Some Effects of Aromatic Hydrocarbons on Sulfur Metabolism and Tumor Induction in Mice

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Compounds of different chemical types have been shown to inhibit the induction of tumors by carcinogenic hydrocarbons (5-8). Their common feature was the property of reacting with sulfhydryl groups by addition, condensation, or oxidative coupling. The inference was drawn that a primary stage of carcinogenic action involved a fixation of the hydrocarbons, or their derivatives, to sulfhydryl-containing cell constituents of unknown nature.

These phenomena were made less easy to interpret on account of the uncertainty of the part played by sulfur in the elimination of the carcinogens. Only indirect evidence of an inconclusive nature was available on this question from the feeding experiments of White (13, 14). Positive chemical evidence that disturbances of sulfur metabolism were a consequence of the administration of carcinogens was lacking.

The experimental work recorded here is part of an approach to the solution of this problem. Studies on the distribution of sulfur in mouse urine and skin after the external application of fairly large doses of 1,2,5,6-dibenzanthracene and 3,4-benzpyrene have been made. For purposes of comparison noncarcinogenic hydrocarbons of lower molecular weight, known to be mercapturate-formers, have been investigated on similar lines. As a consequence of the data accruing from these determinations naphthalene, anthracene, and phenanthrene were tested for their influence on the carcinogenic action of 1,2,5,6-dibenzanthracene and 3,4-benzpyrene.

I. Effect of Aromatic Hydrocarbons, Applied to the Skin, on the Partition of Sulfur in Mouse Urine

MRC/H hybrid mice were used in most experiments, though similar results were obtained by the occasional use of the pure strains R3 and C57. Food consisted solely of a balanced mixture of dietary essentials pressed into small bricks and obtained commercially.

To guard against accidental contamination of the urine with food powder or chance dilution with drinking water the mice were denied access to food and water during the overnight collection of urine. This precaution led to much greater consistency in the absolute values of inorganic, ethereal, and neutral sulfur, though their relative percentages of the total sulfur were not greatly affected.

Fifteen to 20 mice provided ample urine for chemical determinations. It was more concentrated with respect to sulfur than normal human urine and was suitably diluted before sulfur partition was estimated by Fiske's benzidine titration method. Five to tenfold dilution, depending on the color-depth and the apparent density of the urine, was found adequate to give reproducible results.

The same sequence was observed in all experiments. Normal values of urinary sulfur were obtained by daily estimations for 3 days. Then the same group of mice were epilated and treated with the appropriate hydrocarbon solution during the hour preceding their transfer to the cages for urine collection. Hydrocarbon treatment was repeated for 2 more days, and the mice were then left untouched for 3 further days. Thus 6 daily measurements of urinary sulfur partition were made on consecutive days. When significant deviations from normal values were observed after the first dose of hydrocarbon, these persisted during the 3 days of treatment, but normal sulfur partition was quickly attained again in the 2 days following.

Application of hydrocarbons.—Solubility considerations caused variations in the number of applications required to ensure a rough equality of dosage between the different hydrocarbons. The following measured solubilities decided the choice of either ether or acetone as an innocuous solvent.

	Solvent	Solubility at 15° gm./100 ml.
Anthracene	Ether	0.450
	Acetone	0.714
1,2,5,6-Dibenzanthracene	Ether	0.120
	Acetone	0.425
3,4-Benzpyrene	Ether	0.771

Details of the mode of application of the various hydrocarbon solutions used (1 brushful = ca. 0.2 ml.) are included in Table I. Limitation of available substances prevented the study of a more comprehensive range.

Sulfur partition in mouse urine.—The absolute values of the sulfur content and its partition in mouse urine vary considerably, even in successive collections from the same batch of mice. Uncontrolled water intake is probably the chief factor responsible for this. Yet when inorganic, ethereal, and neutral sulfur are expressed as percentages of the total sulfur, the range of values is much narrowed. Over 50 estimations of normal urine showed an average distribution of approximately 70 per cent inorganic, 10 per cent ethereal, and 20 per cent neutral sulfur. A summary of these data is given in Table II.

Typical experiments with 6 hydrocarbons are illustrated in Fig. 1. For the sake of clearness the results are grouped in two graphs, each referring to 3 hydrocarbons. Each curve represents the data from a single experiment in which 6 consecutive daily estimations of sulfur in the urine of the same group of mice were made. The separate controls for each experiment are not shown, but the curves are all referred to the average control values (see Table II).

