The Effect of Carcinogens on the Hepatic Vitamin A Stores of Mice and Rats

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This study of vitamin A depletion occurring with experimentally induced visceral carcinogenesis was undertaken because it was not feasible to estimate accurately the vitamin A content of epidermis in the systematic study of epidermal methylcholanthrene carcinogenesis in mice, which is being carried out in the Barnard Hospital.

Goerner (8) was the first to show that repeated intraperitoneal injections of 1,2,5,6-dibenzanthracene markedly reduced the vitamin A content and increased the total lipid of hepatic mitochondria of rabbits. Enlargement of the liver and loss of body weight accompanied these changes. Goerner and Goerner (9) also demonstrated that the mitochondria of a transplanted rat carcinoma and sarcoma were lacking in vitamin A, although the hepatic stores of the host were normal. Further experiments of these workers (10) showed that 2-amino-5-azotoluene caused a lowering of the hepatic vitamin A when fed to rats. Cells of liver tumors induced by this azo compound were devoid of vitamin A, but surrounding normal hepatic cells still contained the vitamin. While dibenzanthracene injected intraperitoneally did not affect the liver stores of vitamins C or D, the inhibition of growth in young rats was paralleled by a decrease in hepatic vitamin A (11).

Baumann, Foster, and Lavik (1) placed rats with moderate stores of vitamin A on diets low in this factor and injected separate groups of the animals with colloidal suspensions of 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, benzanthracene, and methylcholanthrene. All of these hydrocarbons caused partial depletion of vitamin A from the liver, but dibenzanthracene proved much more effective than the others. These investigators found no correlation between the carcinogenicity of the compounds and the effect upon vitamin A. For example, butter yellow, a carcinogenic compound, and carbon black, a non-carcinogenic substance, were equally without effect upon the vitamin store. Livers of rats bearing methylcholanthrene-induced subcutaneous tumors contained more vitamin A than non-tumorous rats treated with dibenzanthracene.

Since in these earlier studies attention was concentrated on the depletive action of cancer-producing hydrocarbons on hepatic vitamin A content, without reference to their carcinogenic action, it seemed important to us to include the latter effect in this correlation. Methylcholanthrene was therefore injected intraperitoneally at regular intervals over a long period of time to determine whether a relationship exists between the decrease in hepatic vitamin A and the appearance of tumors in the liver and other viscera of mice.

MATERIALS AND METHODS

All vitamin A determinations were made with an improved microphotoelectric colorimeter which will be described in detail elsewhere (3). Briefly, the density of the 620 mμ. band of the Carr-Price reaction was measured as follows: Red light of *constant* intensity was allowed to pass through the blue solution and then to a recording photoelectric cell for exactly 1½ minutes. The current generated by the latter was stored in a condenser and measured by deflection of a ballistic galvanometer. Fig. 1 shows the calibration curve when a vitamin A concentrate [1] of known potency was used

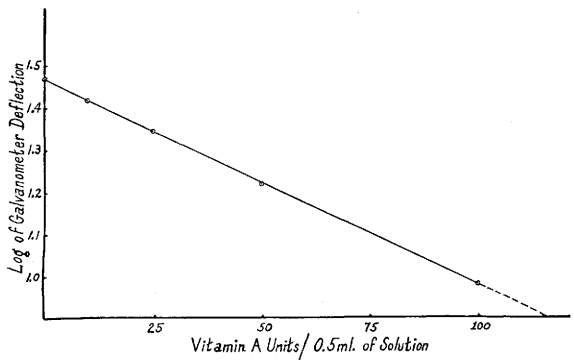

FIG. 1.—Calibration curve for vitamin A.

for reference. The logarithms of the galvanometer deflections are plotted against the U.S.P. units of vitamin A. The units of vitamin A in an unknown could be easily calculated by determining the logarithm of the galvanometer deflection, after which the U.S.P. units of vitamin A could be read directly from the calibration curve.

The entire liver was excised from the animal immediately after killing by a sharp blow on the head. Excess moisture was removed from it by blotting with filter paper, and the tissue then weighed to the nearest

[1] The author is indebted to Dr. Samuel M. Gordon, Endo Products Inc., 395 Fourth Avenue, New York, and to the American Research Products, Minneapolis, Minn., for the supply of the distilled vitamin A concentrate.

milligram. Saponification and extraction with purified ether was carried out according to the procedure of Davies (5). The unsaponifiable fraction was dissolved in chloroform (reagent quality) and diluted to exactly 5 ml. for determination of the vitamin A. Usually 0.5 ml. of this solution sufficed for analysis. To this was added an equal volume of a saturated solution of antimony trichloride in chloroform to develop the color.

