Biochemical Pharmacology, 1967, Vol. 16, pp. 131–141. Pergamon Press Ltd., Printed in Great Britain.

EFFECT OF PRETREATMENT WITH POLYCYCLIC HYDROCARBONS ON THE METABOLISM OF DIMETHYLBENZANTHRACENE-12-^{14}C BY RAT LIVER AND OTHER TISSUES

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(*Received* 27 *June* 1966; *accepted* 15 *August* 1966)

Abstract—Pretreatment of rats with certain polycylic hydrocarbons alters the metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) by hepatic tissue from side-chain to ring hydroxylation. No change in the metabolism of DMBA by the adrenal glands was observed under these conditions, and no 7-hydroxylated metabolite of the carcinogen was formed by this tissue. It is suggested that protection by aromatic hydrocarbons against DMBA-induced adrenal necrosis is achieved by decreasing the yield of the 7-hydroxymethyl derivative of DMBA in the liver. This product, rather than the unchanged carcinogen, may act as the proximal necrotic agent by virtue of its structural resemblance to the adrenocortical steroids. The cytotoxic effects in the adrenals may be due to damage of cellular membranes and release of lysosomal enzymes.

THE CAPACITY to induce selective adrenal necrosis is not a property shared in common by all polynuclear aromatic hydrocarbons and, as far as is known, it resides only in DMBA* and very closely related compounds.[1, 2] It is now well established from the classical experiments of Huggins[3–5] and Dao[6, 7] and their co-workers that the administration of certain aromatic substances to rats prior to the feeding of DMBA completely inhibits the induction of adrenal necrosis by this carcinogen and also gives some protection against the development of mammary cancer. It is also known [8–10] that a small dose of a polycyclic hydrocarbon such as 3-methylcholanthrene can induce a marked increase in hydroxylating enzymes in the liver and other tissues of rats. In addition, Boyland and Sims[11] have found that DMBA is metabolized in the rat mainly by oxidation of the methyl group to the isomeric monohydroxymethyl derivatives in contrast to phenanthrene, benz(a)anthracene, dibenz(a,h)anthracene, and other unsubstituted aromatic hydrocarbons which are oxidized at reactive double bonds in the nucleus.

It was therefore considered of interest to determine whether the metabolism of DMBA in rat liver could be altered from side-chain oxidation to preferential oxidation in the aromatic ring by pretreating the animals with polycyclic hydrocarbons. The effect of such a procedure on the metabolism of DMBA by homogenates of rat adrenals

* The following abbreviations are used: DMBA, 7,12-dimethylbenz(a)anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; 12-OMH-7-MBA, 12-hydroxymethyl-7-methylbenz(a)anthracene; 7,12-DiOHM-BA, 7,12-dihydroxymethylbenz(a)anthracene; MC, 3-methylcholanthrene.

and other tissues was also investigated, and the results are discussed in relation to carcinogenesis and adrenal necrosis.

MATERIALS AND METHODS

Animals and experimental procedure. Sprague–Dawley rats or an inbred hooded strain, 55–65 days old and weighing 120–150 g (females) or 170–210 g (males) were used. All animals had free access to food (Purina laboratory chow) and water. The polycyclic hydrocarbons dissolved in sesame oil by gentle heating were administered by stomach tube, and unless otherwise indicated, the rats received 10 mg hydrocarbon in 1 ml oil.

Preparation of tissue fractions. The animals were killed 42 hr after the oral dose of hydrocarbon by placing them in an atmosphere of carbon dioxide; the liver and other tissues were rapidly removed and weighed. One part by weight of liver in one part by volume of 0·25 M sucrose was then homogenized for 3 sec in a Servall Omni-Mixer and further disrupted in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to a final concentration of 50 mg liver/ml sucrose solution and the nuclei, mitochondria, and cell debris were removed by spinning for 15 min at 8000 *g* to give a supernatant fraction (8000 *g* supernatant) containing microsomes equivalent to 50 mg liver/ml solution. Liver slices (100 mg wet weight) were sectioned in a Stadie–Riggs slicer and, in this case, the incubations were carried out in Krebs phosphate saline.[12] A 5% homogenate of the adrenal glands or other tissues in 0·1 M potassium phosphate buffer, pH 7·4, was also prepared and used without fractionation.

