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Comparative Biologic Activities of 7,12-Dimethylbenz(a)anthracene, 7-Hydroxymethyl-12-methylbenz(a)anthracene, 7,12-Dihydroxymethylbenz(a)anthracene, and 4-Methoxy-7,12-dimethylbenz(a)anthracene in the Sprague-Dawley Female Rat[1]

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SUMMARY

7,12-Dimethylbenz(a)anthracene (DMBA) and the 7-hydroxy-, 7,12-dihydroxy-, and 4-methoxy- derivatives were studied in parallel to determine their effects on the hemopoietic system, adrenal cortex, liver enzymic activity, and body weight in Sprague-Dawley female rats. The monohydroxy compound caused more extensive damage to hemopoietic and adrenocortical tissue than DMBA, whereas the 7,12-dihydroxy- and 4-methoxy- derivatives were inactive. Liver menadione reductase and *N*-2-fluorenylacetamide hydroxylase activities were stimulated by DMBA and 4-methoxy-DMBA, but not significantly by the hydroxymethyl compounds. Mean body weight was not influenced by 4-methoxy-DMBA or 7,12-dihydroxy-DMBA. DMBA induced a temporary loss of body weight, whereas 7-hydroxy-DMBA caused a significant and prolonged loss. These observations strongly support the conclusion that DMBA-induced hemopoietic and adrenocortical damage is due to its metabolite, 7-hydroxy-DMBA.

INTRODUCTION

The isolation of 7-hydroxymethyl-12-methylbenz(a)anthracene (7-hydroxy-DMBA) and 7,12-dihydroxymethylbenz(a)-anthracene (7,12-dihydroxy-DMBA) from rat liver homogenates incubated with 7,12-dimethylbenz(a)anthracene (DMBA) have been reported from two laboratories (3, 13). Both compounds have been synthesized. Earlier investigations by Miller and Miller and their coworkers (17-19) have clearly demonstrated that the *N*-hydroxylated metabolite of *N*-2-fluorenylacetamide is the proximate carcinogen, but there is no definitive evidence that metabolites of the polynuclear hydrocarbons fit into this category. Both DMBA and 7-hydroxy-DMBA induce cancer in rats (6, 13, 16, 20, 24) and mice (4, 6), but the 7-hydroxy derivative appears to be a less effective carcinogen. The derivative is, on the other hand, a more potent adrenocorticolytic agent (6, 20, 23). Since DMBA induces hemopoietic damage in other species (21) but has no influence on the adrenal cortex (8, 15), the two metabolites together with a synthetic derivative of DMBA, 4-methoxy-DMBA, were studied in parallel to determine their *early* effects. Liver enzymic activity, damage to hemopoietic and adrenocortical tissue, changes in body weight, and mortality were the criteria used for evaluation of biologic activity.

MATERIALS AND METHODS

Chemicals. DMBA, m.p. 122–123°C, was purchased from Eastman Organic Chemicals (Distillation Products Industries, Rochester, New York): 4-methoxy-DMBA, m.p. 121–122°C, and 7-hydroxy-DMBA, m.p. 162–163°C, were synthesized as described by Flesher *et al.* (13); and 7,12-dihydroxy-DMBA, m.p. 222–223°C, was prepared by the method described by Badger and Cook (2). Each of the compounds gave a single blue fluorescent spot on thin-layer chromatograms [glass plates coated with silica-gel G of 0.25 mm thickness were developed with benzene or benzene containing 5% (v/v) ethanol] when examined under ultraviolet light.

Liver Enzyme Activities. Determinations were made in quadruplicate on pooled one-gram liver samples from each experimental group of animals. *N*-2-Fluorenylacetamide hydroxylase activity was assessed by the method of Cramer *et al.* (9), using the equivalent of 25 mg of liver per incubation flask and a 1:1 mixture of redistilled hexane and ether for the extraction of *N*-2-fluorenylacetamide. Menadione reductase activity was measured by the method described by Williams-Ashman and Huggins (26) at 20°C. Protein was estimated by the method of Warburg and Christian (22).

