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Testing of New Compounds for Long-Term Toxicity

ELIZABETH K. WEISBURGER, Ph.D.*

Synopsis—Although the long-term testing of new compounds for CHRONIC TOXICITY may superficially appear to be a straightforward matter, many factors can influence the outcome of such studies. These include the SPECIES and STRAIN of test animal, AGE at start of the tests, SEX, VEHICLE and ROUTE of administration, DIET, and other variables. Some materials which are used in cosmetic formulations have the capacity to inhibit or to enhance the response of animals to known carcinogens. Awareness of all these influences is necessary in designing meaningful long-term tests for toxicity.

Introduction

The National Cancer Institute (NCI) is faced with the responsibility of identifying carcinogenic hazards with the greatest possible effectiveness, preferably prior to their widespread introduction into the human environment. Worldwide, thousands of new chemical compounds are proposed each year as entering the environment (patent applications); in actual practice, there may be hundreds introduced each year. The thorough testing of all these materials for safety would become an unmanageable task with respect to the space and budgetary requirements.

Since the risk from each compound varies, depending on production, usage, chemical structure, extent of exposure, and character of the population, priorities for thorough testing of each material can be obtained through a process of successive screening. Eventually, by this scheme a

* Experimental Pathology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

yearly list of approximately 250 compounds, prime candidates for thorough study by NCI, will be produced.

However, although this technique will systematize the selection of compounds for future testing, NCI has been engaged for sometime in a Bioassay Program, designed to determine whether certain materials may or may not be carcinogenic. In addition, under this program NCI scientists are attempting to develop bioassay methods which may indicate more quickly which compounds should be studied thoroughly.

Bioassay Methods

Bioassay methods include cutaneous application (of most interest to the cosmetics field), oral administration, injection, implantation, and inhalation. These techniques have been reported in various monographs on testing of materials for safety (1–7). In these publications information regarding types of tests, numbers of animals to use, treatment, length of test, necropsy, and other factors are thoroughly discussed.

Although cutaneous application appears to be a straigtforward method for the testing of cosmetic ingredients, there are many complicating factors such as age, sex, species and strain of animal, route, vehicle, and diet which may alter the response of the test animals. Other factors being equal, the newborn or neonatal animal, especially the mouse, is generally more sensitive to most carcinogens (8, 9). Usually, but not always, animals treated with carcinogens as newborns or infants show a higher incidence of some types of tumors in a shorter time in comparison with adults (9) (Table I). Part of the effect may be ascribed to a lack of or lower levels of drug metabolizing enzymes in the newborn animal. Consequently, the carcinogen is excreted less rapidly and has a greater opportunity to initiate the processes leading eventually to tumor

Table I

Starting Age and Effects of a Single Subcutaneous Injection of *N*-Nitrosodimethylamine in Rats[a]

Age	Dose (mg/kg)	Sex	Tumor Incidence (%)	
			Renal	Liver
<1 day	21–25	M	40	44
		F	40	44
12–15 weeks	25	F	8	0
	Untreated	M	0	0
		F	4	0

[a] Data from Della Porta and Terracini (9). The animals were kept until they reached 80 weeks of age.

formation. An example is that of urethan (ethyl carbamate) which is excreted more slowly in infant mice (10) and is a more active agent in infants (9).

Despite the apparent enhanced responsiveness of newborn animals to carcinogens, there are still too many unknown factors involved in this technique. Therefore, in routine tests for the safety of new compounds, it is considered best to start with weanling animals or those a few weeks postweanling (9, 11). Starting with animals which are already adult may not leave enough of the animal's life span for tumors to develop, especially in the case of weaker carcinogens.

A second factor which influences the results of tests for chronic toxicity is the sex of the animals. Bates (12) reported that male mice had more skin tumors from painting a typical carcinogen 7,12-dimethylbenz(*a*)-anthracene on the skin than did females (Table II). Some explanations for these differences are given by Bock (13). In many cases male animals also responded to a greater degree to other carcinogens which affect the internal organs.

