at a final DMSO concentration up to 2%, and the cultures were incubated for the desired time interval. To determine initial cell survival, the cultures were washed three times with saline G, and the cells were detached by trypsin (0.05%). Cell number was determined, and 200 to 10,000 cells were plated in 60-mm dishes in regular medium in triplicate for each culture. After seven days of incubation, the resulting colonies were fixed with formalin (3.7%), then stained with crystal violet solution (0.1%); the colonies containing 100 or more cells were counted. Cloning efficiency was determined and is usually expressed in percent relative to the untreated control culture, which usually has 80% or better efficiency (Hsie *et al.*, Somat. Cell Genet. 1: 247, 1975).

For determination of mutation induction, the treated cultures were subcultured every 48 hr. Usually one plate was treated as described above. After trypsinization, an aliquot was removed for cell number determination and single-cell survival, and approximately one-half of the culture was replated. Routine subculture was then performed in F12FCM5 medium. After eight days of culture the cells were plated for selection in the presence of TG. The cultures were washed three times with saline G and removed by trypsinization before the cell number was determined. For cloning efficiency measurement, 200 cells were plated in triplicate in hypoxanthine-free F12 medium containing 5% dialyzed fetal calf serum. For measurements of TG resistance, 2×10^5 cells were plated in 100-mm dishes (5 to 10 plates = 1 to 2×10^6 cells total) in TG medium. After seven days of incubation, colonies were fixed, stained, and counted. The mutation frequency is determined by dividing the total number of

mutant colonies in the TG medium by the total number of cells plated (1 to 2 × 10^6), corrected by the cloning efficiency (Hsie *et al.*, Somat. Cell Genet. 1: 247, 1975).

In vitro microsomal activation assay. CHO-K_1-BH_4 cells were plated at a density of 5 × 10^5 cells/60-mm tissue culture dish and allowed to incubate overnight. Cells were then washed three times with saline G, and sufficient serum-free F12 medium was added to bring the final volume in each to 5 ml after the addition of various amounts of microsomal preparation (up to 1 ml) and 50 μl of test compound in DMSO. Test agents and/or microsomes were omitted from some plates to provide controls. The microsomal preparation (supplied by J. L. Epler) was made according to the method of Ames *et al.* (Mutat. Res. 31: 347, 1975) and contained (per ml) 8 μmoles $MgCl_2$, 33 μmoles KCL, 5 μmoles glucose-6-phosphate, 100 μmoles phosphate buffer (pH 7.4), and 0.2 ml microsome fraction, prepared from Aroclor 1254-induced male Sprague-Dawley rat livers. Cells were then incubated for 5 hr and washed three times with saline G before 5 ml of F12FCM5 was added. Following overnight incubation, cells were trypsinized and plated for survival and mutagenesis according to our standard protocol described previously.

Results. Resin I is relatively nontoxic (Table A.4). It does not have an appreciable lethal effect at a concentration range of 10 to 500 μg/ml without microsome treatment and exerts only slight toxicity at 500 μg/ml in the presence of microsomes.

Resin II is extremely toxic. Without microsome, it kills 50% of the cells at ∿15 μg/ml and over 99% at ∿25 μg/ml. Microsome protects against the lethal effect, as evidenced by the near-normal survival up to ∿100 μg/ml and a mild toxicity at 200 μg/ml.

Table A.4. Cytotoxicity of industrial compounds in CHO cells

Concentration (μg/ml)	Relative survival (%)			
	16 hr, no microsome		5 hr with microsome	
	I[a]	II[a]	I	II
	Bis[2,3-epoxycyclopentyl]ether			
0	100	100	100	100
10	115	116		
25		96		
50	114	103		
100		102		
200	94	99		
300		98	128	92
400	88	102		
500	80	97	56	67
	2,2-Bis(*p*-glycidyloxyphenyl)propane			
0	100	100	100	100
2		110		
5		92		
10	95	86		
15	56	55		
20	13	13		
25		0.05		
50	<0.01		114	110
100	<0.001		85	103
150	<0.001			
200	<0.001		60	72
300	<0.001		1	
400	<0.001			
	1:1 mixture of I and II			
0	100	100	100	100
10	118	99		
20	81	73		
30	52			
40	19			
50	0.6			
100	<0.01			
150	<0.01			
200	<0.01		81	88
300	<0.01		55	55
400	<0.01			
500	<0.01		0.8	
	m-Phenylenediamine			
0	100	100		
10	120	101		
25		94		
50	105	90		
100	95	103		
200	42	48	120	99
300	23	23	62	77
400	15	18		
500	3	2	63	62

[a]I and II denote experiments performed independently by two investigators.