The chloroform extracts of the unsaponifiable fractions of mouse livers practically always gave clear colorless solutions. Occasionally, when a liver had an excessive amount of vitamin A, the chloroform solution was tinged with yellow. In such cases the unsaponifiable fraction had to be diluted to 20 to 25 ml. to give 10 to 90 U.S.P. units of vitamin A in 0.5 ml. of solution. Spectroscopically, the Carr-Price reaction for the unsaponifiable fractions of mouse livers showed only one visible absorption band, that at 620 to 625 mμ.

Exploratory experiments revealed that the intraperitoneal injection of methylcholanthrene resulted in ascites, the severity depending upon the amount of this carcinogen administered. These preliminary experiments indicated that in order to reduce mortality from ascites the total amount of this carcinogen administered in small doses for a long period of time should not exceed one mgm. As a convenient concentration, methylcholanthrene (Edcan Laboratories) was dissolved in the fraction of lard melting at or below 37° C. (2) so that 0.05 ml. contained 0.025 mgm. of the hydrocarbon. As a control, the noncarcinogenic hydrocarbon, phenanthrene (Eastman), was used at the same concentration.

Swiss mice, 3 to 4 months old of both sexes, were employed. One hundred and thirty-two mice were divided into 4 groups as follows: 42 for methylcholanthrene (MC); 30 for phenanthrene (PH); 30 for lard controls (L); and 30 for untreated controls (C). Each mouse of the MC group received 0.025 mgm. methylcholanthrene twice weekly; each of the PH group, 0.025 mgm. phenanthrene twice weekly; each of the lard controls, 0.05 cc. of this substance alone twice weekly. Injections were usually made 4 days apart. When malignant tumors appeared, portions were removed for histological study.

Mice were fed Rockland Mouse Pellets and water ad libitum.

RESULTS WITH MICE, GROUP I

The results are shown in Fig. 2. For analysis, 4 mice from the methylcholanthrene-treated group and 3 from each of the other groups were killed at the times indicated. Livers were removed and the vitamin A contents determined separately on each liver and averaged to the figures given. At and after 91 days, 3 mice from each group were killed for analysis. The total dosage of the hydrocarbons, at the time the analysis was made, is indicated on the graph. It can be seen that there was no significant change in the vitamin A content per gm. of liver in the 4 groups.

The vitamin A stores of the untreated controls varied from 140 to 295 U.S.P. units of vitamin A per gm. of liver. With one exception, that of the phenanthrene-treated group at 110 days, the vitamin A content per gm. of liver for the 4 groups was between 135 and 320 U.S.P. units of vitamin A per gm. of liver.

The first ascites occurred at 38 days even with the small dose (0.30 mgm.) of methylcholanthrene. The first visceral tumors appeared at 68 days, and at 124 days 10 mice had developed malignant growths. Nevertheless, the vitamin A stores were unchanged. Even the occurrence of extensive ascites, together with tumors that bound the intestinal tract together, did not appreciably affect the content of vitamin A in the livers.

Table I-a shows the occurrence of ascites and of visceral tumors in mice in which analyses were made, and Table I-b gives the same data for mice which died before analysis could be performed. Fortunately, the dose of methylcholanthrene administered was small enough to allow the experiment to extend beyond 100 days. From 108 to 124 days the remaining mice had either tumors or ascites. Not a single case of ascites was observed in the phenanthrene- or lard-treated groups. Nor were there any significant sex differences to the response of the mice to methylcholanthrene. Liver tumors were not observed in any of the methylcholanthrene-treated mice.

Therefore, under the conditions of this experiment, methylcholanthrene did not appreciably affect the vitamin A content of the livers of mice. Tumors were produced in other organs but not in the liver with a minimum carcinogenic dose of around 0.5 mgm. This is in agreement with the work of Shear, Stewart, and Seligman (18) who found that the direct implantation of 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, and 20-methylcholanthrene into the livers of 53 mice resulted in only 4 hepatomas and 1 adenocarcinoma.