Incubation. 7,12-Dimethylbenz(a)anthracene-12-^{14}C (0·11 μc in 3 μg) dissolved in ethanol (0·02 ml) was added to the tissue preparation (1 ml) and incubated at 38° for 1 hr under O_2 together with $NADPH_2$ (0·3 mM) or NADP (0·3 mM) and glucose 6-phosphate (3 mM) in 0·1 M potassium phosphate buffer, pH 7·4; total volume 4 ml. Oxygen was bubbled through the mixture before incubation and, unless otherwise stated, the reaction was stopped by the addition of 1 N HCl (1 ml) and the incubation mixture extracted three times with equal volumes of peroxide-free ether. A control tube containing DMBA-12-^{14}C without any tissue was also incubated. After drying over anhydrous Na_2SO_4, the ethereal fractions were evaporated to dryness under N_2 at 40° and the residue dissolved in ethanol for radioactive assay and chromatography.

Measurement of radioactivity. The amount of ^{14}C in 0·1-ml aliquots of the ether-soluble fraction and 0·2 ml of the residual aqueous medium was determined in a Packard Tri-Carb liquid scintillation counter and the percentage of added radioactivity in each phase calculated from these values. The scintillation liquid consisted of a mixture of 1 ml Hyamine hydroxide and 10 ml of toluene:ethanol (3:2) containing 0·4% PPO (2,5-diphenyloxazole) and 0·01% POPOP [1,4-bis-2(5-phenyloxazoyl)-benzene], obtained from Packard Instrument Co., La Grange, Ill.

Protein precipitation. Proteins (and nucleic acids) were precipitated from the aqueous medium (4·5 ml) by the addition of 3 ml of 1·5 N trichloroacetic acid and the turbid solution centrifuged at approximately 1000 *g* for 5 min. The supernatant was decanted and the precipitate washed by resuspension in 2 ml trichloroacetic acid followed by centrifugation. With the same procedure, the precipitate was washed twice with 3 ml 95% ethanol and dissolved by heating in formamide (1 ml) at 180° for 2 hr. An aliquot (0·1 ml) of this solution was then counted in the usual manner.

In some experiments, proteins (and nucleic acids) were precipitated by the addition of an equal volume of 95% ethanol to the extracted aqueous medium at pH 6·5, and the tubes were allowed to stand at −20° for 1 hr before centrifugation. The precipitate was then washed with ethanol. Good agreement was obtained between these two methods.

Purification of DMBA-12-^{14}C. 7,12-Dimethylbenz(a)anthracene-12-^{14}C (9·31 mc/m-mole), obtained from the Radiochemical Centre, Amersham, England, was freed of radioactive decomposition products by washing through a Florisil column (0·7 × 7 cm) with 20 ml purified hexane† and was stored in the dark at − 20°. All subsequent operations with DMBA-12-^{14}C were carried out in diffuse light.

Preparation of reference compounds and chromatography. The reference standards, 7-OHM-12-MBA, 12-OHM-7-MBA, and 7,12-diOHM-BA, were prepared by the method of Boyland and Sims[11] and their systems for thin-layer chromatography (benzene or benzene: ethanol, 19:1) were also used. However, precoated silica gel chromatogram sheets (Eastman Kodak) were found to be more convenient for subsequent autoradiography than coated glass plates. The sheets were stapled onto X-ray film (Kodak No Screen) and kept in close contact in a light-tight cassette for approximately 3 days. Nonradioactive reference compounds were located by their fluorescence in ultraviolet light (254 mμ). For quantitative ^{14}C assay of the ether-soluble DMBA metabolites, the radioactive areas were scraped off the thin-layer sheets into vials, scintillation fluid added, and counting done in the usual manner. Recoveries of 85–95 per cent were obtained by this technique.