Table II

Skin Tumors in Mice after 7,12-Dimethylbenz(*a*)anthracene[a]

Sex	Tumor Incidence (%)	Tumors per Mouse
Male	79	5.90
Female	18	0.26

[a] Data from Bates (12). The tumors were induced by a single dose of 16μg of dimethylbenzanthracene applied to the back, followed by application of 0.2 ml of 1% croton oil in acetone weekly. The experiment was terminated after 23 weeks. Controls treated with acetone alone had <0.06 tumors per mouse.

An illustration of the part played by hormones comes from the report of Morris and Firminger (14). Male ACI rats are much more susceptible to the hepatocarcinogenic effects of 2-diacetylaminofluorene than are females. However, castration of males and administration of estrogen led to a tumor response more nearly like that of females. Conversely, females which had been ovariectomized and then given testosterone responded like intact males. Because of these differing responses, test systems should include both male and female animals.

Likewise, species and strain play an important role in the response. Thus, it was noted in some of the first work on cutaneous response to the pure aromatic hydrocarbons that there was a great range in the reactivity of various species. For example, with benzo(*a*) pyrene, a hydrocarbon

Table III

Species Difference in Response to Benzo(*a*)pyrene[a]

Species	Tumor Incidence (Range, %)	Duration (Range)
Mouse	60–100	75–200 days
Rabbit	2–15	8–55 months
Rat	...	2 years
Guinea pig	...	2 years
Fowl	...	2 years
Monkey	...	Up to 10 years

[a] Data from Hartwell (15). The benzo(*a*)pyrene was applied to the skin by painting an acetone or benzene solution (0.1–0.5%) two or three times weekly.

Table IV

Mouse Strain and Response to Repeated 3-Methylcholanthrene[a]

Strain	Papilloma Incidence (%)	Average Time to Autopsy (Weeks)
A	100	30
BALB/c	100	31
C57BL	97	36
C3H	100	28
DBA/2	100	35
I	100	27
R III	100	43

[a] Data from Andervont and Edgcomb (16). A 0.25% solution of 3-methylcholanthrene was applied weekly to the skin of the interscapular area by a single brush stroke.

often used as a standard carcinogen, the mouse was most sensitive, the rabbit next, while some species did not respond within a useful time period (15) (Table III).

Within a given species, the response to any given carcinogen may vary greatly. Although repeated skin painting with 3-methylcholanthrene presents a sizable carcinogenic dose, mice of some strains such as the C57BL or RIII either took a longer time period to die from the tumors induced or else did not develop tumors. On the other hand, a high percentage of strain I mice developed multiple papillomas and died early (16) (Table IV).

A study with the hepatocarcinogen ethionine exhibits clearly the wide variation in response. Holtzman male rats fed ethionine for 5 months developed no liver cancer; under identical conditions Carworth Wistars had an 86% incidence of these tumors (17) (Table V).

Table V
Strain Differences in Induction of Liver Cancer by Ethionine[a]

Rat Strain	Sex	Duration of Ethionine (Months)	Liver Cancer (%)
Carworth Farms Wistar	M	5	86
Holtzman	M	5	0
Carworth Farms Wistar	M and F	7.5	100
Holtzman	M	7.5	60
Holtzman	F	7.5	25
Fischer	M	7.5	100
Fischer	F	7.5	90

[a] Data from Farber (17). Ethionine was fed at 0.25% of the diet.

Table VI
Mammary Cancer in Female Rats After Single Dose of 7,12-Dimethylbenz(*a*)anthracene[a]

Strain	Dose (mg)	Per Cent with Mammary Cancer	Median Induction Time (Days)
Long-Evans	20	16	82
Marshall	30	0	...
Sprague-Dawley	20	100	41
Wistar	45	100	50
August	30	90	90
Chester Beatty	45	100	50

[a] Data from Boyland and Sydnor (18) and Sydnor *et al.* (19).

In most stains of rats, oral administration of a single large dose of 7,12-dimethylbenz(*a*)anthracene to 40–50 day old females represents an overpowering carcinogenic potential at a sensitive time period. Usually 100% of these animals develop mammary cancer in 25–80 days. However, some strains such as the Long-Evans or the Marshall hardly responded or did not respond at all (18, 19) (Table VI).

Thus, in planning long-term toxicity (or carcinogenicity) tests, it is important to use strains of animals which respond to carcinogens and are not totally resistant, but which are not so extremely responsive that misleading results are obtained, due to their high spontaneous tumor incidence. In our usual practical, this is accomplished by including a positive control (known carcinogen) in each test series. If the animals do not respond to the appropriate known carcinogenic agent, the results of the test series are then in doubt.

