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The Carcinogenicity of Multiple Intragastric Doses of Aromatic and Heterocyclic Nitro or Amino Derivatives in Young Female Sprague-Dawley Rats

D. P. Griswold, Jr., A. E. Casey, E. K. Weisburger, and J. H. Weisburger

Southern Research Institute[1] *(D. P. G.) and Birmingham Baptist Hospital (A. E. C.), Birmingham, Alabama 35205, and National Cancer Institute (E. K. W., J. H. W.), Bethesda, Maryland 20014*

SUMMARY

The carcinogenicity of 35 compounds was evaluated in young female Sprague-Dawley rats 9 months after the oral feeding of 10 doses at the maximally tolerated level. The following compounds were definitely active in causing breast cancer: 2-anthramine, 2,7-fluorenediamine, benzidine, *N*-6-(3,4-benzocoumarinyl)acetamide, [α-(2-methylhydrazino)toluoyl]urea, and 7,12-dimethylbenz[*a*]anthracene, the positive control. Weaker responses were elicited by tolidine, thiodianiline, and 1-chloro-2,4-dinitronaphthalene. Biphenyltetramine, 1,3,7-tribromo-2-fluorenamine, 1-anthramine, and nitrofurazone led to a borderline response. One of 132 negative control rats had breast cancer.

Single lesions at sites other than the breast, not present in controls, were observed in rats administered: 4,4′-oxydianiline, 4,4′-sulfonyldianiline, 1,3,7-tribromo-2-fluorenamine, 2-anthramine, 1-anthramine, 1-methylaminoanthraquinone, *N*-methyl-*N*-2,4,6-tetranitroaniline, *N*-6-(3,4-benzocoumarinyl)acetamide, diphenylthiohydantoin,[α-(2-methylhydrazino)toluoyl]urea and 7,12-dimethylbenz[*a*]anthracene.

2-Aminoanthraquinone induced cystic changes in the kidneys in a high percentage of the treated rats.

Mammary cancer induction in young female Sprague-Dawley rats is a rapid and sensitive technic for detection of the carcinogenicity of polynuclear aromatic hydrocarbons, of polycyclic nitro and amino derivatives, and also of select heterocyclic compounds. Multiple dosing was no more sensitive than a single large dose for pinpointing active compounds. However, repeated treatments increased the percentage of tumor-bearing animals and the multiplicity of the tumors with active compounds.

INTRODUCTION

The exquisite sensitivity of the mammary gland of female Sprague-Dawley rats to certain chemical carcinogens, developed by Huggins (12) from the observation of Shay *et al.* (28), has been a useful tool for the penetrating study of controlling factors in breast neoplasia. We have utilized the system for a rapid evaluation of the carcinogenicity of select chemicals. A single large dose of compound was administered, and the mammary tumor incidence after 6 months was taken as the endpoint in Phase I of this study (6).

The question arose whether the sensitivity of the technic could be increased further by multiple dosing (7, 14, 33) and by extending the period of observation. Huggins *et al.* (13) demonstrated that the response of the rats to an identical challenge with carcinogen was a function of age. It was low in weanlings, increased sharply in rats 40 days old, reached a peak with rats about 55 days old, and dropped off sharply in rats older than 70 days. Thus, the sensitive region extended over a 30-day span, from 40 to 70 days in rats.

The present paper reports on the carcinogenicity of 35 compounds of a variety of chemical structures given as 10 predetermined maximally tolerated doses every 3 days to female Sprague-Dawley rats 40 days old at the start. The period of observation was extended to 9 months.

MATERIALS AND METHODS

Animals

Female rats (Sprague-Dawley, Inc., Madison, Wisconsin) were 40 days of age at the beginning of the test. They were housed in stainless steel cages, 16 x 16 x 5 inches, with no more than 5 rats per cage, with free access to Purina Laboratory Chow and water.

Chemicals

Rationale for Selection of Compounds. In the first phase of this experiment (6), 2-anthramine gave a high incidence of breast tumors after a single dose, and was used as reference. The isomeric 1-anthramine was included to pinpoint the active structural features in this series of compounds. Other related anthraquinone derivatives, namely 1-methylaminoanthraquinone, 2-aminoanthraquinone, and 2,6-diaminoanthraquinone are either dyes or dyestuff intermediates.