RESULTS

Table 1 shows the effect of different hydrocarbons on the metabolism *in vitro* of DMBA-12-^{14}C by rat liver microsomes (8000-*g* supernatant). A marked increase in the yield of water-soluble products was observed in animals pretreated with MC, dibenz(a,h)anthracene, benzo(a)pyrene, and DMBA but the noncarcinogenic hydrocarbons, anthracene, phenanthrene, and naphthalene, gave little or no increase. A good correlation was obtained between the median protective dose of the hydrocarbons against adrenal necrosis[7] and their ability to induce liver enzymes that metabolize DMBA. An increase in the yield of water-soluble products was always accompanied by an increase in the more polar ether-soluble metabolites and a decrease in the amount of unchanged DMBA and of the hydroxymethyl derivatives. Some of the more polar products formed by MC-treated rats were phenolic since they could not be removed from the incubation medium at pH 12 with ether (Fig. 1). These products, however, could be extracted from saturated $NaHCO_3$ solution and are therefore not strongly acidic.

No sex differences in the hepatic metabolism of DMBA was observed in either Sprague–Dawley or hooded rats and similar results were obtained with slices or unfractionated liver homogenates (Table 2).

It can be seen (Fig. 2) that the effect of MC (10 mg) first became apparent about 6 hr after feeding and reached a maximum at 24–48 hr. The dose–response curve for MC is shown in Fig. 3.

A weight of 50 mg liver was chosen for the experiments because increasing the tissue concentration above this value produced no increase in the yield of water-soluble

† R. G. Harvey and J. W. Flesher, personal communication.

metabolites when they were incubated with 3 μg DMBA-12-^{14}C, and, furthermore, any change produced by MC would be at a stable point on the curve (Fig. 4). The weight of substrate was fixed by the amount needed to give good autoradiograms. The MC effect, however, could also be demonstrated at lower concentrations of

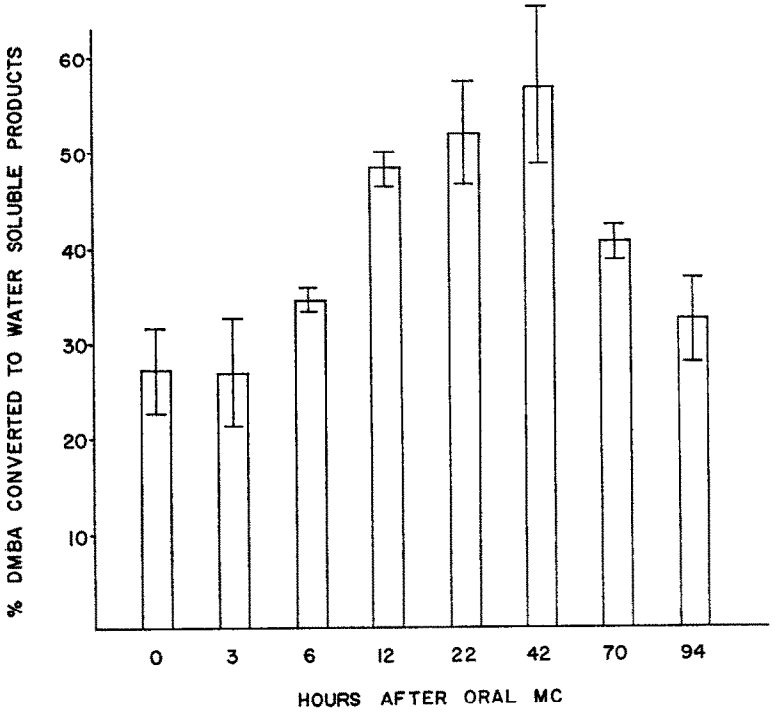

FIG. 2. Effect of time after oral administration of 10 mg 3-methylcholanthrene (MC) on the conversion of DMBA-12-^{14}C to water-soluble metabolites by rat liver microsomes (8000-*g* supernatant).

DMBA, as shown in Table 3, and decreasing the incubation time to 30 min did not influence the yield of water-soluble products.