The mixture of I and II produces nearly the expected survival, as if it were a half strength of II, since I is virtually nontoxic over the concentration range tested. Resin I does not appear to affect the cytotoxicity of II either additively, synergistically, or antagonistically.

Mutagenicity testing of all four compounds remains at a qualitative stage, since more experiments are required to reach a quantitative conclusion. All that can be said is that I, II, the mixture, and *m*-phenylenediamine are weakly mutagenic in the CHO-HGPRT system (Table A.5).

Conclusions and recommendations. Cytotoxicity testing establishes that II is highly toxic and that III is toxic. Mutagenicity testing suggests that all four materials are weakly mutagenic (which remains to be confirmed). Prudent handling of all compounds, especially II and III, should be enforced. It appears that long-term animal carcinogenicity testing is necessary to further assess the possible health effects of such compounds. Meanwhile, a search for nontoxic substitutes, especially for II and III, should be considered.

Table A.5. Mutagenicity of industrial compounds in CHO cells

Concentration (μg/ml)	Mutation frequency ($\times 10^{-6}$)			
	16 hr, no microsome		5 hr with microsome	
	I[a]	II[a]	I	II
	Bis[2,3-epoxycyclopentyl]ether			
0 (control)	<1	2	5	<1
200	3	1	9	1
500	6	3	<1	
	2,2-Bis(*p*-glycidyloxyphenyl)propane)			
10	4	14		
15	<1			
20	8	<1		
50			14	
100			13	1
200			8	12
	1:1 mixture of I and II			
20	10			
30	6	2		
40	18	<1		
100			8	<1
200			2	1
400			1	<1
	m-Phenylenediamine			
200	4	<1	<1	
400	21	8		
500			13	

[a] I and II denote experiments performed independently by two investigators.

Appendix B

CHEMICAL ANALYSIS AND AIR SAMPLING

N. J. Williams J. G. Dorsey E. T. Creech

Y-12 Plant Laboratory Product Certification Division

Determination of m-Phenylenediamine and Bis[2,3-epoxycyclopentyl]ether in Mouse Treatment Room Air

Air samples were taken in the mouse treatment room to determine the concentrations of *m*-phenylenediamine (III) and bis[2,3-epoxycyclopentyl]ether (I) in the air during the mouse painting procedure. Room air was pulled through silica-gel tubes at the rate of 1 liter/min during the mouse treatment period. Immediately after sampling, the contents of the tubes were quenched in 2 ml of 40% ethanol/60% water solution (v/v) to preserve the trapped components. The resulting solutions were then analyzed for III by liquid chromatography and for I by gas chromatography.

Silica-gel tube construction

The silica-gel adsorption tubes were constructed from 7.6 cm × 8 mm ID Pyrex tubing containing approximately 0.5 g of 45/60 mesh silica gel between 300 mesh stainless steel screens. The screens were held in position by rings cut from 0.635-mm-OD Teflon tubing. Each sampling unit consisted of two silica-gel tubes connected with Teflon tubing.

m-Phenylenediamine determination by liquid chromatography

The silica-tube quench solutions were analyzed for III by reverse-phase high-pressure liquid chromatography. Technical-grade III was used as received to prepare calibration standards in the 0 to 5 μg of III in the 2-ml volume of quench solution from each tube section. The instrument conditions were as follows:

Instrument: Waters model ALC-202 liquid chromatograph

Column: 1.2 m × 3.18 mm OD stainless steel packed with Bondapak Phenyl/Corasil (37 to 50 μ)

Column temperature: Room temperature, ∿25°C

Solvent: 6% ethanol–94% water

Flow rate: 1.5 ml/min

Detector: UV (254 nm)

Bis[2,3-epoxycyclopentyl]ether determination by gas chromatography

The silica-tube quench solutions were analyzed for I by temperature-programmed gas chromatography. Two isomeric forms of I existing at room temperature were measured, the solid isomer and the liquid isomer. A 50/50 mixture by weight of the solid and liquid isomers was used to prepare calibration standards in 40% ethanol–60% water solvent. The detection limit of the method was 1 μg/ml for each isomer, or 2 μg of each isomer in the 2-ml volume of quench solution from each tube section. The instrument conditions were as follows:

Instrument: Varian 1400 gas chromatograph

Column: 2 m × 2 mm ID Pyrex glass packed with 5% Dexsil-300 GC on Chromasorb WHP 80/100 mesh

Column temperature: 2 min at 120° then 120 → 170° at 12°/min

Nitrogen carrier: 50 cc/min at 60 psig

Injector temperature: 200°C

Flame ionization detector temperature: 200°C

Injection volume: 2 ml

Discussion of the methods

The reactive nature of III and I makes air sampling for these materials very difficult. During the air sampling process, III and I on the silica-gel adsorbant can react to form adducts, and III undergoes rapid oxidation in flowing air. Resin III loss through air oxidation is the more rapid of these reactions.