Observations on these results.—Many of the findings conform to those of previous workers who have studied the detoxication of hydrocarbons in rabbits and rats. With the doses of benzene used, increased ethereal sulfur excretion was clear, but no mercapturate formation could be demonstrated. Zbarsky and Young (18) have shown that benzene mercapturate occurs in rat urine after administration of benzene, though only as a minor metabolic product.

With naphthalene the large increase of neutral sulfur and the more moderate rise of ethereal sulfur correspond to the findings of Bourne and Young (1) in rabbits and of Stekol (11) in rats.

Relatively smaller increases in the urine content of ethereal and neutral sulfur occur after the administration of anthracene, though the changes found are in keeping with the work of Boyland and Levi (2-4) on rats.

Phenanthrene appears to be excreted preponderantly in ethereal combination, yet considerable mercapturate formation is also indicated.

In contrast with these straightforward demonstrations of metabolic change, no certain indications of disturbed metabolism were revealed in the urine of mice treated with approximately the same doses of the carcinogens 1,2,5,6-dibenzanthracene and 3,4-benzpyrene. The slight rise in the level of ethereal sulfur shown in Fig. 1 when dibenzanthracene was used, may have been fortuitous, since 2 further experiments yielded equivocal results. The recorded variations in the level of neutral sulfur after administration of either of these carcinogens are well within the range found for normal urines.

Three possible deductions may be drawn from the data presented: (a) The carcinogens do not utilize sulfur by mercapturate formation during their elimination. (b) They use sulfur at too slow a rate to permit certain detection by analyses of urine. (c) Detoxication mechanisms involving sulfur lead to their excretion by a route different from that used by the hydrocarbons of lower molecular weight.

All these possibilities emphasize differences in the behavior of the carcinogenic and noncarcinogenic hydrocarbons used. But the results do not provide final proof that sulfur does not play a role in the detoxication of carcinogens. The conception of White (13, 14) that growth inhibition in young rats receiving dietary supplements of carcinogens is caused by carcinogen-mercapturate formation with consequent depletion of the available sulfur-amino acids essential for growth, is not disproved, but is not supported.

TABLE I

	Solution	No. brushfuls per mouse during 3 days	Amount hydrocarbon per mouse, mgm.
Benzene	Pure	3	ca. 50
Naphthalene	2.7% in ether	3	" 16
Phenanthrene	" " "	3	" 16
Anthracene	0.71% in acetone	10	" 14
1,2,5,6-Dibenzanthracene	0.42% in acetone	14	" 12
3,4-Benzpyrene	0.77% in ether	10	" 15

TABLE II: VARIATIONS IN CONTENT AND PARTITION OF SULFUR IN NORMAL MOUSE URINE

	mgm./100 ml.	% total S	Av. % total S
Total S	316.8-451.2		
Inorganic S	216.0-324.8	65.0-72.0	ca. 70
Ethereal S	40.0- 49.8	9.1-12.5	" 10
Neutral S	60.8- 76.8	17.1-22.3	" 20

II. Effect of Aromatic Hydrocarbons on the Glutathione (GSH) Content of Mouse Skin

It has been shown previously (7) that bromobenzene applied to the backs of mice causes a notable fall in the normal level of skin GSH, indicating mercapturate formation in the skin itself. Fixation of sulfhydryl groups in the skin by unsaturated dicarboxylic acids is also revealed by a similar reduction of its GSH content (8). The effects of the aromatic hydrocarbons mentioned in section I were studied from this point of view to see if mercapturate formation in the skin was a general process. It was also hoped to obtain information on possible local disturbances of sulfur metabolism following treatment with carcinogens.

EXPERIMENTAL

The control mice used for these estimations were necessarily distinct from the treated ones. By careful selection on the basis of age, color, and strain it was found possible to narrow the normal variations of GSH between 2 batches to no more than 5 per cent.

Doses of hydrocarbons comparable in amount to those shown in Table I were applied to the skin as described earlier; *i.e.,* spread over 3 days. Only uncertain indications of altered sulfur metabolism could be detected by this procedure. Yet when the same amount of hydrocarbons was used over 1 to 2 hours, and GSH determinations were made during the following hour, changes, when these occurred, could readily be detected.