Approximately 10–20 per cent of the ^{14}C in the extracted aqueous fraction of the liver incubations was bound to protein (or nucleic acid) in a form which could not be dissociated by washing the precipitate with ethanol. Generally, more radioactivity was associated with protein after treating the animals with MC, but the results were variable and this problem as well as the nature of the water-soluble material and of the unidentified ether-soluble metabolites of DMBA is being further investigated.

Other tissues tested, which included kidney, spleen, lung, brain, adrenal, ovary, and testis, were less active than liver forming water-soluble and other metabolites of DMBA and only kidney and lung showed some change in the yield of ether-soluble products (visualized by autoradioagraphy) as a result of the administration of MC *in vivo*.

The adrenals gave the same yield of ether-soluble products in both control and MC-treated rats (Table 4), and although the main metabolite formed by this tissue had a mobility similar to that of 7-OHM-MBA in the benzene–ethanol system, it was shown to differ from it by mixed chromatography with the reference compound in

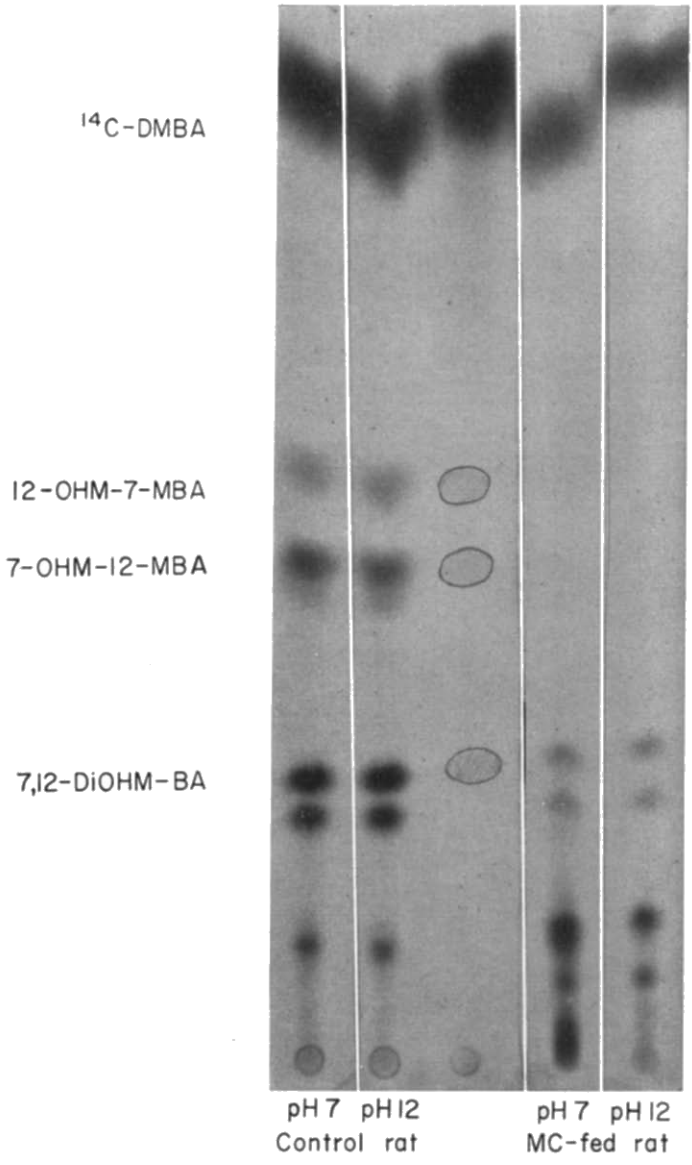

FIG. 1. Autoradiogram of ether-soluble DMBA-12-^{14}C metabolites formed by rat liver microsomes (8000-*g* supernatant) before and after administration of 10 mg 3-methylcholanthrene (MC). The incubation mixtures were extracted at pH 7 or 12 and the nonradioactive standards located by their fluorescence.

facing page 134 B.P.