The following aromatic amines or derivatives are important in the manufacture of polymers: 4,4′-oxydianiline, 4,4′-methylenedianiline, biphenyltetramine, tetraaminodiphenyl ether (4,4′-oxybis-*o*-phenylenediamine), octafluorobenzidine, and 3,3′-dimethoxy-4,4′-biphenylene diisocyanate. Some of these had been

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tested in Phase I and had given 1 tumor each, admittedly a background level. Other amines examined were 4,4′-sulfonyldianiline, now an important antimalarial drug, and its analog 4,4′-thiodianiline. Various benzidine derivatives, namely benzidine, 3,3′-dichlorobenzidine, and 3,3′-dimethylbenzidine (tolidine), are useful as reagents or dyestuff intermediates. Auramine O, a dyestuff, is hepatomogenic in mice, but was inactive in Phase I.

2,3-Diaminophenazine, a reagent for the determination of various metals, is a structural analog of certain drugs, while 3,6-diamino-2,7-dimethylacridine is the dimethyl derivative of proflavine, an antiseptic.

2,7-Fluorenediamine was found active in Phase I and was included as another positive control. Other fluorenamine compounds, 1,3,6,8-tetrachloro-2,7-fluorenediamine, 1,3,7-trichloro- and 1,3,7-tribromo-2-fluorenamine have shown some promise against animal tumors in a CCNSC[2] screening program for anticancer drugs.

Hexanitrostilbene and *N*-methyl-*N*,2,4,6-tetranitroaniline are heat-resistant explosives and rocket propellants. The structure of HNS makes it interesting, since it is related to the aminostilbenes, known carcinogens, which are active in this system. Likewise, tetryl has an *N*-nitro-*N*-methylaniline structure, similar to the carcinogenic *N*-methyl-*N*-nitrosoaniline.

In Phase I, *N*-6-(3,4-benzocoumarinyl)acetamide was found to be fairly potent while 6-acetamidocoumarin was not. Therefore, these compounds were retested to determine the effect of repeated doses. The important drug diphenylhydantoin gave a hint of activity in Phase I. Accordingly, this compound, along with the structural analogs, diphenylthiohydantoin and dimethylhydantoin, were investigated to pinpoint the responsible structural features of the molecule.

Likewise, carbarsone and 1-chloro-2,4-dinitronaphthalene were reexamined, since, in Phase I, these materials gave a few tumors at a background level. In the meantime, an extensive feeding study of carbarsone has failed to detect carcinogenicity (23).

[α-(2-Methylhydrazino)toluoyl]urea is an analog of MIH, an experimental cancer drug, which has been reported to cause breast tumors in rats (10, 15). Nitrofurazone is a useful antiseptic against many Gram-positive and Gram-negative bacteria.

Source of Compounds. Dr. T. Lloyd Fletcher, University of Washington Medical School, furnished 1,3,7-tribromo- and 1,3,7-trichloro-2-fluorenamine, tetrachloro-2,7-fluorenamine and *N*-6-(3,4-benzocoumarinyl)acetamide. The HNS and tetryl were obtained through Dr. James Ablard, Naval Ordnance Laboratory, White Oak, Maryland. Nitrofurazone was donated by Norwich Pharmacal Co., Norwich, New York. Dr. Frank Riel, Whittaker Corporation, San Diego, California, provided the biphenyltetramine. Initial supplies of 1-chloro-2,4-dinitronaphthalene came from Dr. Julius Hyman, Fundamental Research Co., Berkeley, California, while later samples were synthesized. The [α-(2-methylhydrazino)toluoyl] urea was kindly supplied by Dr. Harry Wood, Jr. and Mr. Robert Ing of CCNSC. The initial batch of 1-methylaminoanthraquinone was procured through Dr. Hans Falk; later it was purchased commercially. 3,3′-Dichlorobenzidine · HCl was acquired from the National Aniline Division, Allied Chemical Co. The other compounds were available from the earlier study (6) or were purchased commercially. All chemicals were tested for purity by appropriate methods.

Toxicity Determinations. Each compound was dissolved or suspended in sesame oil, usually in 1 ml/dose. The MTD was determined as described (6), except that the test compounds were administered repeatedly by gastric tube. The first dose was given to 40-day-old rats. Initially, it was desired to establish whether there was a difference in the maximal amounts of compound which could be administered to rats over the 30-day period with a regimen of 6 doses every 5 days or of 10 doses every 3 days. Evaluation of the 6-dose versus the 10-dose trials with all of the compounds studied gave no clearcut advantage to either system based on the endpoint of 60-day mortality and body weight change. The final studies were, therefore, designed on the basis of a 10-dose scheme, primarily because compounds of relatively low toxicity could be given in greater total amounts.

