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STUDIES ON THE METABOLISM OF 7-METHYLBENZ-[a]ANTHRACENE AND 7,12-DIMETHYLBENZ[a]-ANTHRACENE AND ITS HYDROXYMETHYL DERIVATIVES IN RAT LIVER AND ADRENAL HOMOGENATES

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Abstract—Liver homogenates from rats pretreated with 3-methylcholanthrene converted 7-hydroxymethyl-12-methyl and 12-hydroxymethyl-7-methylbenz[a]anthracene into phenols and dihydrodiols: some hydroxylation of the methyl groups also occurred. Qualitative and quantitative studies of the metabolism of 7,12-dimethylbenz[a]anthracene, 7-methylbenz[a]anthracene and the above hydroxymethyl derivatives showed that with liver homogenates from normal rats products were formed that arise either by ring-hydroxylation or by hydroxylation of the methyl groups, whereas with homogenates of the adrenals from the same animals ring-hydroxylation but little or no hydroxylation of the methyl groups occurred. Adrenal homogenates were more efficient than liver homogenates in effecting the ring hydroxylations of these compounds. Large increases in the amounts of metabolites formed from the above substrates occurred when homogenates of the livers of rats that had been pretreated with 3-methylcholanthrene were used: no such increases were found when homogenates of the adrenals of these animals were used. The metabolism of the above substrates by liver homogenates from mice, guinea pigs, hamsters and rabbits is briefly described. The significance of the results in relation to the induction of adrenal necrosis in rats is discussed.

EARLIER work[1] on the metabolism of DMBA* by homogenates of the livers of normal rats, showed that it is metabolised mainly into the hydroxymethyl derivatives, 7-OHM-12-MBA and 12-OHM-7-MBA: this was confirmed by other workers.[2-4]

* The following abbreviations are used: DMBA, 7,12-dimethylbenz[a]anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; 12-OHM-7-MBA, 12-hydroxymethyl-7-methylbenz[a]anthracene; 7,12-DiOHMBA, 7,12-dihydroxymethylbenz[a]anthracene; 4-OHDMBA, 4-hydroxy-7,12-dimethylbenz[a]anthracene; 3-OHDMBA, 3-hydroxy-7,12-dimethylbenz[a]anthracene; 8,9-DiHOHDMBA, 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene; 8,9-DiHOH-7-OHM-12-MBA, 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene; 8,9-DiHOH-12-OHM-7-MBA, 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]anthracene; 10,11-DiHOH-7-OHM-12-MBA, 10,11-dihydro-10,11-dihydroxy 7-hydroxymethyl-12-methylbenz[a]anthracene; 12-MBA-7-COOH, 12-methylbenz[a]anthracene-7-carboxylic acid; 7-MBA-12-COOH, 7-methylbenz[a]anthracene-12-carboxylic acid.

7-MBA, 7-methylbenz[a]anthracene; 7-OHMBA, 7-hydroxymethylbenz[a]anthracene; 8,9-DiHOH-7-MBA, 8,9-dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene; 10,11-DiHOH-7-OHMBA, 10,11-dihydro-10,11-dihydroxy-7-hydroxymethylbenz[a]anthracene; BA-7-COOH, benz[a]anthracene-7-carboxylic acid.

7-OHM-12-MBA induces mammary cancer in rats and both it and 12-OHM-7-MBA induce local sarcomas after subcutaneous injection into mice.[5] The overall rate of metabolism of the hydrocarbon is increased if the rats are pretreated with 3-methylcholanthrene: the products are then more polar metabolites[4,6] that were identified as dihydrodiols.[7] Quantitative aspects of DMBA metabolism have been discussed.[8] The metabolism of DMBA in adrenal homogenates has been investigated:[9] those from mice and infant rats failed to metabolise the hydrocarbon, whereas those from adult rats converted it into a product that was not 7-OHM-12-MBA.

The metabolism of 7-OHM-12-MBA and 12-OHM-7-MBA in liver homogenates from both normal and pretreated rats showed that the major products were phenols and dihydrodiols.[7] It has been reported[10] that ^{14}C-labelled 7-OHM-12-MBA is metabolised by rat liver, but not by rat adrenal homogenates.

The induction of adrenal necrosis in rats treated with DMBA[11] or with 7-OHM-12-MBA[5] is well established: 12-OHM-7-MBA is inactive. DMBA-induced adrenal necrosis can be prevented by pretreating the rats with any one of a number of compounds,[12] and this effect has been related[3,6,7] to the stimulation of hepatic microsomal enzymes that convert DMBA into non-toxic products. This paper describes the metabolism of tritiated DMBA, 7-MBA, 7-OHM-12-MBA and 12-OHM-7-MBA in homogenates of the livers and adrenals of normal rats and of rats that had been pretreated with 3-methylcholanthrene.

EXPERIMENTAL

Materials. 7-OHM-12-MBA,[1] 12-OHM-7-MBA,[1] 7,12-diOHMBA,[1] 12-MBA-7-COOH,[7] 7-MBA,[13] 7-OHMBA[13] and BA-7-COOH[13] were prepared as described. 4-OHDMBA was the gift of Dr. J. W. Flesher.

DMBA (sp. act. 81·5 mc/m-mole), generally labelled with tritium, was prepared by diluting a more highly labelled sample of the hydrocarbon (tritiated at the Radiochemical Centre, Amersham, Bucks) with unlabelled carrier DMBA. The mixture was purified by column chromatography on a silica gel-packed column in benzene–light petroleum (b.p. 60–80°) (1:1, v/v). The hydrocarbon was finally recrystallized from ethanol in plates, m.p. 122°.