TABLE 1. EFFECT OF PRETREATMENT WITH POLYCYCLIC HYDROCARBONS ON THE FORMATION OF DMBA-12-^{14}C METABOLITES BY RAT LIVER MICROSOMES

PD_{50}* (mg)	Hydrocarbon†	Per cent of added ^{14}C remaining in aqueous medium after ether extraction	No. of expts.	Ether-soluble fraction after chromatography‡		
				Unchanged DMBA (10^3 counts/min)	Hydroxymethyl-MBA region (10^3 counts/min)	Polar products near origin (10^3 counts/min)
	No tissue	<0·4	8	7·7		0·2
	Control (oil)	27·1 ± 4·3	8	2·1	3·0	1·5
1·5	3-Methylcholanthrene	56·9 ± 8·4	8	0·9	1·1	5·4
2·2	Dibenz(a,h)anthracene§	48·5 ± 9·0	5	1·0	1·6	3·9
2·5	Benzo(a)pyrene	47·1 ± 7·9	4	0·9	1·9	3·8
3·0	DMBA	53·3 (48·6–57·9)	2	1·0	2·5	3·0
3·0	DMBA (30 mg)	62·1 (59·3–64·9)	2	1·0	1·7	3·7
18·5	Anthracene	28·1 (26·3–29·9)	2	2·2	2·0	1·3
36·0	Phenanthrene	32·5 (31·0–33·9)	2			
>100	Naphthalene	27·3 (24·7–30·0)	2	1·9	3·2	1·5

* Dose to protect 50 per cent of rats against DMBA-induced adrenal necrosis.[7]

† Hydrocarbon (10 mg) in oil given orally 42 hr before removing liver.

‡ Mean of two or more experiments with results adjusted to a constant value for the controls.

§ Dissolved in 1·5 ml oil.

TABLE 2. EFFECT OF SEX, STRAIN, AND PRETREATMENT WITH METHYLCHOLANTHRENE ON THE METABOLISM OF DMBA-12-^{14}C BY RAT LIVER *IN VITRO*

Tissue preparation	Sex	Strain*	Pretreatment with MC (10 mg)	Per cent of added ^{14}C remaining in aqueous medium after ether extraction	Ether-soluble metabolites†	
					Unchanged DMBA (10^3 counts/min)	Polar products near origin (10^3 counts/min)
8000-*g* Liver supernatant (50 mg)	♀	IH	−	27·1 ± 4·3		
	♂	IH	−	32·9 ± 5·1		
	♀	SD	−	25·3 (20·5–30·1)		
	♂	SD	−	28·9 (26·7–31·1)		
Liver homogenate (50 mg)	♀	IH	−	14·5		
	♀	IH	+	48·0		
Liver slices (~120 mg)	♂	IH	−	28·5 (28·0–29·1)	4·0	1·2
	♂	IH	+	54·7 (50·5–58·9)	0·6	4·7

* IH: Inbred hooded; SD: Sprague–Dawley.

† No qualitative or quantitative differences in untreated animals were observed by autoradiography.

TABLE 3. EFFECT OF SUBSTRATE CONCENTRATION ON THE METABOLISM OF DMBA-12-^{14}C BY RAT LIVER MICROSOMES

Wt. of ^{14}C-DMBA (μg)	Per cent of added ^{14}C remaining in aqueous medium after ether extraction	
	Control	MC-fed
0·3	32·1 (31·9–32·4)	61·3 (59·4–63·2)
1·0	25·9 (25·7–26·1)	58·5 (56·4–58·6)
3·0	25·9 (23·8–28·1)	48·7 (47·8–49·7)
6·0	23·3 (23·2–23·4)	41·0 (39·8–42·3)

DMBA-12-^{14}C incubated with the 8000-*g* supernatant of rat liver (♂ hooded) for 1 hr. Other conditions as described in text.