Carcinogenicity Studies. The experimentally determined MTD was administered by gastric intubation in 10 equal doses to 20 rats beginning at age 40 days. The tests were performed in 7 groups, each consisting of 5 compounds, generally classified by chemical type. With each of the groups there was a series of negative controls fed the sesame oil vehicle. Positive control groups received a single dose of 18 mg of DMBA, as a check on the constancy of responsiveness of the animals. In cases where there was excessive mortality, additional groups of rats were given select lower doses of compound so that a sufficient number of survivors were available at the end of the experiment.

The observation period was extended to 9 months in order to maximize the sensitivity of the test with respect to mammary tumor formation and to discover lesions in other sites for which the previously used experimental period of 6 months might have been insufficient.

The animals were weighed and inspected weekly. Any abnormalities, particularly masses in the breast region, were noted. If animals appeared ill for any reason, they were examined twice daily and sacrificed when death appeared imminent.

All animals were carefully autopsied. Grossly apparent lesions were recorded. The pituitary and adrenal glands, kidneys, spleen, and liver were weighed. Any diseased tissues as well as mammary tissue, intestinal tract, pituitary, liver, ovaries, and adrenals were fixed in 10% formalin and processed for histologic examination. In addition, representative portions of other viscera were fixed for later examination if indicated. Hematoxylin and eosin-stained sections were studied microscopically without knowledge of the observer (A. E. C.) of the experimenal history of the donor rat.

RESULTS

The dosages for the carcinogenicity tests were selected on the basis of preliminary determinations of the MTD over a

[2] Abbreviations: HNS, hexanitrostilbene; MIH, *N*-isopropyl-1-(2-methylhydrazino)-*p*-toluamide; tetryl, *N*-methyl-*N*,2,4,6-tetranitroaniline; CCNSC, Cancer Chemotherapy National Service Center, National Cancer Institute; MTD, maximally tolerated dose; DMBA, 7,12-dimethylbenz[*a*]anthracene.

period of 60 days (Table 1). Nevertheless, it was found in several instances that the values thus secured resulted in inordinate mortality. In these cases, appropriate lower dosages were administered to additional animals. In a general screening program, isolated early losses of rats due to excessive dosage are not necessarily undesirable. One can be certain that the subsequently chosen slightly lower levels will lead to critical evaluation of the drug under conditions of maximal loading of the animal. This scheme is particularly relevant in cases such as the present, where the animals are exposed to agent only for a limited time. Continuing administration of toxic levels of a test material in the diet, on the other hand, might result in an inhibition of tumor formation. The dose rate would have to be selected carefully to avoid this eventuality (39).

The mortality at 45 days, shortly after termination of the administration of the compound, reflects toxicity resulting from the treatment (Table 1). The survivors at the end of the 9-month experiment reveal the chronic effects. In these experi-

Table 1

Classification and compound	Total dosage[a] (mg/rat)	Effective numbers of rats			No. autopsied	No. of rats with mammary lesions[b]	No. & type of mammary lesions	Other lesions
		0 days	45 days	9 months				
Aromatic amines								
3,3',4,4'-Biphenyltetramine	1000	20	20	20	20	1	Carcinoma 1	
Octafluorobenzidine	3000	20	20	17	18	1	Hyperplasia 1	
3,3'-Dichlorobenzidine·2HCl	300	20	16	14	15	0		
Tolidine	500	20	16	16	16	3	Carcinoma 4; hyperplasia 1	
Benzidine	50	20	14	4	5	4	Carcinoma 17	Lymph node hyperplasia 1
	35	20	20	0				
	25	10	10	8	9	7	Carcinoma 12	
	12	10	10	10	10	5	Carcinoma 5; fibroadenoma 1	
4,4'-Oxydianiline	400	20	11	8	11	0		Squamous metaplasia of uterus 1
	300	10	10	10	10	0		
4,4'-Thiodianiline	400	20	12	12	12	3	Carcinoma 3; hyperplasia 1	
	300	10	9	7	8	2	Carcinoma 1; hyperplasia 1	
4,4'-Sulfonyldianiline	300	20	20	19	19	1	Hyperplasia 1	Hyperplasia & lymphocytic infiltration of spleen & liver 1
4,4'-Methylenedianiline·2HCl	300	20	16	14	14	1	Hyperplasia 1	
Auramine (4,4'-Imidocarbonyl-bis(N,N-dimethylaniline)·2HCl)	800	20	16	14	15	1	Hyperplasia 1	
3,3',4,4'-Tetraaminodiphenylether·4HCl (4,4'-Oxybis-*o*-phenylenediamine)	500	20	17	16	16	0		
2,7-Fluorenediamine	400	20	19	16	17	15	Carcinoma 37; hyperplasia 6; fibroadenoma 1; papilloma 1	
1,3,7-Trichloro-2-fluorenamine	20	20	10	10	10	0		
	15	10	10	0				
	14	5	5	5	5	2	Hyperplasia 2	Cystic change of adrenal 1
	10	10	10	9	9	0		Cystic change of adrenal 1
1,3,7-Tribromo-2-fluorenamine	5.0	20	12	12	12	1	Hyperplasia 1	Hyperplasia of lymph nodes 1
	4.0	20	14	14	14	4	Carcinoma 1; hyperplasia 3	Fibrolipoma of subcutis 1; lymphocytic infiltration of liver 1
	2.0	15	15	15	15	1	Hyperplasia 1	Plasma cell infiltration of salivary gland 1