The twice-minced skins from the whole backs of 5 to 8 mice were weighed (3 to 5 gm.) and ground with pure silver sand; 25 ml. of 10 per cent sulfosalicylic acid solution was added and the whole reground. After standing, with occasional regrinding, for 12 minutes, the mass was filtered at the pump; the residue was re-treated with 15 ml. of acid for 6 minutes and again filtered. The combined filtrates were normally clear, but sometimes were rid of a slight turbidity by a final filtration at atmospheric pressure. Aliquots were used for the estimation of total reducing substances (GSH and ascorbic acid) with $N/1{,}000$ iodine, and ascorbic acid with 0.01 per cent phenol-indo-2,6-dichlorophenol. In iodine equivalents 1 mgm. ascorbic acid equals 3.485

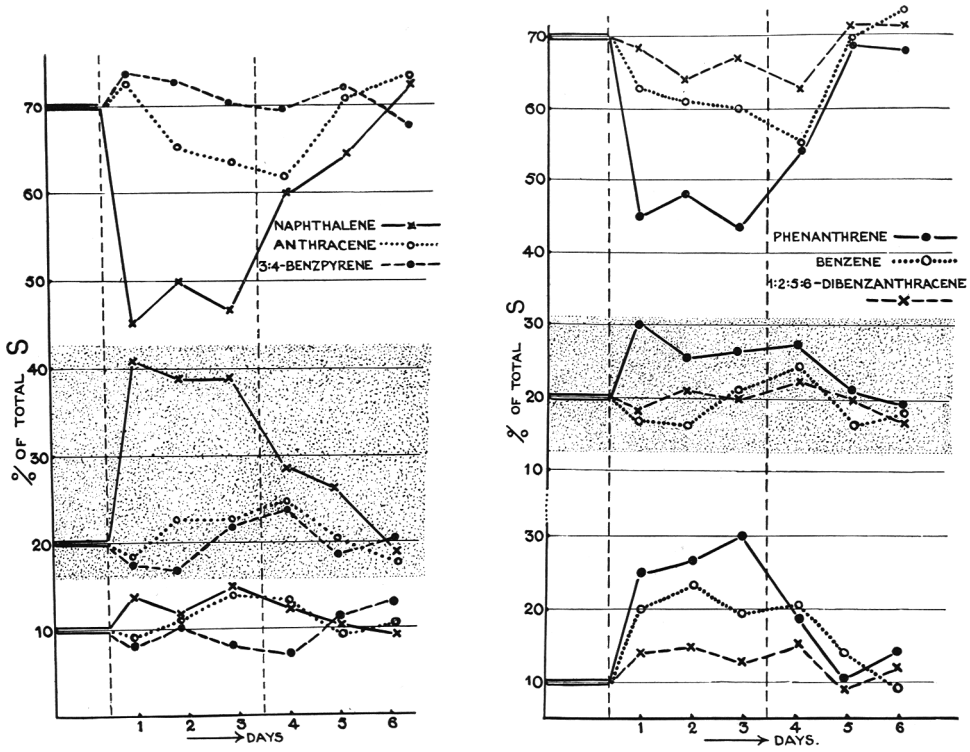

Fig. 1.—Changes in the partition of sulfur in mouse urine produced by the application of aromatic hydrocarbons to the skin.

Curves in upper sections = Inorganic sulfur
" " shaded " = Neutral "
" " lower " = Ethereal "

mgm. GSH; the GSH concentration was obtained by difference.

RESULTS

	Effect on GSH level
Benzene	Little detectable change
Naphthalene	Well marked fall in GSH level during first hour after applications ended; normal value re-attained after 4 hours.
Anthracene	*Slighter* falls found during same period
Phenanthrene	As with naphthalene
1,2,5,6-Dibenzanthracene	No change detected
3,4-Benzpyrene	No change detected

A selection of results obtained with naphthalene and phenanthrene are included in Table III to indicate the quantitative features of the measurements.

There is an exact parallelism between these results and those obtained for urinary sulfur partition. They indicate that the known mercapturate formers —naphthalene, anthracene, and phenanthrene—can be detoxicated in the skin. Again, the different behavior of the 2 carcinogens is noteworthy. No indication is given that sulfur is concerned in their passage through the skin, and once more it is necessary to invoke other possibilities to sustain the view of White (13), that their growth-inhibiting action is related to their removal of sulfur amino acids.

III. The Influence of Mercapturate-Forming Hydrocarbons on the Rate of Induction of Tumors by Carcinogenic Hydrocarbons

Since the aromatic hydrocarbons of lower molecular weight are excreted as mercapturates, and proof has been given that this detoxication process can take place in the skin, these substances should behave like other inhibitors of normal sulfur metabolism (5-8) in delaying or preventing the induction of tumors by carcinogenic hydrocarbons. The experiments described below demonstrate the truth of this prediction.