TABLE 4. EFFECT OF SEX, STRAIN, AND PRETREATMENT WITH METHYLCHOLANTHRENE ON THE METABOLISM OF DMBA-12-^{14}C BY RAT ADRENAL HOMOGENATES

Sex	Strain*	Pretreatment with MC (10 mg)	Per cent of added ^{14}C remaining in aqueous medium after ether extraction	Ether-soluble metabolite† (10^3 counts/min)
♀	IH	−	7·4 ± 1·1	1·6
♀	IH	+	7·9 ± 1·0	1·7
♂	IH	−	8·3 ± 0·9	
♂	IH	+	8·0	
♀	SD	−	9·1	3·7
♀	SD	+	8·2	3·9

A homogenate of both adrenals (35–45 mg) was used. Other conditions as described in text.

* IH: Inbred hooded; SD: Sprague–Dawley.

† With same R_f as 7-OHM-MBA in benzene–ethanol but moving faster in the benzene system.

the benzene system. Under these conditions, the corresponding liver metabolite whose identity had already been established by Boyland and Sims[11] still migrated with 7-OHM-MBA.

DISCUSSION

Several mechanisms have been proposed[13] to explain the protective action of polycyclic hydrocarbons against selective damage of the adrenals by DMBA. One theory suggests that the markedly increased concentration of hydroxylating enzymes in the liver following treatment with polycyclic hydrocarbons makes possible the metabolic destruction of DMBA before it can reach the adrenal cortex in sufficient quantity to inflict damage upon the gland.

Our results support this theory, but we should like to extend it by proposing that the main effect may be due to a shift from the formation of the hydroxymethyl derivatives by the liver to ring-hydroxylated phenolic metabolites. These are then converted to water-soluble products by a reaction that may be analogous to the conversion of estrogens to water-soluble derivatives by rat liver preparations.[14] However, in the case of DMBA, no sex-difference was observed. Recently Boyland *et al.*[15] have

reported a decrease in the yield of the hydroxymethyl derivatives of DMBA after treating rats with substances that induce increases in liver microsomal enzymes, while Conney and Levin,[16] using a fluorometric technique, showed an increase in a phenolic metabolite under these conditions.

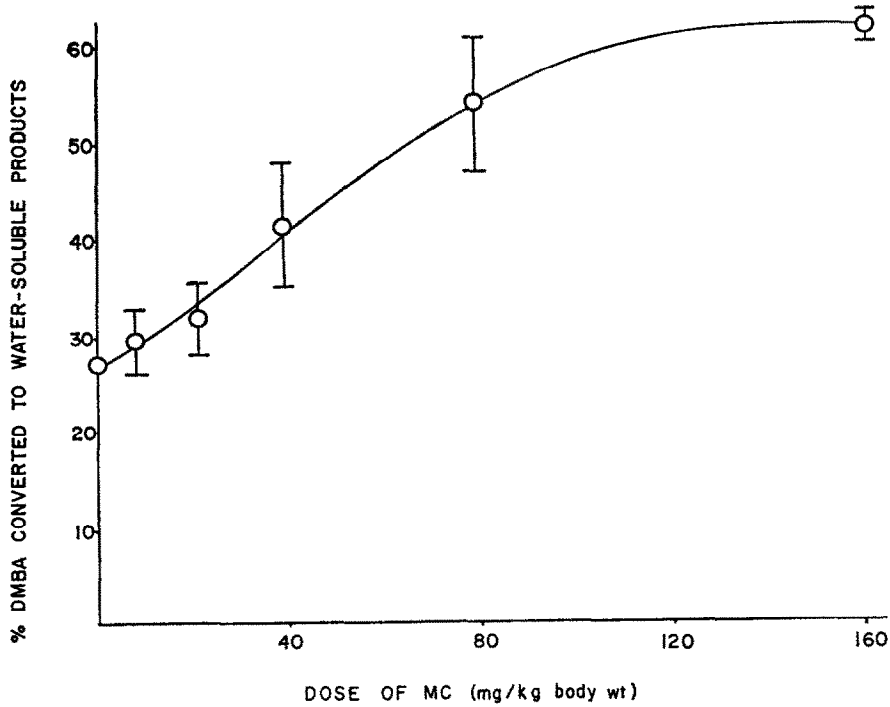

FIG. 3. Effect of dose of 3-methylcholanthrene (MC) given orally 42 hr prior to killing animals on the conversion of DMBA-12-^{14}C to water-soluble metabolites by rat liver microsomes (8000-*g* supernatant).