The route of administration may also influence the site at which tumors appear. Although most of the polycyclic hydrocarbon carcino-

gens such as benzo (*a*)pyrene, dimethylbenz(*a*)anthracene, or 3-methylcholanthrene cause tumors when painted on mouse skin, feeding these materials to infant mice can lead to hepatomas and numerous other tumors (9). However, in adult mice feeding of the hydrocarbons usually leads to relatively few tumors, generally of the forestomach, since much of the carcinogen passes through the gastrointestinal tract unchanged (3). A more striking example is that oral administration of dibutylnitrosamine to rats caused mostly liver cancer while repeated subcutaneous injection led to bladder tumors in all rats (20, 21) (Table VII).

The solvent or vehicle employed for administration by gavage or skin painting can alter the response dramatically. An example is the work of Bingham and Falk (22) who found that if dodecane, noncarcinogenic by itself, were added as a solvent, the skin tumor response of mice to benzo(*a*)pyrene was increased tremendously (Fig. 1).

The fact that diet may also radically affect the outcome of experiments in carcinogenicity was emphasized almost 30 years ago by

Table VII

Carcinogenic Effect of Nitrosodibutylamine in Rats[a]

Route	Dose (mg/kg)	Frequency	Tumor Incidence (%)		Average Induction Time (Days)
			Liver	Bladder	
Oral	10–75	Daily	58	35	150–540
Subcutaneous	200–400	Weekly	10	100	208–334

[a] Data from Druckrey *et al.* (20, 21).

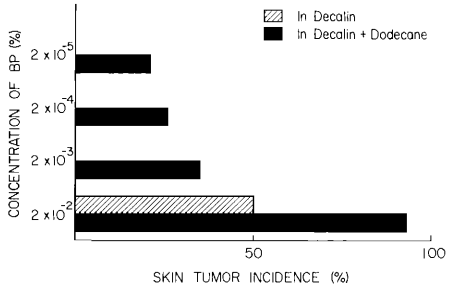

Figure 1. Skin tumor incidence in mice after repeated cutaneous application of low concentrations of benzo(*a*)pyrene (BP) in decalin or *n*-dodecane and decalin. Data from Bingham and Falk (22)

Tannenbaum (23). Mice were fed either a low or high calorie diet during cutaneous application of benzo(*a*)pyrene or during the subsequent holding period when the tumors appeared. The animals kept on a restricted low calorie intake during the entire experiment had a tumor incidence less than half that of mice fed *ad libitum* on the high calorie diet (Table VIII).

Another illustration of the effect that diet may have on carcinogenicity comes from the work of Kawachi *et al.* (24). Addition of 1% tryptophan, an essential amino acid, to the diet of rats receiving a relatively low level of the hepatocarcinogen *N*-nitrosodiethylamine increased the carcinogenic action on the liver almost fourfold (Table IX).

Heated (thermally oxidized) oil, a common dietary component, may also have a synergistic effect on a carcinogen and enhance its action. Thus, Sugai *et al.* (25) found that rats fed a low level of the carcinogen 2-acetylaminofluorene developed virtually no tumors. However, simultaneous feeding of this low level of 2-acetylaminofluorene with fractions of oxidized corn oil led to an 80–100% tumor incidence (Table X).

The influence of ultraviolet light on the response to topical application of a test substance should also be kept in mind. Previously, the

Table VIII

Induced Skin Tumors and Diet in Mice[a]

Diet during Benzopyrene Application (10 Weeks)[b]	Subsequent Diet (52 Weeks)	Tumor Incidence (%)
High calorie	High calorie	69
High calorie	Low calorie	34
Low calorie	High calorie	55
Low calorie	Low calorie	24

[a] Data from Tannenbaum (23).

[b] A 0.05-mg dose of benzo(*a*)pyrene was applied to the skin twice weekly during this period.

Table IX

Effect of L-Tryptophan on *N*-Nitrosodiethylamine Carcinogenesis[a]

L-Tryptophan (%)	*N*-Nitrosodiethylamine (mg/day)	Tumor Incidence (%)
1	None	0
None	0.79	17
1	0.77	62

[a] Data from Kawachi *et al.* (24). All the animals were killed and examined for tumors after 192 days on experiment.