Both benzpyrene and dibenzanthracene were used for test purposes, but since the results with either compound were the same in character only those obtained with benzpyrene are illustrated. The carcinogen was applied in the usual way on Mondays and Thursdays, and the noncarcinogen (in ethereal solution containing 2 per cent liquid paraffin) on Tuesdays, Wednesdays, Fridays, and Saturdays. Fig. 2 shows the degree of inhibition of papilloma development in two groups of experiments.

Phenanthrene proved the most potent inhibitor and anthracene the least. The low solubility of anthracene in ether made two applications of a saturated solution necessary to give approximately "1 per cent," and for the same reason it was not used at the "4 per cent" level. This property probably also determines its rate of absorption by the skin and hence its relatively small checking action on benzpyrene.

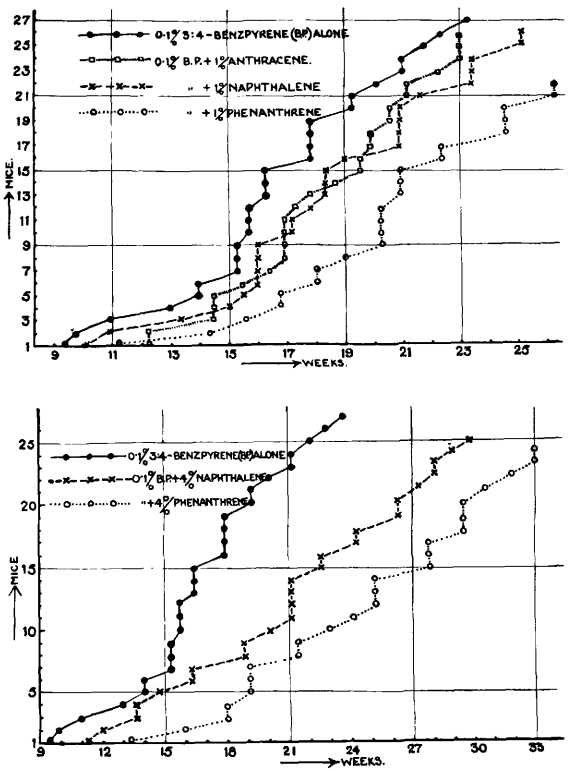

Fig. 2.—Retarding effect of naphthalene, anthracene, and phenanthrene on the rate of tumor induction by 0.1 per cent 3,4-benzpyrene.

(a) 1 per cent naphthalene, anthracene, and phenanthrene.
(b) 4 per cent naphthalene and phenanthrene.

DISCUSSION

The metabolism of sulfur has been associated with three aspects of cancer research: (a) The bearing of sulfur deprivation on the percentage incidence of spontaneous mammary tumors in susceptible strains of mice (15). (b) The growth-retarding influence of carcinogenic hydrocarbons and azo dyes fed to young rats (13, 14). This was linked with the work of Haddow and Robinson (10) on the inhibition of normal and tumor growth induced by many carcinogenic and some noncarcinogenic substances. (c) The checking effect on skin tumor induction produced by substances that specifically lower the local level of sulfur metabolism by a variety of mechanisms (5-8).

In the experiments on spontaneous tumor incidence (15) the dietary sulfur was inadequate for even limited growth, and among the profound physiological changes

TABLE III: CHANGES IN THE GLUTATHIONE CONTENT OF MOUSE SKIN PRODUCED BY EXTERNAL APPLICATIONS OF NAPHTHALENE AND PHENANTHRENE