HISTOPATHOLOGY OF TUMORS INDUCED BY METHYLCHOLANTHRENE AND FACTORS INVOLVED IN ASCITES PRODUCTION [2]

Thirteen malignant tumors were produced by the intraperitoneal injection of methylcholanthrene. All of these tumors were spindle cell sarcomas, the origin of which was difficult to determine. Most of them were rich in mitotic figures, indicating rapid growth. Invasion of musculature and of adipose tissue gave

[2] The author wishes to express his gratitude to Dr. Zola K. Cooper for histological classification of these tumors.

evidence of malignancy. The tumors also had occasional giant cells and large cells with irregularly shaped nuclei. Tumors appeared in 4 animals in the neighborhood of the kidneys, one of which was invaded. In 3 other mice, there were malignant growths invading respectively the pancreas, the small intestine, and the prostate. Histological examination of the liver did not reveal any abnormal growths, either primary, metastatic, or invasive.

One might note with interest the differences in the production of ascites by the intraperitoneal injection of these hydrocarbons. Methylcholanthrene has a decided tendency to induce ascites even when small amounts are administered over a rather short period of time. Benzpyrene has the same property, but experiments with dibenzanthracene, although limited, indicate that this hydrocarbon has the characteristic to but a slight degree, if at all. Phenanthrene was not

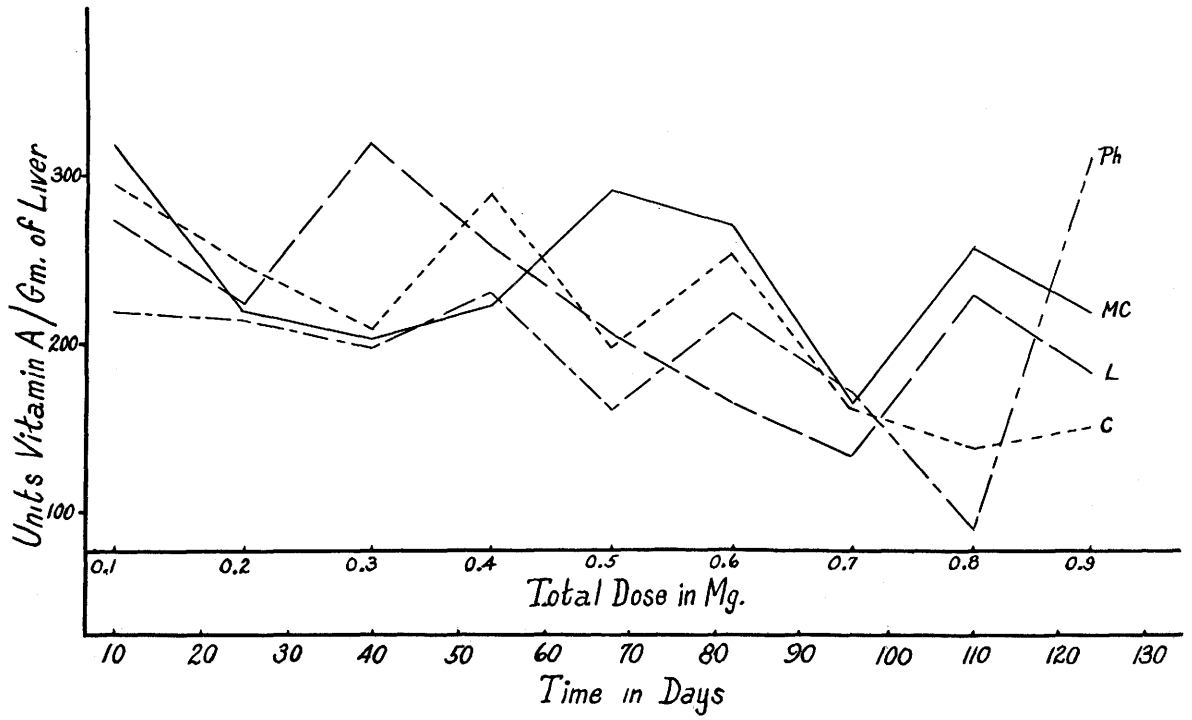

FIG. 2.—The influence of lard solutions of hydrocarbons and of the solvent upon hepatic vitamin A content of mice. PH = phenanthrene; MC = methylcholanthrene; L = lard; C = untreated controls.