Table X
Tumor Incidence from 2-Acetylaminofluorene (AAF) and Oxidized Corn Oil Fractions in Rats[a]

AAF (%)	Oxidized Oil (%)	Sex	Tumor Incidence at 30 Months (%)		
			Liver	Ear Duct	Mammary Gland
0.005	...	M	0	0	0
0.005	...	F	0	0	12.5
0.005	2.5	M	96	21	70
0.005	2.5	F	89	21	94
Controls ...	...	M	0	0	0
...	...	F	0	0	0

[a] Data from Sugai *et al.* (25).

greatest concern was that photosensitization might occur (26). However, fairly recent results point toward the need for reassessing whether certain compounds may not enhance the weak carcinogenic action of ultraviolet radiation sufficiently so that tumors are produced. Thus, Bingham and Falk showed that three separate optical brighteners had such capability (27) (Fig. 2).

Other factors to be noted are that percutaneous absorption of various materials, including carcinogens, does occur (28). In certain such instances tumors did not develop on the skin but in various internal organs (29, 30). Thus, despite efforts of Hoffmann and Graffi (30) to elicit tumors at the site of cutaneous application of the strong hepatocarcinogen, *N*-nitrosodiethylamine, none were found. One liver tumor, and many tumors of the nasal cavity were produced, however.

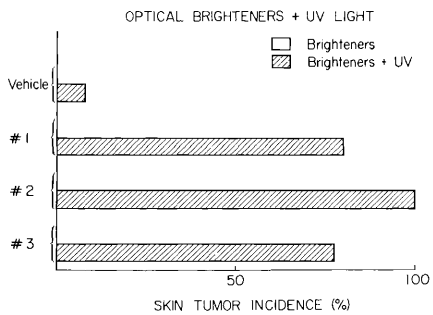

Figure 2. Tumor incidence in mice from repeated topical application of three optical brighteners and exposure to ultraviolet light. Data from Bingham and Falk (27)

Contrarily, carcinogens applied to the skin may cause epithelial tumors but not at the site of application. An example is *N*-nitrosomethylbutylamine which, when applied to the skin of the back, caused carcinomas of the eyelids and of the nasal cavity in mice (31). These diverse results illustrate that one must not merely inspect the test site for skin tumors. To be certain of the results, it is necessary to do complete necropsies of the experimental animals.

Enzyme Induction

In view of the fact that certain materials which are present in many perfumery agents may act as enzyme inducers, thus influencing substantially the response to carcinogens, this subject should be mentioned.

In most animal species, enzymes which are located in the microsomal fraction of the cell can detoxify a large number of compounds not normally present in the organism. Such systems have been studied extensively by pharmacologists because of their effects on drugs and because of the effects of drugs on each other (32). In addition to their capacity to detoxify drugs, these enzymes can convert a number of carcinogens such as polycyclic hydrocarbons, aromatic amines, or azo dyes to less active or inactive compounds such as their ring-hydroxylated derivatives. An important characteristic of these microsomal detoxification systems is that administration of the appropriate compounds, both *in vivo* or *in vitro,* can induce an increase in the activity of these systems.

Among the types of compounds which have this property of enzyme induction are numerous polycyclic aromatic hydrocarbons, both carcinogenic and noncarcinogenic (33). Other inducers are phenothiazines and a sizable number of derivatives (34), certain other drugs including phenobarbital, certain insecticides, numerous natural products such as safrole (35), some terpenes, as found in cedarwood (36, 37), and synthetic and naturally occurring flavones (38). Terpenes, safrole, and flavones are of special interest since they are representative compounds which are present in small amounts in many perfumery and flavoring materials currently in use.

In many experiments, it has been shown that such materials can lessen the action of carcinogens. An example is the decrease in number of skin tumors induced by benzo(*a*)pyrene in mice which were also treated with 5,6-benzoflavone (β-naphthoflavone) (38) (Table XI). The terpenes present in sweet orange oils inhibited considerably the activity of benzo(*rst*)pentaphene (dibenzopyrene) (39).