Control C Treated T	Strain of mice	Treatment	Interval before measurement, hrs.	Glutathione + Ascorbic acid	Ascorbic acid mgm. per 100 gm.	Glutathione	% Fall of glutathione
			PHENANTHRENE				
C	Mixed	—	—	140.2	13.8	92.0	
T		4%	0.5	111.5	12.5	67.9	26
C	"	—	—	143.0	17.3	82.5	
T		6%	0.5	103.0	14.0	54.7	33
C	"	—	—	134.2	12.3	91.2	
T		4%	1.0	112.2	11.4	72.2	21
C	"	—	—	138.2	14.0	89.4	
T		6%	1.0	105.7	12.3	62.8	30
C	"	—	—	140.2	14.3	90.3	
T		"	4.0	135.6	13.9	87.1	4
C	"	—	—	133.4	12.4	90.1	
T		"	4.0	128.2	12.8	83.5	7
C	R 3	—	—	97.5	9.7	63.7	
T		4%	1.0	88.3	9.6	54.8	14
C	"	—	—	102.7	9.7	68.8	
T		6%	1.0	82.3	9.6	48.8	29
C	"	—	—	95.3	10.1	60.1	
T		"	4.0	91.8	10.3	55.8	7
C	Simpson	—	—	110.1	9.8	75.9	
T		4%	1.0	98.3	9.7	64.4	15
C	"	—	—	102.3	10.1	67.0	
T		6%	1.0	81.4	10.3	45.4	32
			NAPHTHALENE				
C	Mixed	—	—	136.2	14.5	85.6	
T		4%	0.5	110.7	12.1	68.5	20
C	"	—	—	140.0	13.9	91.5	
T		6%	0.5	101.7	12.9	56.7	38
C	"	—	—	132.6	14.2	83.0	
T		4%	1.0	104.4	12.8	59.8	28
C	"	—	—	139.8	12.9	94.8	
T		6%	1.0	102.8	11.8	61.6	35
C	"	—	—	144.2	14.8	92.5	
T		4%	4.0	136.5	14.2	87.0	6
C	"	—	—	135.0	15.1	82.3	
T		6%	4.0	125.1	14.6	74.1	10
C	R 3	—	—	95.8	9.9	61.2	
T		4%	1.0	80.9	9.7	47.1	23
C	"	—	—	104.6	10.2	69.0	
T		6%	1.0	78.0	8.9	46.9	32
C	"	—	—	100.1	9.7	66.3	
T		6%	4.0	96.2	9.5	63.0	5
C	Simpson	—	—	108.5	10.6	71.5	
T		4%	1.0	92.5	10.3	56.5	21
C	"	—	—	103.6	9.9	69.0	
T		6%	1.0	84.6	9.6	51.1	26

that ensued endocrine disturbances led to loss of ovarian function (17). This was the direct cause of the failure of spontaneous tumors to arise. Certainly lack of sulfur indirectly caused this sequence of biological abnormalities, but no specific intimacy between sulfur metabolism and tumor incidence is revealed. The similar reduction of the incidence of both spontaneous and induced tumors in mice subjected to other types of dietary deficiency (12, 16) lends support to this view.

The mechanisms underlying the inhibition of growth by carcinogens are little understood. White (13, 14) suggested that growth failure was correlated with the loss of sulfur-amino acids used up in detoxication processes, since growth was resumed when supplements of cystine and methionine were given. Similar effects on growth were produced when other substances, known to be excreted as mercapturates, were substituted for the carcinogens, and by analogy the latter were therefore assumed to use sulfur for their excretion. No chemical proof of this has been offered and the present work, together with that of Elson, Goulden, and Warren (9), tends to negate this supposition. Carcinogens, administered by injection in rats or by skin painting in mice, have failed to give any indication of sulfur utilization either at the site of application or in the end products of their metabolism. By contrast the mercapturate-forming hydrocarbons naphthalene, anthracene, and phenanthrene caused significant changes in urinary sulfur partition and skin-glutathione content when applied to mice in the same way and in similar doses. To sustain the view of White (13, 14) it would be necessary to assume that any affect of these carcinogens on sulfur metabolism proceeds at too slow a rate for detection by the methods used, or that sulfur fixation is manifested in other unknown ways.

All the work on the retardation of tumor induction by inhibitors of sulfur metabolism indicates a direct association between sulfur-containing cell constituents and carcinogens during the primary phases of carcinogenesis. This is fortified by the demonstration, recorded here, that mercapturate-forming hydrocarbons oppose the biological action of the carcinogenic hydrocarbons. Direct evidence for this hypothetical process is not yet available. The metabolic transformations of carcinogenic hydrocarbons so far recorded are all of an oxidative character, and seem to be concerned with processes of elimination, though intermediate phases of these detoxication reactions may well be the true initiators of carcinogenic action. Sulfur conceivably plays a role in the fixation of any such potent derivatives within the cells as a preliminary to the biological changes that culminate in malignant transformation.

SUMMARY

1. Estimations of the partition of sulfur in the urine of mice receiving skin applications of benzene, naphthalene, anthracene, phenanthrene, 3,4-benzpyrene, or 1,2,5,6-dibenzanthracene have been made. The non-carcinogenic hydrocarbons produced such variations of sulfur distribution as were anticipated on the basis of previous work. By contrast the two carcinogens, administered in like amounts, caused no significant changes. In particular the value for neutral sulfur was not altered, indicating that mercapturate formation plays no part in their detoxication.