Animals. Sprague-Dawley female rats were purchased from Sprague-Dawley Farms, Inc., Madison, Wisconsin, and acclimatized to the laboratory for one week prior to use at age 50 days. They were fed Purina rat chow *ad libitum* and given tap water to drink throughout the experiment. Chemicals were dissolved in sesame oil, 10 mg/ml, and fed as a single dose via a No. 8 soft rubber catheter on Day 0. Control animals were fed a comparable volume of sesame oil. Blood for microhematocrit, leukocyte, and platelet determinations was obtained from

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the tail vein on Day 0 before feeding the compound, and on Days 5, 9, 14, 19, and 24 after feeding the compound. Hematologic indices were determined by standard methods. Differential blood counts were estimated by counting 500 consecutive oil immersion fields of a Wright-stained blood film. Body weight was determined at 24-hour intervals until sacrifice by cervical fracture. At necropsy, the liver, thymus, spleen, and adrenals were weighed and prepared for microscopic examination.

RESULTS

Hemopoietic Damage. Each experimental group consisted of 8 or 9 rats; the control group, of 6. All experimental groups received 20 mg (approximately 124 mg/kg) of the compound. The data presented in Chart 1 indicate that 7-hydroxy-DMBA causes a more profound thrombocytopenia than DMBA. The values obtained for rats given 4-methoxy-DMBA or sesame oil alone are in agreement with those shown for 7,12-dihydroxy-DMBA. Both DMBA and the 7-hydroxy-derivative induce a comparable degree of granulocytopenia and lymphocytopenia on Days 5 and 9 after treatment (Table 1), but anemia, as measured by the hematocrit, was more severe in the group fed 7-hydroxy-DMBA. In contrast to the group fed DMBA, a relative and absolute granulocytosis had developed in the rats fed 7-hydroxy-DMBA by Day 14 and persisted throughout the observation period of 24 days. This type of response is similar to that described by Elson (11, 12) for rats treated with chlorambucil, an alkylating agent. Changes in the peripheral

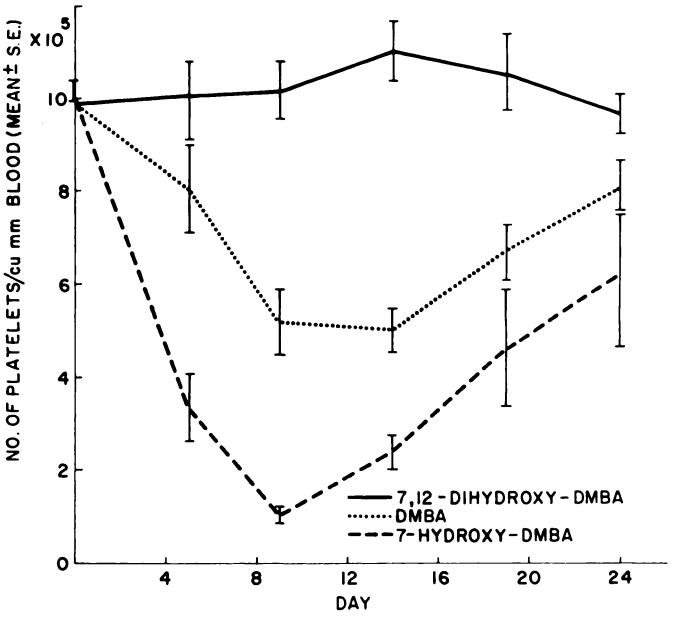

Chart 1. Serial platelet counts (mean ± S.E.) in Sprague-Dawley female rats given a single 20-mg feeding of the benz(a)anthracene derivative in sesame oil by gastric tube on Day 0 (age 50 days). The values obtained for the groups given sesame oil and 4-methoxy-DMBA are in agreement with those shown for 7,12-dihydroxy-DMBA. 7,12-Dihydroxy-DMBA, 7,12-dihydroxymethylbenz(a)anthracene; 7-hydroxy-DMBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; DMBA, 7,12-dimethylbenz(a)anthracene.