Laboratory tests with pure III deposited on silica gel from solution indicate that approximately 60% is lost through oxidation within 2 hr in flowing room air. Taking this into consideration, along with uncertainties in collection efficiency, the reported III concentrations in the treatment room air may be low by a factor of 2 or more.

Similar laboratory tests with I deposited on silica gel from solution show that greater than 90% is recovered after 2 hr of exposure to flowing air. In tests involving III-I mixtures, the I recovery was also greater than 90%. These results indicate that the reported I values accurately reflect the concentrations of I in the mouse treatment room air.

Experimental data

Representative air sampling data are presented in Tables B.1 and B.2. The data in Table B.1 were taken to determine the concentrations of III and I in the mouse cages immediately after applying acetone solutions of these materials to the mice. Room air samples taken above the mouse treatment cart during application of the solutions, Table B.2, were intended to measure the exposure the technicians received.

Discussion of results

Resin I was detected in all samples of room air and of mouse cage air. Therefore, all the animals in the room, including the control group, were exposed to I at about the 0.14 μg/liter level for several hours after each mouse treatment period. No long-term air samples were taken to measure the length of time required for the I concentration in air to decrease to the detection limit of the method.

The resin I air concentration in the 10% I cage (1.55 μg/liter of air) was about the same as in the 50% I cage (1.75 μg/liter of air). This indicates that sufficient I was present even at the lower dose to establish an equilibrium level in the cage air. The air sampling rate of 1 liter/min probably increased the air exchange in the 9-liter cages. If the natural exchange rate is low, then the sampling process could have caused a low bias in the I values.

Resin III was detected only in the cage where the mice were treated with 2% III in acetone. No III was detected in either the room air or in other cages adjacent to the III-treated mice.

Table B.1. Mouse cage air sampled for epoxy volatiles

Component concentration in acetone	Air sampling rate (liters/min)	Volume of air sampled (liters)	Total micrograms found			Micrograms/liter of air		
				I			I	
			III	Solid isomer	Liquid isomer	III	Solid isomer	Liquid isomer
2% III	1	110	1.4	10.5	4.5	0.02[a]	0.10[b]	0.04[b]
50% I	1	110	<0.5	139	54	<0.01	1.26	0.49
50% I	1	110	<0.5	122	53	<0.01	1.11	0.48
10% I	0.8	88	<0.5	96	41	<0.01	1.09	0.46

[a]Based on a 60-min sampling period after the mice were treated with 2% III solution. The 2% III cages were treated at about the midpoint of the treatment period.

[b]Based on the full 10-min sampling period.

Table B.2. Room air samples for epoxy volatiles[a]

Compound	Micrograms per liter of air		
	-1	-2	Average
III	<0.006	<0.006	<0.006
I solid isomer	0.095	0.099	0.092
I liquid isomer	0.039	0.036	0.038

[a]Air samples were taken in Room 5025 of Building 9207 on Dec. 15, 1976, to determine the concentrations of bis[2,3-epoxycyclopentyl]ether and *m*-phenylenediamine in the room air during the mouse treatment process. Two 160-min (320-liter) samples were taken using silica-gel tubes mounted approximately 6 in. above the mouse treatment table.

Chemical Analysis of Bis[2,3-epoxycyclopentyl]ether, 2,2-Bis(*p*-glycidyloxyphenyl)propane, and a 1:1 Mixture of Each

The three epoxy resin samples listed below were analyzed to determine whether impurities were present which could influence the animal test results.

Bis[2,3-epoxycyclopentyl]ether. No impurities were detected by NMR.

2,2-Bis(*p*-glycidyloxyphenyl)propane. This material is estimated to contain 10 wt % of two components other than II. All lots of II that have been analyzed by NMR over the past two years contained these components. IR and NMR analyses of fractions isolated by gel permeation chromatography (GPC) and by vacuum distillation have identified the "impurities" as an expoxidized glycol and a small amount of phenyl glycidyl ether $\left(\bigcirc\!\!-O-CH_2-\overset{O}{\overset{/\backslash}{CH-CH_2}}\right)$. The NMR data indicate that the epoxidized glycol in the condensate is $CH_3CH_2O(CH_2CH_2O)_nCH_2\underset{O}{\underset{\backslash/}{CH-CH_2}}$ with n = 1. However, GPC data and the low volatility of this polymer in the sample indicate that the bulk of the material is of a higher molecular weight (>500).

I + II. NMR analysis showed the sample to be 50 wt % I and 50 wt % II. A small amount of impurities associated with resin II was detected in the mix.

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