2. The influence of the same aromatic hydrocarbons on the glutathione content of mouse skin has been investigated. The falls from normal levels following treatment with naphthalene, anthracene, and phenanthrene show that detoxication involving sulfur occurs in the skin itself. Equal amounts of the two carcinogenic hydrocarbons produced no detectable changes of a similar nature.

3. Naphthalene, anthracene, or phenanthrene, when applied to the skin in conjunction with either carcinogen reduced the rate of tumor induction.

REFERENCES

1. Bourne, M. C., and Young, L. The Metabolism of naphthalene in Rabbits. Biochem. J., **28**:803-808. 1934.
2. Boyland, E., and Levi, A. A. Metabolism of Polycyclic Compounds. 1. Production of Dihydroxydihydroanthracene from Anthracene. Biochem. J., **29**:2679-2683. 1935.
3. Boyland, E., and Levi, A. A. Metabolism of Polycyclic Compounds. 2. Production of Dihydroxydihydroanthraceneglycuronic acid from Anthracene. Biochem. J., **30**: 728-731. 1936.
4. Boyland, E., and Levi, A. A. Metabolism of Polycyclic Compounds. 3. Anthrylmercapturic Acid. Biochem. J., **30**:1225-1227. 1936.
5. Crabtree, H. G. Retardation of the Rate of Tumour Induction by Substances Which Inhibit Glycolysis. J. Path. & Bact., **51**:303-309. 1940.
6. Crabtree, H. G. Retardation of the Rate of Tumor Induction by Hydrolyzing Chlor-Compounds. Cancer Research, **1**:39-43. 1941.
7. Crabtree, H. G. Influence of Bromobenzene on the Induction of Skin Tumors by 3,4-Benzpyrene. Cancer Research, **4**:688-693. 1944.
8. Crabtree, H. G. Influence of Unsaturated Dibasic Acids on the Induction of Skin Tumors by Chemical Carcinogens. Cancer Research, **5**:346-351. 1945.
9. Elson, L. A., Goulden, F., and Warren, F. L. The Urinary Partition of Sulphur in Rats Treated with Aromatic Hydrocarbons, with Special Reference to Growth Retardation. Biochem. J., **39**:301-307. 1945.
10. Haddow, A., and Robinson, A. M. Influence of Various Polycyclic Hydrocarbons on the Growth Rate of Transplantable Tumours. Proc. Roy. Soc., s.B, **122**:442-476. 1937.

11. Stekol, J. A. Studies on the Mercapturic Acid Synthesis in Animals. V. The Effect of Naphthalene on the Growth of Rats as Related to Diets of Varying Sulfur Content. J. Biol. Chem., **121**:87-91. 1937.
12. Tannenbaum, A. The Genesis and Growth of Tumors. II. Effects of Caloric Restriction per se. Cancer Research, **2**: 460-467. 1942.
13. White, J., and White, A. Inhibition of Growth of the Rat by Oral Administration of Methylcholanthrene, Benzpyrene, or Pyrene, and the Effects of Various Dietary Supplements. J. Biol. Chem., **131**:149-161. 1939.
14. White, J. Retardation of Growth of the Rat Ingesting *p*-Dimethylaminoazobenzene (Butter Yellow). I. The Effect of Various Dietary Supplements. J. Nat. Cancer Inst., **1**:337-341. 1940.
15. White, J., and Andervont, H. B. Effect of a Diet Relatively Low in Cystine on the Production of Spontaneous Mammary-Gland Tumors in Strain C3H Female Mice. J. Nat. Cancer Inst., **3**:449-451. 1943.
16. White, Florence R., and White, J. Effect of a Low Lysine Diet on Mammary-Tumor Formation in Strain C3H Mice. J. Nat. Cancer Inst., **5**:41-42. 1944.
17. White, Florence R., and White, J. Effect of Diethylstilbestrol on Mammary Tumor Formation in Strain C3H Mice Fed on a Low Cystine Diet. J. Nat. Cancer Inst., **4**:413-415. 1944.
18. Zbarsky, S. H., and Young, L. Mercapturic Acids. III. The Conversion of Benzene to Phenylmercapturic Acid in the Rat. J. Biol. Chem., **151**:487-492. 1943.