Table 1 (continued)

Classification and compound	Total dosage[a] (mg/rat)	Effective numbers of rats			No. autopsied	No. of rats with mammary lesions[b]	No. & type of mammary lesions	Other lesions
		0 days	45 days	9 months				
1,3,6,8-Tetrachloro-2,7-fluorenediamine	100	20	18	16	16	2	Hyperplasia 3	
2-Anthramine	25	20	13	3	10	10	Carcinoma 20; fibroadenoma 5; cyst 1	Xanthomatosis of lymph node 1
	18	10	10	0				
	10	10	10	10	10	6	Carcinoma 6; fibroma 1; hyperplasia 1	
	5.0	10	10	8	10	5	Carcinoma 5	Lymphocytic infiltration of liver & lymph nodes 1; cystic adrenal 1
1-Anthramine	800	20	18	18	18	4	Carcinoma 1; adenoma 1; hyperplasia 3	Squamous metaplasia of uterus 1
1-Methylaminoanthraquinone	5000	19	18	14	18	1	Hyperplasia 1	Tubular adenocarcinoma of kidney, 1
2-Aminoanthraquinone	1000	20	20	13	19	0		Cystic changes in kidney 13 (see text)
2,6-Diaminoanthraquinone	1000	20	20	20	20	1	Fibroadenoma 1	
3,6-Diamino-2,7-dimethylacridine	800	20	20	19	19	1	Hyperplasia 1	
2,3-Diaminophenazine	120[c]	20	14	14	14	0		
Nitro compounds								
1-Chloro-2,4-dinitronaphthalene	3000	20	19	17	17	4	Carcinoma 2; fibroadenoma 2; hyperplasia 1	
2,2',4,4',6,6'-Hexanitrostilbene	800	20	20	20	20	0		
N-Methyl-*N*,2,4,6-tetranitroaniline	400	20	19	17	19	1	Hyperplasia 1	Adenoma of stomach 1
Isocyanates								
3,3'-Dimethoxy-4,4'-biphenylenediisocyanate	400	20	17	17	17	0		
Heterocyclic compounds								
N-6-(3,4-Benzocoumarinyl)-acetamide	1000	20	19	16	19	11	Carcinoma 6; adenoma 3; hyperplasia 8	Parotid gland carcinoma 1
6-Acetamidocoumarin	1000	20	20	19	20	0		
Diphenylhydantoin	1000	20	19	19	19	2	Fibroadenoma 1; hyperplasia 1	
Dimethylhydantoin	3000	20	20	20	20	1	Fibroma 1	
Diphenylthiohydantoin	400	20	18	16	18	2	Fibroadenoma 1; hyperplasia 1	Carcinoma of kidney 1
Carbarsone (*p*-Ureidobenzenearsonic acid)	3800[d]	20	12	8	8	1	Hyperplasia 1	
	3000	10	9	8	9	0		
5-Nitro-2-furaldehyde semicarbazone (nitrofurazone)	500	20	15	5	5	1	Carcinoma 1	
	350	10	10	10	10	1	Hyperplasia 1	
	200	10	10	9	9	1	Fibroadenomatous change 1	

Table 1 (continued)

Classification and compound	Total dosage[a] (mg/rat)	Effective numbers of rats			No. autopsied	No. of rats with mammary lesions[b]	No. & type of mammary lesions	Other lesions
		0 days	45 days	9 months				
(α-(2-Methylhydrazino)-toluoyl) urea·HBr	100	20	20	16	17	11	Carcinoma 12; acanthoma 1; fibroadenoma 1; hyperplasia 4	Hyaline change of kidney 2; adenocarcinoma of ileum 1; adenocarcinoma of stomach 1; epidermal cyst 1
Controls								
Positive control								
7,12-Dimethylbenz[a]anthracene	18	40	35	19	29	29	Carcinoma 75; fibroadenoma 10; hyperplasia 47	Hyperplasia of lymph nodes 2; carcinoma of kidney 1; carcinoma of pancreas 1; lymphocytic infiltration of liver & lymph node 2; abdominal reticulum cell granuloma 1
Sesame Oil		140	134	127	132	5	Carcinoma 3; fibroadenoma 1; hyperplasia 5	

Mortality and histopathologic description of lesions in female Sprague-Dawley rats fed multiple doses of compounds.