TABLE I: OCCURRENCE OF ASCITES AND TUMORS IN MICE INJECTED INTRAPERITONEALLY WITH METHYLCHOLANTHRENE *

Number of mice	Male	Female	Dose of MC, mgm.	Number of tumors	Occurrence of ascites and tumors (days)	Degree of ascites			
						+++	++	+	o
			A. HEPATIC VITAMIN A DETERMINED						
4	4	0	0.3	0	38	0	0	1	3
4	4	0	0.4	0	52	2	0	1	1
3	0	3	0.5	1	66	1	0	0	2
4	4	0	0.6	2	80	1	0	2	1
3	2	1	0.7	2	94	2	0	1	1
3	1	2	0.8	3	108	0	1	1	1
2	1	1	0.85	2	124	1	0	0	1
			B. HEPATIC VITAMIN A NOT DETERMINED						
2	0	2	0.7	2	72- 96	2	0	0	0
3	2	1	0.58-0.65	0	79-107	1	1	0	1
2	2	0	0.5	0	87- 96	0	1	1	0
4	1	3	0.85	1	108-123	0	0	1	3

* Letters and symbols indicate the following: MC = methylcholanthrene; +++ = extensive; ++ = moderate; + = slight; o = none.

found to have induced a single case of ascites. Lorenz and Stewart (15), on the other hand, reported that dibenzanthracene after oral administration could produce ascites in mice and a low grade inflammatory lesion of the small intestines more frequently and more severely than did methylcholanthrene.

In another experiment the carcinogenic action of methylcholanthrene choleic acid, phenanthrene choleic acid, and desoxycholic acid was investigated. The complexes of the hydrocarbons were made according to the method of Fieser and Seligman (7). They were dissolved by triturating 100 mgm. of each with 1 ml. of 0.2 N sodium hydroxide and solution maintained by adding 100 ml. of phosphate buffer of pH 8. One mgm. injections (subcutaneously) were given mice of strain C57 black twice weekly for 14 weeks. At the end of this time one group had received 28 mgm. of methylcholanthrene choleic acid (equivalent to 5.2 mgm. methylcholanthrene); another group had received 29 mgm. phenanthrene choleic acid (equivalent to 3.6 mgm. phenanthrene); and a final group had received 30 mgm. of desoxycholic acid. However, no ascites was observed, and although methylcholanthrene choleic acid induced subcutaneous sarcomas at the site of injection, liver tumors were not found. Phenanthrene choleic acid and desoxycholic acid did not produce any tumors. The lowering of the pH of these choleic acids when injected under the skin of the mice probably caused precipitation of some of the choleic acids. It would seem that the liver has the property of removing cancer-producing hydrocarbons either by detoxification or by metabolizing them so rapidly that an abnormal growth response is not elicited. The results suggest that irritating action of the hydrocarbons when injected intraperitoneally might play a part in the production of ascites.

RESULTS WITH RATS

In attempts to lower the vitamin A stores of young rats, each of 3 groups of 10 animals was given one of the following: methylcholanthrene (MC); phenanthrene (PH); 3,4-benzpyrene (BzP). Doses of 3 mgm. in 0.5 ml. lard were injected intraperitoneally twice weekly for 2 weeks, and then once weekly for one week. On the 4th and final week the remaining rats (which had not yet been sacrificed for analysis) received an additional mgm. of hydrocarbon, or a total of 16 mgm. At 14, 28, 41, and 55 days, 2 rats from each group were killed, the livers removed, and the vitamin A content determined as mentioned above. Ten untreated animals served as controls. Rats of both sexes (96 to 118 gm.) were employed. The diet was Purina Dog Chow with unlimited water. Body weights were recorded every 5 days. The results of this experiment are shown in Table II.

TABLE II: INFLUENCE OF HYDROCARBONS ON THE GROWTH AND HEPATIC VITAMIN A STORAGE IN RATS *

Hydrocarbon	Mgm. injected	Time in days: Of treatment	Time in days: After last injection	U.S.P. units of vitamin A per gm. liver	Per cent hepatic vitamin A †	Per cent of normal growth	Rats with ascites: Males	Rats with ascites: Females
C	0	14	..	68-238 (153)	100	100	..	..
PH	9	14	4	60-178 (119)	78	92	0	0
BzP	9	14	4	64 (64)	52	77	0	1+
MC	9	14	4	13- 89 (51)	33	80	1+	0
C	0	28	..	153-247 (200)	100	100	..	..
PH	16	28	1	136-196 (166)	83	86	0	0
BzP	16	28	1	60-119 (89)	48	72	1+	0
MC	16	28	1	90-102 (96)	45	70	0	1+
C	0	41	..	135-160 (147)	100	100	..	..
PH	16	41	14	87-148 (117)	79	87	0	0
BzP	16	41	14	24- 56 (40)	27	64	1+++	0
MC	16	41	14	0- 16 (8)	5.4	68	1+++	2+
C	0	55	..	106-249 (177)	100	100	..	..
PH	16	55	28	150-213 (182)	102	89	0	0
BzP	16	55	28	123-137 (130)	73	64	0	0
MC	16	55	28	73-111 (92)	46	74	0	0

* Average figures are indicated in parentheses. Letters indicate the following: C=control; PH = phenanthrene; BzP = 3,4-benzpyrene; MC = methylcholanthrene; + = trace; +++ = extensive.
† Indicates per cent of normal where normal is considered 100 per cent.