Since no change in the yield of DMBA metabolites by the adrenals was observed after treatment with polycyclic hydrocarbons, and since no 7-hydroxylated derivative is formed by this tissue even under normal conditions, it seems unlikely that protection against necrosis is brought about by an alteration in DMBA metabolism within this gland. It seems more probable that the protective action of the hydrocarbons is mediated by the liver.

Huggins and Sugiyama[2] and Morii and Huggins[17] found that the adrenal cortex must be in a susceptible state to be vulnerable to necrosis by DMBA, and have inferred that this type of adrenal apoplexy was related to the adrenal gland's capacity to synthesize steroids. This was supported by the findings of Currie *et al.*[18] and others,[6, 19] who prevented adrenal damage from DMBA by the concurrent administration of SU 4885 [2-methyl-1,2-bis-(3-pyridyl)-1-propanone], an amphenone analog which inhibits 11β-hydroxylation and hence corticosterone and cortisol synthesis.[20] The suggestion has also been made[3, 17, 19] that the cytolytic effect in the adrenal may be related to steric resemblance between DMBA and the corticosteroid hormone molecules produced in the adrenal cortex.

In the light of the findings[11] that DMBA, unlike other carcinogenic hydrocarbons, is oxidized preferentially in the side chain, it seems reasonable to suggest that it is the hydroxymethyl derivative rather than the unchanged carcinogen that interferes

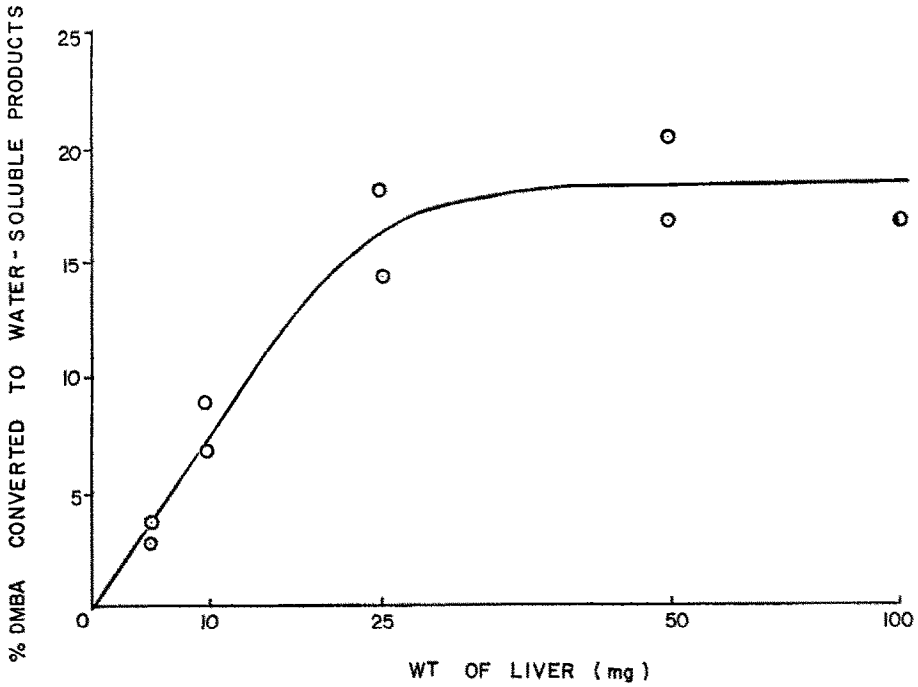

FIG. 4. Tissue concentration curve. DMBA-12-^{14}C (3 μg) was incubated with microsomes (8000-g supernatant) derived from different weights of rat liver.