[a] In most cases each animal received 10 doses at 3-day intervals, starting at 45 days of age. The amount shown is the total given in 10 doses, with exceptions as noted, over a 30-day period.

[b] Lesions are classified as carcinoma, fibroadenoma or adenoma, and hyperplasia. The hyperplasias were divided between adenomatous hyperplasia and fibroadenomatous hyperplasia, respectively, in a 1:2 ratio. Only the most advanced lesion is listed where there were multiple masses.

[c] These animals received 6 doses of 20 mg each due to a shortage of the compound.

[d] The animals received 500 mg/rat 4 times and then 6 doses of 300 mg/rat because of early mortality.

ments poor long-term survival appeared to be associated with an early development of tumors, indicative of potent carcinogenicity of the chemical tested.

Table 1 also shows the total number of gross and microscopic lesions and the number of rats affected. In the mammary tumor system, the multiplicity of the lesions is an additional indicator of carcinogenic potency (29), similar to the pulmonary tumor system of the mouse (31). The palable mammary masses agreed reasonably well with the microscopically established tumor incidence. Diagnosis of some grossly visible lesions as cysts was balanced by the discovery of tumors only upon microscopic examination. In addition, as has been observed previously, some of the palpable masses regressed spontaneously and, therefore, were not present at autopsy. Not only did individual rats sometimes bear more than one tumor, but these lesions were of different histologic type, such as lobular carcinoma, milk-secreting or nonsecreting adenoma, fibroadenoma, or adenomatous hyperplasia. Even a single mass might contain several distinct histologic entities. Others (4, 8, 12, 32) have recorded similar observations, but, nevertheless, the interrelations in the pathogenesis of these lesions merit continuing attention.

Striking differences in activity among closely related compounds were found. Thus, benzidine was quite active, but tolidine was considerably less so, especially if the total dosages administered are taken into account. Dichlorobenzidine, octafluorobenzidine, and biphenyltetramine, all industrial products of some importance, appeared to be inactive in this test system. The dichloro compound was reported carcinogenic under other conditions (24). Even though so closely related to benzidine, the inactivity of diaminobenzidine or biphenyltetramine is of some interest. It could be, however, that this compound is truly negative, inasmuch as 3,4-diaminobiphenyl is also much less carcinogenic than 4-aminobiphenyl (20); the underlying rationale deserves investigation.

Derivatives or analogs of methylenedianiline (cf. 19) also seemed to be devoid of carcinogenic potency in this system, except for thiodianiline which was weakly active. Possibly the sulfur atom is a better conductor of electrons than any of the other connecting elements. The analog, an *ortho*-bis(diamino)aryl derivative, tetraaminobiphenyl ether, was also inactive.

Although 2,7-fluorenediamine was quite potent, substitution by chlorine practically abolished carcinogenicity. Halogen derivatives of 2-fluorenamine, itself active, also showed virtually no carcinogenic activity, but these compounds were very toxic.

The aminoanthracene and anthraquinone series also exhibited drastic changes in carcinogenic potency as a function of structure. 2-Anthramine was among the most powerful carcinogens

in these tests. In contrast, the 1-isomer can be considered inactive. There was a sizable spread in tolerated dosages between these two compounds, 1-Methylaminoanthraquinone, a commercially used dyestuff, had little toxicity and no carcinogenicity. Although 2-aminoanthraquinone, the quinone corresponding to highly carcinogenic 2-anthramine, was not carcinogenic at all and showed relatively low toxicity, there were cystic changes in the kidneys in a high proportion of the treated animals. These ranged from cystic dilation of the renal tubules to actual kidney cysts. On the other hand, 2,6-diaminoanthraquinone, in which the amino groups are symmetrically located, had no pathologic effect at the same dose level. A compound with similar structure, but based on the heterocyclic acridine ring system, 2,7-dimethyl-3,6-diaminoacridine, also proved inactive at fairly high dose levels. This drug is noteworthy, since it and the closely related 3,6-diaminoacridine (proflavine) can bind to DNA (17, 27). A similar heterocyclic derivative with an *ortho*-diamine structure, 2,3-diaminophenazine, failed to induce tumors.