Table XI

5,6-Benzoflavone as Skin Tumor Inhibitor[a]

Primary treatment	Carcinogen	Tumor Incidence (%)	Tumors per Mouse
Sesame oil[b]	None	0	0
5,6-Benzoflavone[b]	None	0	0
Sesame oil	Benzopyrene[c]	40	0.7
5,6-Benzoflavone	Benzopyrene[c]	17	0.3

[a] Data from Wattenberg and Leong (38).

[b] 5,6-Benzoflavone, 6 mg/ml of sesame oil, or sesame oil alone, was painted on the backs of 9-week old mice once daily for 3 days.

[c] Two doses of 5 μg of benzo(*a*)pyrene in acetone with 1% mineral oil were applied to the skin 24 hr after the last inducer or vehicle administration.

Cocarcinogens

In contrast to the effects of compounds which can induce enzymes, thereby ultimately reducing the potency of many carcinogens, other natural products have the ability to enhance or promote the action of carcinogens. These so-called "cocarcinogens" generally have no carcinogenic activity. At most, they have an extremely weak effect (40). The best known of these agents are the phorbol esters, isolated from croton oil, from the seeds of *Croton tiglium L.* The structure of phorbol is shown in Fig. 3. In croton oil the phorbol is esterified by various fatty acids C_8 (caprylic) to C_{14} (lauric) (41). Other materials which have this capability are linalyl oleate and linalyl acetate (42), perfumery agents, and certain of the Tweens and Spans which have a lipophilic-hydrophilic structure similar to the phorbol esters. However, with the Tweens and Spans, extremely high doses are necessary to show any promoting effect (41). In some cases, irritant materials, when applied after a carcinogen, had promoting action in skin carcinogenesis, but not consistently so.

An illustration of the action of the phorbol ester fractions in enhancing the effect of the strong carcinogen 7,12-dimethylbenz(*a*)anthracene

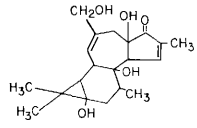

Figure 3. Structure of phorbol, parent compound of the phorbol esters

Table XII
Cocarcinogenic Effect of Phorbol Esters[a]

Primary Treatment[b]	Secondary Treatment	Time to Papilloma (Days)	Papilloma Incidence (%)
DMBA	None	266	15
DMBA	Croton ester A, low dose[c]	222	40
	Croton ester A, high dose[d]	51	95
DMBA	Croton ester C, low dose[c]	104	45
	Croton ester C, high dose[d]	51	100

[a] Data from Van Duuren and Orris (43).

[b] A single application of 300 μg of 7,12-dimethylbenz(*a*)anthracene was used in each case. The animals were Swiss Millerton female mice.

[c] The croton ester was applied at a dose of 0.5 μg twice weekly beginning 14 days after primary treatment.

[d] The material was applied to the skin at a dose of 5.0 μg 3 times weekly beginning 14 days after primary treatment.

comes from the report of Van Duuren and Orris (43). The esters decreased the time for tumor formation but increased markedly the tumor incidence (Table XII).

From this brief survey it is concluded that many factors are capable of influencing the actual response of an animal system to a carcinogen. Some of these factors cancel the effects of each other, while in other cases the net effect may be an enhancement of the response.

Compounds Under Test

Following this survey of some of the possible influences on carcinogenic response, the types of compounds now under test in the NCI bioassay program are next considered. A number of these compounds are being investigated because (a) they are known to be commercially important, and (b) adequate data on these materials are not available in the scientific literature. Many of these compounds are being administered in the feed but not because they are necessarily food additives. However, this route simulates entry of dusts into the system.

They include dyestuff intermediates, mostly aromatic amines, some of which are employed in cosmetic formulations (hair dyes), while others are industrial intermediates for dyes or polymers. Also being tested are pesticides, polymers, and intermediates to prepare them; chemotherapeutic agents, perfumery and flavoring agents, tobacco smoke components, and possible food contaminants.

Conclusion

In summary, a survey has been made of the methods generally employed in testing compounds for long-term toxicity or carcinogenicity. The factors which should be kept in mind when selecting animal test systems and the influence these factors can have on the outcome have been mentioned. Under current NCI bioassay programs, many compounds of environmental importance are being tested for possible carcinogenicity. It is NCI policy to encourage publication of the results in the scientific journals so that the data are available for all.

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