Table 1

Day	DMBA	7-Hydroxy-DMBA	7,12-Dihydroxy-DMBA	4-Methoxy-DMBA	Sesame oil
Serial hematocrit %, mean ± S.E.					
0	46.1 ± 0.6	44.8 ± 0.7	44.7 ± 0.5	46.0 ± 0.6	47.5 ± 0.9
5	40.6 ± 2.8	42.2 ± 0.7	44.9 ± 0.9	44.3 ± 0.9	46.7 ± 0.9
9	40.1 ± 1.4	19.9 ± 3.9	44.8 ± 0.9	44.3 ± 0.5	46.8 ± 1.1
14	42.0 ± 2.2	21.2 ± 1.2	46.0 ± 1.0	46.8 ± 0.8	47.8 ± 1.2
19	44.2 ± 0.9	36.8 ± 4.3	44.7 ± 1.0	44.3 ± 0.9	50.2 ± 1.2
24	45.7 ± 1.0	46.0 ± 1.5	44.7 ± 0.8	44.3 ± 0.5	47.8 ± 0.8
Lymphocytes + monocytes ($\times 10^{-3}$), mean ± S.E.					
0	12.91 ± 0.88	14.59 ± 1.30	14.60 ± 1.46	12.06 ± 1.00	14.30 ± 1.38
5	6.87 ± 0.86	7.17 ± 1.25	14.88 ± 1.83	10.02 ± 0.64	16.36 ± 1.35
9	4.58 ± 0.95	6.18 ± 1.04	9.58 ± 1.04	9.19 ± 1.49	13.69 ± 2.76
14	6.52 ± 0.89	8.24 ± 1.04	11.87 ± 1.32	8.74 ± 0.64	11.47 ± 0.48
19	8.85 ± 0.62	12.08 ± 1.17	12.90 ± 1.08	11.77 ± 0.72	12.15 ± 1.71
24	9.79 ± 0.40	11.67 ± 0.69	11.55 ± 1.01	12.46 ± 0.96	11.94 ± 2.35
Granulocytes ($\times 10^{-3}$), mean ± S.E.					
0	1.19 ± 0.14	1.23 ± 0.15	1.45 ± 0.14	1.16 ± 0.10	1.13 ± 0.17
5	0.33 ± 0.07	0.38 ± 0.07	2.24 ± 0.35	1.82 ± 0.31	1.47 ± 0.21
9	0.47 ± 0.09	0.79 ± 0.18	1.95 ± 0.46	1.21 ± 0.14	2.67 ± 0.27
14	1.38 ± 0.12	4.04 ± 0.61	1.75 ± 0.29	1.04 ± 0.07	2.07 ± 0.26
19	0.71 ± 0.11	5.24 ± 2.47	0.68 ± 0.10	0.96 ± 0.16	1.01 ± 0.17
24	0.66 ± 0.11	2.48 ± 0.48	0.82 ± 0.15	0.87 ± 0.15	0.83 ± 0.17

Serial hematocrit, lymphocytic and monocytic, and granulocytic elements per cu mm blood from tail vein of Sprague-Dawley female rats. Blood indices were determined on Day 0 (prior to) and on Days 5, 9, 14, 19, and 24 following administration of a single 20-mg feeding of the benz(a)anthracene derivative dissolved in sesame oil by gastric tube. Values are expressed as the mean ± S.E. DMBA, 7,12-dimethylbenz(a)anthracene; 7-hydroxy-DMBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; 7,12-dihydroxy-DMBA, 7,12-dihydroxymethylbenz(a)-anthracene; 4-methoxy-DMBA, 4-methoxy-7,12-dimethylbenz(a)anthracene.