Another carcinogen which was positive, even after a single dose, was *N*-6-(3,4-benzocoumarinyl)acetamide. The activity of this compound is not necessarily related, however, to the coumarin skeleton. Indeed, 6-acetaminocoumarin was completely negative. Also after a single dose, diphenylhydantoin seemed to have borderline tumorigenic potency (6). Reexamination under the present conditions did not increase the incidence. The thio analog, diphenylthiohydantoin, acted alike, and dimethylhydantoin certainly was inactive.

Carbarsone was inactive. Nitrofurazone, at 3 different dose levels, had only questionable activity. A highly carcinogenic compound was methylhydrazinotoluoylurea. In addition to mammary tumors, an adenocarcinoma of the stomach and of the ileum were found in 1 rat. Such tumors were not seen in control animals in this or in the previous series. In addition, 2 rats had hyaline changes in the renal cortex.

A high total dosage of chlorodinitronaphthalene gave borderline carcinogenic activity. Hexanitrostilbene and tetryl appeared to be inactive for the breast, but the latter compound gave a gastric adenoma, a tumor not observed in controls. Curiously, the highly reactive dimethoxybiphenyldiisocyanate, derived from the carcinogenic dimethoxybenzidine (24, 38), was entirely negative.

Several groups of animals administered DMBA gave the expected response throughout the series of experiments, with an average of 86% incidence of actual mammary carcinoma or sarcoma, and isolated tumors in other organs.

The negative control rats administered sesame oil had a distinct but low tumor incidence. Of a total of 132 rats, 2 had 1 each and 1 showed 2 fibroadenomatous hyperplasias, 1 had a giant fibroadenoma, and 1 rat had multiple lesions detected microscopically, namely, 3 lobular carcinomas *in situ* and 1 area of fibroadenomatous hyperplasia. The affected rats were killed between 279 and 295 days, except 1 which was sacrificed after 231 days.

Table 2 lists animals with palpable lesions as a function of time with several of the more active carcinogens. With DMBA, 2-anthramine, benzidine, and methylhydrazinotoluoylurea, the first lesions appeared approximately 60 days after the first exposure and somewhat later with fluorenediamine and benzocoumarinylacetamide. In all these cases, there was a progressive increase of tumor-bearing animals and of tumor multiplicity. Also, with compounds fed at several levels, there was a dose-related response.

DISCUSSION

The present effort was part of a deliberate search for better technics to assess the carcinogenic potency of chemicals. As in the previous study (6), advantage was taken of the apparent high sensitivity of the mammary gland of female Sprague-Dawley rats under specific experimental conditions, particularly with respect to the age of the animal being treated and the vehicle used. A more severe regimen of exposure, 10 doses given over a 30-day period compared to a single dose, and a longer period of observation, 9 months compared to 6 months, was expected to yield a more pronounced carcinogenic effect.

Table 3 presents a comparison between the two series with select compounds tested under both conditions. A striking increase in tumor yield following multiple dosing was observed with the two most active compounds in this series, 2,7-fluorenediamine and 2-anthramine. With another active compound, benzocoumarinylacetamide, the tumor incidence was almost identical by the two methods, of which the single dose intubation is much more convenient. Chlorodinitronaphthalene also gave a similar weak response after 1 dose of 500 mg or 3 gm divided in 10 doses. With the remainder of the compounds, all weak or negative, no advantage was seen with the more extensive treatment. With some agents, perhaps an even more strenuous exposure is required. For example, nitrofurazone had only borderline activity in our experiments, but Price *et al.* (25) found 35 of 44 rats had fibroadenomas when they were fed the maximally tolerated level, 0.1–0.3% of compound in the diet for 44 weeks, and were held to 15 months of age.

Despite the 9-month period of observation, there was little effect in organs other than the mammary gland with most compounds. Under other conditions, chemicals such as fluorenediamine or anthramine have affected the liver or other organs. Halogenated fluorenamines are highly hepatotoxic (19, 22). 4,4′-Methylenedianiline, in addition to its effect on steroid metabolism (35, 40), also leads to liver, kidney, and eye damage in several species even after single exposure (11, 16, 36). Some of these materials affect male rats more than females, accounting perhaps for the absence of lesions in our series. Also, our rats were killed 8 months after cessation of treatment, allowing time for repair of reversible damage.