Because of the wide variation of the hepatic vitamin A content of the normal rat (68 to 249 U.S.P. units per gm. of liver), a depletive effect of the hydrocarbons would have to be extreme to be significant. At 14 and 28 days, after each rat had received 9 and 16 mgm. of each hydrocarbon, respectively, the vitamin A stores of the benzpyrene- and methylcholanthrene-treated groups were nearly half of the normal, while in the phenanthrene-treated group they were about 78 per cent of that of these untreated animals. At 41 days, 14 days after the last injection, the stores of the methylcholanthrene-treated group were almost

exhausted, those of the benzpyrene-treated group being 27 per cent of the controls, while those of the phenanthrene-treated group were still nearly 80 per cent of that of the control rats. At 55 days, 28 days after the last injection, the vitamin A contents were approaching normal, although the methylcholanthrene-treated group was still below 50 per cent. However, the inhibitory effect of methylcholanthrene and benzpyrene on the growth rate persisted.

The unsaponifiable fraction of the rat livers, which was diluted to 20 to 25 ml. for analysis, was always light yellow in color unless vitamin A content was low or absent. However, after 30 seconds (when the determinations were made), no interference due to other carotenoids in the rat livers could be detected by spectroscopic examination of the Carr-Price reaction for vitamin A.

Goerner found that the retardation of growth induced in the rat by 1,2,5,6-dibenzanthracene was paralleled by the decrease in the vitamin A content of the liver. Such was not the case with the hydrocarbons used in this study, although there was some correlation in the phenanthrene-treated group. On the contrary, the rate of depletion of the vitamin A stores in the benzpyrene- and methylcholanthrene-treated groups was greater than the inhibition of growth which was nearly the same for these hydrocarbons. Five rats which received methylcholanthrene and 3 which received benzpyrene developed ascites. Other investigators, Haddow *et al.* (13), Lee (14), and White and White (19) have demonstrated an inhibitory effect of hydrocarbons on the growth of the rat.

RESULTS WITH MICE, GROUP 2

Since it was found that methylcholanthrene and phenanthrene had no appreciable effect upon the vitamin A stores of the mouse (Fig. 2) in the doses given, larger amounts of phenanthrene (PH), 3,4-benzpyrene (BzP), methylcholanthrene (MC), and 1,2,5,6-dibenzanthracene (DiBz) were injected in an attempt to affect the vitamin A stores. Thirty male Swiss mice, 3 to 4 months old, were divided into 5 groups of 6 each. One group served as untreated controls, while each mouse of the other 4 groups received bi-weekly injections of 0.3 mgm. of one of the above-named hydrocarbons dissolved in lard for 7 weeks, and 2 injections of 0.6 mgm. during the 8th week. Each mouse also received 1 drop of Mead's Oleum Percomorphum (containing about 1,400 U.S.P. units of vitamin A per drop) 3 times weekly. It was hoped that by maintaining a large hepatic reservoir of vitamin A, some depletive action of the hydrocarbons could be demonstrated. Vitamin A analysis was carried out as before.

Because of the wide variation in the stores of vitamin A, it is rather difficult to draw any definite conclusions (Table III). At 41 days, when the remaining mice of each group had received a total of 4.5 mgm. of hydrocarbon (1.2 mgm. during the last week), the liver stores of vitamin A were large, but the variation again was rather great. Goerner (8) has already shown that dibenzanthracene in some way prevents the storage of vitamin A in the liver of rabbits, even when accompanied by an excess of carotene or vitamin A administered parenterally. Apparently this does not hold for mice with the hydrocarbons we used and when massive doses of vitamin A are administered orally. Although the mice received large doses of the hydrocarbons, ascites occurred only in the benzpyrene- and methylcholanthrene-treated mice, and this was slight.