blood smears after DMBA during the period of marrow depression and recovery have been described in detail elsewhere (7). The changes induced by 7-hydroxy-DMBA were similar; they differed only in the occurrence of a higher percentage of polychromatophilic macrocytes and normoblasts, granulocytes in the ring form, and large bilobed lymphocytes in the latter group. These changes are interpreted as a reflection of intense regenerative activity by precursor cells in the reticuloendothelial system. Tissues from rats that died during the experiment as a consequence of 7-hydroxy-DMBA and from rats killed at 24 days provide further evidence that damage due to this metabolite was most severe. At 40 hours, the marrow was hemorrhagic and contained few cellular elements other than erythrocytes, normoblasts, and degenerated megakaryocytes. Lymphoid follicles in the spleen were small, and megakaryocytes were not observed. The splenic pulp was characterized by a prominent reticular framework and general decrease in all cellular elements. There was an overall decrease in cellular elements in the thymus and focal hemorrhagic areas in the liver. By Day 10, the spleen and marrow were quite cellular and could not be distinguished from that of rats given 133 mg/kg DMBA (7). By Day 24, spleens from rats given 7-hydroxy-DMBA, though highly cellular with an over-abundance of megakaryocytes, were still disorganized with regard to normal architecture. Despite the small number of animals in this series which survived, evidence of extramedullary hemopoiesis was reflected in the mean spleen and liver weights, which were significantly heavier ($P <$ 0.02 and 0.05) than those of control animals (Table 2). Liver architecture was normal. It is noteworthy that the mean number of granulocytes and lymphocytes in the control group fed sesame oil was not constant, but the percentage change is in agreement with earlier observations (7). Analysis of the data by the method of variance showed that only 7-hydroxy-DMBA and DMBA had a significant influence on the hematologic indices.

Body Weight and Mortality. The changes in mean body weight are shown in Chart 2. The mean weights of the groups given 4-methoxy-DMBA and sesame oil are identical to the group given 7,12-dihydroxy-DMBA. Both DMBA and 7-hydroxy-DMBA induced an immediate loss of body weight, which reached its peak on the second day. But the weight curve for the group given the latter compound is distinctly different. It slowly rises during the first week, then falls until

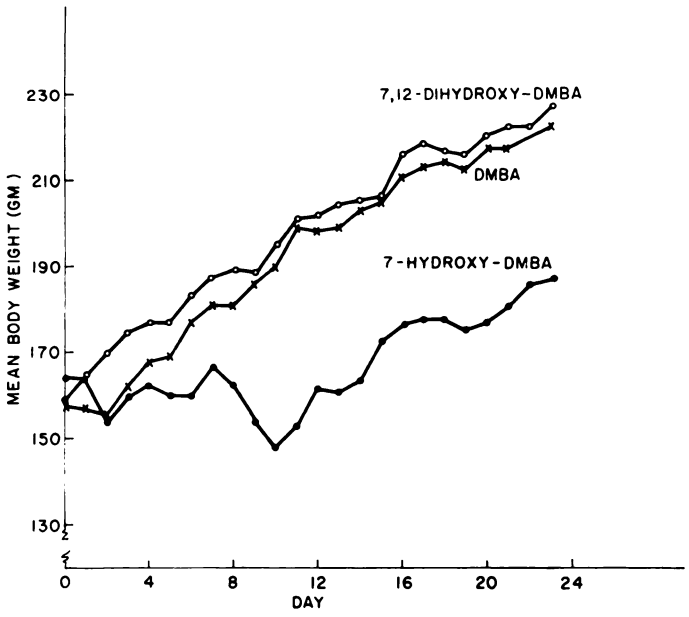

Chart 2. Mean daily body weight of Sprague-Dawley female rats given a 20-mg feeding of the benz(a)anthracene derivative in sesame oil by gastric tube on Day 0. Body weights of the groups given sesame oil or 4-methoxy-DMBA are identical with those shown for 7,12-dihydroxy-DMBA. For abbreviations, see legend to Chart 1.