Some structure-activity correlations in this experimental system deserve emphasis. 2-Fluorenamine was found to be definitely carcinogenic in this system (C. Huggins, University of Chicago, personal communication). By other tests, 7-chloro-2-fluorenylacetamide is toxic and carcinogenic; but in Phase I, this compound was fairly nontoxic and had borderline or no carcinogenicity. In our present tests, tri- and tetrachloro derivatives of fluorenamine or of the more active fluorenediamine did not lead to breast cancer. Tribromofluorenamine, which was administered in only small quantities because of toxicity, seemed weakly active. Of related interest is the inactivity of dichlorobenzidine and octafluorobenzidine, both halogenated

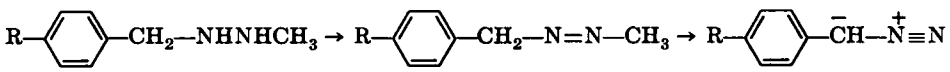
Table 2

Dosage[a]	2-Anthramine								
	2.5 mg			1.0 mg			0.5 mg		
Days after 1st exposure	% survivors	% survivors with masses	Mean No. of masses	% survivors	% survivors with masses	Mean No. of masses	% survivors	% survivors with masses	Mean No. of masses
0	100	0	0	100	0	0	100	0	0
30	65	0	0	100	0	0	100	0	0
60	65	38	0.4	100	10	0.1	100	0	0
90	65	77	1.3	100	20	0.3	100	10	0.1
120	50	80	1.2	100	20	0.3	100	20	0.3
150	45	78	1.9	100	20	0.3	100	20	0.3
180	40	88	2.6	100	30	0.4	100	10	0.2
210	40	100	3.1	100	40	0.5	100	10	0.2
240	30	100	4.0	100	60	0.7	100	30	0.4
270	15	67	3.7	100	60	0.8	80	25	0.4
	Benzidine								
	5.0 mg			2.5 mg			1.2 mg		
0	100	0	0	100	0	0	100	0	0
30	30	0	0	100	0	0	100	0	0
60	30	17	0.6	100	20	0.2	100	10	0.1
90	25	60	1.0	100	60	0.8	100	20	0.2
120	25	60	1.0	100	70	1.3	100	30	0.3
150	25	40	0.8	90	67	1.3	100	30	0.3
180	25	60	2.0	90	56	1.3	100	40	0.4
210	25	60	1.8	90	56	1.4	100	50	0.5
240	25	80	3.4	90	67	1.6	100	60	0.6
270	20	100	2.8	70	71	1.0	100	50	0.6
	4,4'-Thiodianiline						DMBA		
	40 mg			30 mg			18 mg		
0	100	0	0	100	0	0	100	0	0
30	60	0	0	100	0	0	88	0	0
60	60	0	0	80	0	0	88	34	0.4
90	60	8	0.08	80	0	0	88	60	1.3
120	60	17	0.2	80	0	0	83	73	1.9
150	60	8	0.08	80	13	0.1	83	79	2.2
180	60	17	0.2	80	13	0.1	80	91	2.8
210	60	25	0.3	80	13	0.1	78	97	3.8
240	60	25	0.3	70	29	0.3	60	96	4.5
270	55	27	0.4	70	29	0.3	55	100	5.0
	2,7-Fluorenediamine			N-6-(3,4-Benzo-coumarinyl)acetamide			(α-(2-Methylhydrazino) toluoyl)urea·HBr		
	40 mg			100 mg			10 mg		
0	100	0	0	100	0	0	100	0	0
30	95	0	0	100	0	0	100	0	0
60	95	0	0	95	0	0	100	5	0.05
90	95	16	0.2	95	26	0.3	100	30	0.3
120	95	32	0.3	90	28	0.3	95	21	0.3
150	95	74	0.7	90	28	0.5	85	29	0.4
180	95	79	1.8	85	33	0.6	80	31	0.6
210	95	95	2.5	80	44	0.6	80	50	0.8
240	90	89	3.0	80	44	0.6	80	56	1.1
270	80	88	3.1	80	44	0.7	80	56	1.1

Development of mammary tumors as a function of time and dose following select compounds.

[a] The dose listed is the individual level given 10 times every 3 days, except that DMBA was administered as a single dose of 18 mg.

derivatives of the carcinogenic benzidine. Steric problems relative to carcinogenicity of halogenated fluorenamines have been discussed (37). While molecular dimensions play a role in mammary carcinogenesis, lipid solubility may be even more germane. Possibly, there is an inverse relationship between relative polarity of the molecule and lipid or related solubility, relevant to the effective concentration available at the mammary gland (cf. 3). Also, biochemical *N*-oxidation of the arylamines may be required to yield an active carcinogen, and this reaction is structure-sensitive.



R = $CONHCH(CH_3)_2$ in MIH; R = $CONHCONH_2$ in methylhydrazinotoluoylurea.