with the metabolism or action of the corticosteroids. It is the presence of the hydroxymethyl group, characteristic of the adrenocortical steroids, that should make 7-OHM-12-MBA into a more potent and selective inhibitor of corticosteroid synthesis and of the biochemical reactions influenced by these hormones. The necrotic effect on the adrenals may be secondary and perhaps due to a disturbance in the corticosteroid content of this gland. Under these conditions, the membrane of the adrenal lysosomes may be rendered exceptionally susceptible to polycyclic hydrocarbons, and the cytotoxic effects could then be due to release of lysosomal enzymes. Certainly, marked changes in the appearance of the lysosomes and of the endoplasmic reticulum were visible in electronmicrographs of cells of the zona fasciculata within 12 hr after feeding DMBA. There was marked vesiculation of the membrane component of the endoplasmic reticulum with degranulation, and the lysosomes showed fragmentation of the membrane and osmiophilic aggregates.

It is well established[21] that corticosteroids stabilize the lysosomal membrane and markedly inhibit the inflammatory response in small blood vessels after tissue injury. It is also known[22] that polycyclic hydrocarbons are concentrated inside these organelles and that the membrane of rat adrenal lysosomes but not those of liver or kidney are highly susceptible to 7-OHM-12-MBA.

According to this theory, 12-OHM-7-MBA, which shows less structural similarity to the corticosteroids than the 7-hydroxyl analog (Fig. 5) and has little effect on the release of enzymes from adrenal lysosomes,[23] should be less active as an adrenal

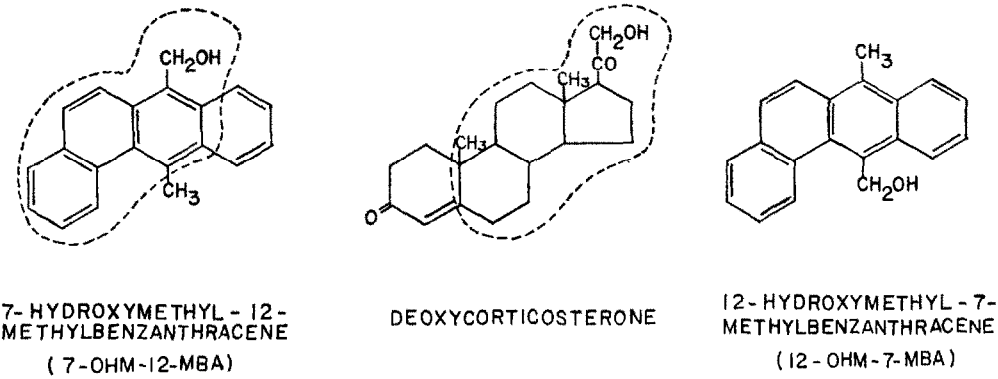

FIG. 5. Molecular structures illustrating points of similarity between the corticosteroids and 7-OHM-12-MBA. The 12-hydroxylmethyl derivative does not easily fit the same receptor sites.

necrotic agent. This, in fact, has been found,[15] since 5 mg of 7-OHM-12-MBA caused about the same amount of adrenal damage as 30 mg DMBA, while 12-OHM-7-MBA was inactive even in 60 mg doses.

Although the relationship of these effects of DMBA and its metabolites to carcinogenesis is not yet clear, it has been suggested by Allison *et al.*[22–24] that the increased membrane permeability accompanied by the gradual release of enzymes from lysosomes over long periods may be implicated in malignant transformation. They have also presented evidence that lysosomal enzymes can react with and produce lasting changes in genetic material and can release cells from mitotic inhibition. Carcinogenesis by DMBA may therefore differ only qualitatively from the sudden and massive release of lysosomal enzymes which is probably involved in adrenal necrosis.

Acknowledgements—We thank Dr. W. L. Dunn for the histology and electron microscopy of the adrenals and Drs. R. L. Noble and J. W. Jull for their interest and valuable discussions. We are also grateful for the collaboration of Miss Sheila Coles and Miss Sharon Dougan. This work was supported by the Medical Research Council and National Cancer Institute of Canada.

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