Table 2

Administered compound	Weight (gm)				
	Body	Liver	Spleen	Thymus	Adrenals
Sesame oil	230	9.53	0.587	0.375	0.068
DMBA	227	10.39	0.581	0.354	0.070
7-Hydroxy-DMBA	199[a]	12.01[a]	0.721[b]	0.316	0.071
7,12-Dihydroxy-DMBA	229	8.82	0.526	0.377	0.068
4-Methoxy-DMBA	228	9.26	0.595	0.376	0.067

Mean body and organ weight of female Sprague-Dawley rats 24 days after a single 20-mg dose of the benz(a)anthracene derivative in 2 ml sesame oil by gastric tube. For abbreviations, see legend to Table 1.

[a]Significantly different from controls, $P \leq 0.05$.

[b]Significantly different from controls, $P \leq 0.02$.

Day 10, and returns to the pretreatment level on Day 14. Diarrhea was first observed on Day 6 and recurred intermittently in all but one rat until Day 18. The blood indices of the animal that escaped this syndrome were least affected by the compound. The weight curve parallels the period of severe thrombocytopenia and more closely resembles that reported for X-irradiation and other alkylating drugs than for chlorambucil (11). These observations are not in agreement with the statement of Wheatley *et al.* (23) that 7-hydroxy-DMBA does *not* influence body weight. These investigators concluded that doses up to 20 mg are less toxic than DMBA. In our experiment, death occurred only in the group given 7-hydroxy-DMBA. After treatment, two rats died between 36 and 40 hours, two on Day 10, and one on Day 23. The cause of the early deaths could not be established with certainty; the late deaths can be attributed to severe anemia, but no gross hemorrhagic diathesis was observed.

Liver Enzymic Activity. Each experimental group consisted of 6 rats. A 1-gram sample of liver was taken from each rat, and the samples were pooled for each experimental group. Menadione reductase and *N*-2-fluorenylacetamide hydroxylase activities were determined on the pooled samples from rats killed 24 hours after administration of the compound. In one experiment (200 mg/kg body weight) and in another, 20 mg per rat (approximately 125 mg/kg) were administered. The larger dose, 200 mg/kg, did not further increase enzymic activity, and the results of the two experiments were in agreement. Table 3 indicates that DMBA and 4-methoxy-DMBA, but not the hydroxymethyl compounds, stimulate microsomal hydroxylase activity. Substitution of a hydroxyl group for hydrogen in the 7- and 12-positions abolishes the stimulatory

Table 3

Administered compound	Menadione reductase[a]	*N*-2-Fluorenylacetamide hydroxylase[b] 30 min	60 min
Sesame oil	76	3.5	5.3
DMBA	278	10.9	19.9
7-Hydroxy-DMBA	187	4.3	8.2
7,12-Dihydroxy-DMBA	119	3.7	5.8
4-Methoxy-DMBA	357	16.1	23.4

Menadione reductase and *N*-2-fluorenylacetamide hydroxylase activities of liver from female rats, age 50 days, 24 hours after feeding a single 20-mg dose of the benz(a)anthracene derivative in 2 ml sesame oil by gastric tube. For abbreviations, see legend to Table 1.

[a] Millimicromoles of oxidized nicotinamide adenine dinucleotide per mg protein per minute at 20°C.

[b] Millimicromoles of *N*-2-fluorenylacetamide per mg protein hydroxylated at 37°C.

effect. Substitution in the 7-position alone is weakly stimulatory. The observations reported by Boyland and Sims (5) indicate that rat liver homogenates metabolize 7-hydroxy-DMBA as efficiently as it metabolizes DMBA. When rats are pretreated with drugs that stimulate liver microsomal activity, the rate of metabolism is increased. After pretreatment with DMBA, the amount of DMBA metabolized to other hydroxylated compounds was approximately twice that of rats pretreated with arachis oil. Observations presented in this report for *N*-2-fluorenylacetamide hydroxylase activity are in general agreement. (When the data presented in Table 3 are calculated on the basis of micrograms of substrate hydroxylated per gram of liver for 30 minutes, the values are 18, 45, 26, 21, and 76 micrograms for sesame oil, DMBA, 7-hydroxy-DMBA, 7,12-dihydroxy-DMBA, and 4-methoxy-DMBA, respectively.) The soluble menadione reductase enzyme is stimulated to some extent by all compounds with the 4-methoxy- derivative being the most active. The values for menadione reductase activity after feeding DMBA and 7-hydroxy-DMBA are in agreement with those reported by Boyland *et al.* (6).