Table 3

Compounds	Phase I: Dosage (mg/rat)	Phase I: No. of rats with tumor/No. observed	Phase II: Total dosage (mg/rat)	Phase II: No. of rats with tumor/No. observed
3,3',4,4'-Biphenyl-tetramine	300	0/17	1000	1/20
Octafluorobenzidine	500	0/20	3000	0/18
4,4'-Oxydianiline	150	0/18	400	0/11
			300	0/10
4,4'-Sulfonyldianiline	100	0/19	300	0/19
Auramine	150	0/19	800	0/15
2,7-Fluorenediamine	100	6/32	400	15/17
2-Anthramine	10	7/20	25	10/10
			10	6/10
			5	5/10
1-Chloro-2,4-dinitro-naphthalene	500	2/20	3000	3/17
N-6-(3,4-Benzo-coumarinyl)acetamide	500	6/20	1000	7/19
6-Acetaminocoumarin	200	0/17	1000	0/20
Diphenylhydantoin	150	1/19	1000	1/19
Carbarsone	500	1/19	3800[a]	0/8
			3000	0/9
Sesame oil controls		0/89		2/132

Comparison of results of administration of single or multiple doses of compounds to female Sprague-Dawley rats. In Phase I, the rats received a single dose at 50–55 days of age; they were autopsied after 6 months. In Phase II, the rats received a total of 10 doses every 3 days from age 40 to age 70 days; they were autopsied after 9 months. Tumor refers to mammary carcinoma, fibroadenoma, or adenoma.

[a] Carbarsone was fed at 500 mg/rat four times and then at 300 mg/rat six times because of mortality.

The lower carcinogenicity of 3,3'-dimethylbenzidine (tolidine) relative to benzidine may not hinge on steric factors or lipid solubility (polarity) but might be explained by the metabolic detoxification at the *ortho*-methyl substituent, for example by oxidation to a carboxyl. It is true that 3-methyl-2-naphthylamine was a powerful mammary carcinogen in rats (6, 30, 38), but in this case the 2- and 3-positions of naphthalene are poorly connected electronically, while the opposite holds in the biphenyl series.[3]

The high potency of methylhydrazinotoluoylurea, a relative of the active MIH (Natulan, Ibenzmethyzin) (10, 15), suggests that the active center for carcinogenicity is based on the presence of the methylhydrazinomethyl side chain (21, 25). The carcinogenicity might be expressed subsequent to metabolic transformation to structures akin to *sym*-dimethylhydrazine, azoxymethane, and related intermediates which ultimately are converted into alkylating agents of the diazoalkane type (1, 5, 18, 26).

Although 2-aminoanthraquinone was noncarcinogenic, the production of cystic changes in the kidneys following a total dose of 1 gm is noteworthy. Recently, Thomas *et al.* (34) reported that rats on 0.1% or more of diphenylamine in the diet over a 2-year period (a total of approximately 7.2 gm) developed cystic kidney changes. Booth *et al.* (2) fed 1.5 gm of biphenyl over 30 days and obtained minimal kidney changes; after 120 days more pronounced effects were noted, and males were affected more than females. The fact that a relatively low dose of 2-aminoanthraquinone led to kidney changes in our females which persisted 7.5 months later indicates that the compound may be a relatively strong nephrotoxin.

Specific conclusions on the value of the breast tumor induction system of Huggins for the assessment of the carcinogenicity of chemicals can be based on this extensive test series involving 2 modes of compound administration, single dose (Phase I) and 10 doses (Phase II) (Table 3). The judgment reached earlier that this technic is useful for the detection of carcinogens of the polynuclear aromatic type, such as hydrocarbons, nitro derivatives, and amines, and of certain coumarins, still stands. Additionally, some limitations can be developed. Lipid solubility to transfer and maintain sufficient dosages of compound, particularly in the fat pads of the mammary gland, seems required. Substitution by halogen on the ring decreased the response, yet, by other criteria, some of these compounds may be carcinogenic. Although with most of the compounds no great advantage resulted from the administration of divided doses during the sensitive period, with some there was a higher tumor yield.

For a preliminary and rapid evaluation of the potential carcinogenic hazard in a large series of compounds falling within the scope of responsiveness of this method, the technic can be recommended. Also the autochthonous mammary tumor system, produced by the variety of carcinogens described herein, may be a superior tool for detection of chemotherapeutic drugs (6, 7, 9, 13).

[3] New data on the effect of methyl substitution on carcinogenicity have appeared. Whereas 4,4'-methylenedianiline had no or only borderline carcinogenicity in rats, the 3,3'-dimethyl derivative was quite potent (A. Munn. Occupational Bladder Tumors and Carcinogens: Recent Developments in Britain. *In:* K. F. Lampe, (ed.), Bladder Cancer, Chap. 16, pp. 187–193. Birmingham: Aesculapius Publishing Co., 1967).

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