TABLE III: INFLUENCE OF LARGE DOSES OF HYDROCARBONS ON THE HEPATIC VITAMIN A CONTENT OF MICE WHEN LARGE DOSES OF VITAMIN A ARE ADMINISTERED ORALLY *

Number of mice	Time of treatment, days	Dose of hydrocarbon, mgm.	Time after last injection, days	U.S.P. units of vitamin A per gm. of liver					Occurrence of ascites	
				C	PH	DiBz	BzP	MC	Number of BzP	Number of MC
2	9	0.9	3	310	203	165	210	505	0	0
2	23	2.1	3	392	221	243	450	293	0	1++; 1+
2	41	4.5	1	992	1704	1392	1841	1066	2+	2+

* Letters indicate the following: C = control; PH = phenanthrene; DiBz = 1,2,5,6-dibenzanthracene; BzP = 3,4-benzpyrene; MC = methylcholanthrene; + = trace; ++ = moderate.

Further investigation will have to be undertaken to explain the differences in response of the mouse and rat to these hydrocarbons with respect to their effect on the hepatic vitamin A stores. A species difference in the metabolism of cancer-producing hydrocarbons, as suggested by the work of Dobriner, Rhoads, and Lavin (6), or a species variation in the chemical configuration of the vitamin A may account for the difference.

In the last experiment the total amount of the hydrocarbons given mice was comparable to that given to the rats on a body weight basis. It has already been noted that the chloroform extract of the unsaponifiable fraction of the liver of mice was invariably colorless unless very rich in vitamin A. On the other hand, colorless solutions from the unsaponifiable fraction of rat livers (which were usually of a light yellow color) meant little or no vitamin A. It does not seem possible that this color difference can be due to wide variation

in vitamin A content since the unsaponifiable fraction of rat livers of average weight of 8 to 10 gm. was diluted to 20 to 25 ml., whereas the same fraction from a mouse liver of average weight 1.5 gm. was diluted to 5 ml. Furthermore the U.S.P. units of vitamin A per gm. of normal mouse liver for 30 normal mice averaged 224 (extremes 140 to 290), while for 10 normal rats the average was 169 (extremes 68 to 249) per gm. of liver. It might also be noted that these hydrocarbons induced not only an increased storage of hepatic vitamin A, but also did not interfere with their assimilation when large doses of vitamin A were administered orally to mice (Table III). The opposite is true for the rat and rabbit according to Baumann (1) and Goerner (8), respectively.

The importance of using more than one species of animals for cancer research is emphasized by an experiment of this type. Whether or not the process of carcinogenesis by hydrocarbons *in situ* differs in various species remains to be investigated.

On the other hand, the work of Claude (4), Polson (16) on rabbits, of Schabad (17) on guinea pigs, and of Goerner and Goerner (12) on rats showed that pronounced liver damage may result from treatment with methylcholanthrene and dibenzanthracene. Apparently this is not the case with mice, since the livers did not show pronounced degenerative changes either in our studies or in those of Lorenz and Stewart (15).

SUMMARY AND CONCLUSIONS

1. Repeated biweekly intraperitoneal injections of mice with lard solutions of methylcholanthrene and phenanthrene for 18 weeks did not affect the hepatic stores of vitamin A. Methylcholanthrene induced rapidly growing sarcomas and the production of ascites. Tumors of the liver were not observed. Neither phenanthrene nor lard produced tumors or ascites.

2. The influence of large doses of intraperitoneally administered phenanthrene, 3,4-benzpyrene, and methylcholanthrene on both hepatic vitamin A storage and growth in young rats was also studied. Methylcholanthrene produced a marked reduction in hepatic vitamin A, and 3,4-benzpyrene had a comparable but somewhat less pronounced effect. Phenanthrene exhibited the least potency, only reducing the hepatic stores about 20 per cent of the normal. With methylcholanthrene and benzpyrene, the depletion of hepatic vitamin A was much greater than the inhibition of growth. In the case of phenanthrene, there was some correlation between the decrease in hepatic vitamin A and retardation of growth. Methylcholanthrene and 3,4-benzpyrene produced ascites in some of the rats.

3. The intraperitoneal administration of large amounts of phenanthrene, 3,4-benzpyrene, methylcholanthrene, and 1,2,5,6-dibenzanthracene in the presence of massive doses of vitamin A administered orally neither appreciably affected the vitamin A stores of the liver of mice nor interfered with the assimilation of the vitamin. Ascites induced by methylcholanthrene was less pronounced in the presence of added vitamin A. Although 3,4-benzpyrene produced ascites in several mice, this reaction did not occur in mice treated with dibenzanthracene or phenanthrene.

4. Possible explanations for the differences in response of the mouse and rat to these hydrocarbons are discussed. The use of more than one species of animal, at least for certain phases of experimental cancer research, would seem to be indicated.

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