Adrenal Damage. There was no significant difference in the weight of the paired adrenal glands from the five groups 24 days after treatment. No microscopic damage was observed in the adrenals from rats given 7,12-dihydroxy-DMBA or 4-methoxy-DMBA. All animals given DMBA or 7-hydroxy-DMBA had calcified adrenals, but the calcification was more extensive in the groups fed the monohydroxy compound. From these observations we assume that adrenocortical hemorrhage had occurred earlier. The adrenals from the two animals that died at 40 hours were grossly hemorrhagic. On microscopic examination only the medullary cells and a thin connective tissue capsule could be recognized; there were no morphologically identifiable adrenocortical cells. The mean weights of the adrenal glands from the five groups of rats fed 20 mg of the compound under test and killed 24 hours later for determination of liver enzyme activity were in agreement. Only the glands from the group fed 7-hydroxy-DMBA had microscopic evidence of injury. In addition to focal necrosis of the parenchyma of the zonae *reticularis* and *fasciculata*, the capillaries were widely dilated and filled with erythrocytes. These observations suggest that the earliest morphologic lesion is vascular hyperemia.

To further evaluate the potency of the two compounds, three groups of rats were fed DMBA or 7-hydroxy-DMBA at doses of 25, 50, and 100 mg/kg body weight and killed 72 hours later. The highest dose inhibited normal weight gain. The mean weight gain for the group fed sesame oil was 12 gm, and 2 gm for the groups given the hydrocarbon. This dose caused a weight reduction in both the thymus and spleen. Thymus weights (mean ± S.E.) were 344 ± 15, 306 ± 30, and 269 ± 21 mg, and spleen weights (mean ± S.E.) were 571 ± 41, 462 ± 4, and 376 ± 20 mg for the groups fed sesame oil, DMBA, and 7-hydroxy-DMBA, respectively. Thymus weights were significantly lower than the controls ($P \leq 0.02$) for the group fed 7-hydroxy-DMBA. Spleen weights, on the other hand, were significantly different from the controls for both groups ($P \leq 0.05$ for DMBA and 0.01 for 7-hydroxy-DMBA). The mean spleen, but not thymus, weight was significantly lower ($P \leq 0.01$) in the rats given 7-hydroxy-DMBA when compared with those given DMBA. As shown in Table 4, 7-hydroxy-DMBA is a more powerful inducer of adrenocortical damage, which has been described in detail by Huggins and Morii (14) for DMBA. With smaller doses, the damage is less severe in that more adrenocortical tissue is normal when hematoxylin and eosin-stained sections are examined. With larger doses, massive hemorrhagic adrenocortical necrosis occurs and frequently involves the zone *glomerulosa*. The adrenals from rats given 100 mg/kg of 7-hydroxy-DMBA fall into the latter category. No precise estimate of adrenocorticolytic potency for the two compounds can be made from our observations since the slopes of the dose-response curve for DMBA and for 7-hydroxy-DMBA are different. The most likely explanation for this difference is that 7-hydroxy-DMBA exerts a direct effect on the adrenal, whereas DMBA must be converted to this metabolite to be effective. The data indicate that the monohydroxy metabolite is *at least* twice as potent as the parent compound.

DISCUSSION

Sufficient experimental data have been accumulated to establish the fact that 7-hydroxy-DMBA, a metabolite of DMBA, is a more powerful adrenocorticolytic agent than the parent compound. The available evidence indicates that the liver plays a major role in its formation, but this does not preclude the possibility that other tissues may participate in the metabolism of DMBA (1). Earlier work in this field has been summarized by Boyland and Sims (5). Our observations on hemopoietic damage, body weight, and mortality indicate that it is a more toxic compound, presumably a consequence of its strong radiomimetic properties, and support the concept that DMBA affects these biologic indices via this metabolite. 7-Hydroxy-DMBA is not converted to DMBA by rat liver homogenates but to other ring-hydroxylated compounds (unpublished observations). Since it is a poor inducer of liver enzymic activity, a higher concentration of the unchanged compound might be expected to be present at the tissue level. Moreover, Wheatley *et al.* (23) have shown that adrenocortical damage is less severe in rats given DMBA when their livers are damaged by carbon tetrachloride or after partial hepatectomy. The adrenals are not protected by these experimental procedures after 7-hydroxy-DMBA.

Table 4

Treatment	No. of rats	Mean body weight (gm)	Weight of both adrenals (mean ± S.E.)	Rats with adrenal damage
Sesame oil (1 ml)	6	155	0.053 ± 0.002	0
DMBA (mg/kg)				
25	6	152	0.052 ± 0.005	2
50	6	161	0.058 ± 0.009	2
100	6	160	0.057 ± 0.004	3
7-hydroxy-DMBA (mg/kg)				
25	6	160	0.059 ± 0.004	4
50	5	152	0.069 ± 0.002	5
100	6	157	0.086 ± 0.004	6

Adrenocortical damage 72 hours after DMBA or 7-hydroxy-DMBA. Sprague-Dawley female rats, age 50 days, were given a single dose of the compound dissolved in sesame oil by gastric tube. Adrenocortical necrosis was assessed by microscopic examination of serial sections. For abbreviations, see legend to Table 1.

Boyland *et al.* (6) and Pataki and Huggins (20) concluded that the methyl group in the 12-position of the benz(a)anthracene molecule was essential for adrenocortical necrosis. Failure to induce adrenal damage by 7,12-dihydroxy-DMBA supports this conclusion. 4-Methoxy-DMBA has a 12-methyl substituent, yet it causes no detectable injury to adrenocortical or hemopoietic cells despite its stimulatory effect on liver enzymic activity. When incubated with rat liver homogenates, 4-methoxy-7-hydroxymethyl-12-methylbenz(a)anthracene and 4-hydroxy-7,12-dimethylbenz(a)anthracene are formed; neither DMBA nor 7-hydroxy-DMBA is detectable under these experimental conditions (unpublished observations). That the structure-activity relationship between polynuclear hydrocarbons and rat adrenocortical tissue is highly specific is demonstrated by the data presented by Pataki and Huggins (20). These investigators tested 30 benz(a)anthracene derivatives for adrenocorticolysis in female Sprague-Dawley rats. Eight of the compounds, all dialkyl compounds with a methyl group at C-12 and a hydroxyalkyl, chloralkyl, methyl, or formyl group at C-7, were adrenocorticolytic. Of the 23 inactive compounds, 5 were derivatives of 7,12-DMBA with the third substituent at another site in the molecule. 4-Methoxy-DMBA has a third substituent which is not readily removed by the liver. This fact alone may alter the structure-activity relationship between the active site(s) at the tissue level. Alternatively, the compound may be transformed so rapidly to inactive ring-hydroxylated products *in vivo* that very little of the "active" metabolite reaches other tissues.

Damage to the hemopoietic system by a metabolite of DMBA is a new observation. We do not know at this time whether this is peculiar to the rat or whether it occurs in other species not susceptible to adrenocortical injury by DMBA. Our preliminary observations indicate that the structure-activity relationship between vulnerable "receptor" sites in hemopoietic tissue and derivatives of DMBA is highly specific, but additional studies on metabolic transformations and hemopoietic damage in other species are necessary before more definitive conclusions can be made.

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