7-OHM-12-MBA and 12-OHM-7-MBA, generally labelled with tritium, were prepared by oxidizing a tritiated sample of DMBA with lead tetraacetate in acetic acid as previously described.[1] Only a relatively small amount of the mixed acetoxy derivatives of the hydroxymethyl compounds was obtained, the major product being a resinous material that was not identified: this material was not formed when unlabelled hydrocarbon was oxidized. The mixture of acetoxy methyl derivatives was hydrolysed with potassium hydroxide in methanol as before[1] and the hydroxymethyl derivatives separated by thin-layer chromatography as described below using solvent (a). The hydroxymethyl derivatives were eluted from the silica gel with ether and each was recrystallized from ethanol in the presence of the appropriate unlabelled compound to give tritiated 7-OHM-12-MBA (sp. act. 38·2 mc/m-mole) and tritiated 12-OHM-7-MBA (sp. act. 31·5 mc/m-mole).

Thin-layer chromatography. Thin-layer chromatograms (TLCs) were prepared by coating glass plates (20 × 20 cm) with a layer of silica gel G (E. Merck A.-G., Darm-

stadt, Germany) of 0·25 mm thickness. The chromatograms were developed for 15 cm in either (a) benzene, (b) benzene–ethanol (9:1 v/v) or (c) chloroform. They were inspected in u.v. light both before and after exposure to ammonia.

Two-dimensional acid-treated TLCs were developed in the first direction with solvent (b), sprayed with hydrochloric acid and heated in an oven at 105° for 5 min and developed in the second direction in the same solvent.

Ultra-violet absorption spectra of metabolites were measured on a Unicam SP 800 recording spectrophotometer. The solutions were obtained by removing the appropriate bands from TLCs and eluting the absorbed material from the silica gel with ethanol.

Experiments with rat-liver and adrenal homogenates. Male rats of the Chester Beatty strain (body wt. approx. 180 g) were used. The mice, guinea pigs, hamsters and rabbits were adult males. The pretreated rats were given 3-methylcholanthrene (5 mg) in arachis oil (0·5 ml) by intraperitoneal injection 48 hr before they were killed. In the

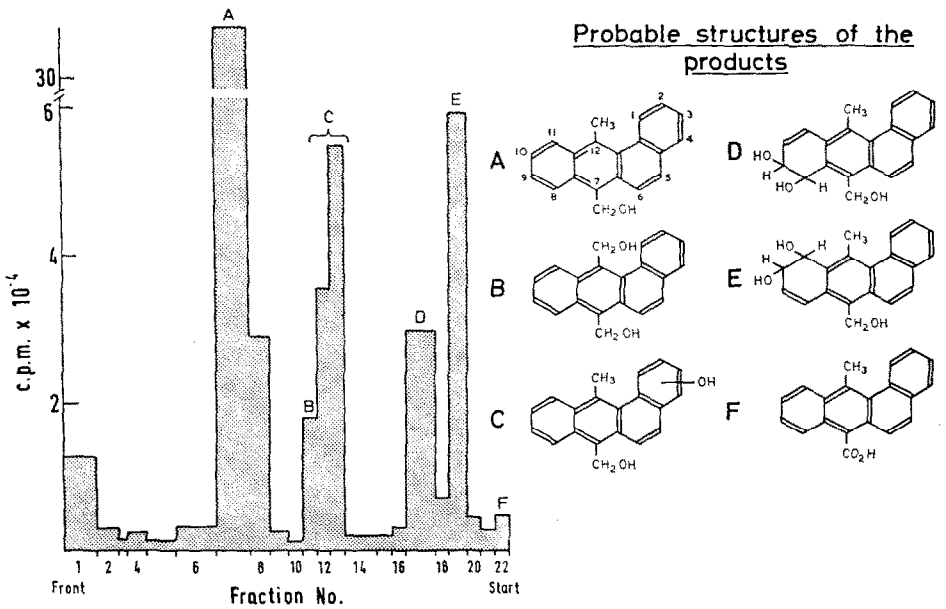

FIG. 1. The metabolism of 7-hydroxymethyl-12-methylbenz[a]anthracene in homogenates of the livers of rats pretreated with 3-methylcholanthrene.

comparative experiments described below, liver and adrenals from the same animals were used in each experiment. Liver homogenates in the amounts indicated below were prepared in 0·1 M phosphate buffer, pH 7·4, as described:[8] adrenal homogenates were similarly prepared. Cofactors, in the amounts indicated,[8] were added to both types of homogenates: in addition glucose 6-phosphate dehydrogenase (0·7 unit) [Boehringer Corporation (London) Ltd., London, W.5] was added to each adrenal homogenate.

(a) *Large scale incubations.* These were carried out as described[14] using homogenates prepared from rat-liver (40 g) obtained from pretreated animals. The substrates were either tritiated 7-OHM-12-MBA or tritiated 12-OHM-7-MBA (5 μmole in 5 ml of acetone). The results are shown in Figs. 1 and 2.

Similar incubations of unlabelled 7-MBA, DMBA, 7-OHM-12-MBA or 12-OHM-7-MBA (2 mg in 5 ml of acetone) were also carried out to obtain mixtures of metabolites for use as chromatographic markers in the experiments described below. Incubations of the above substrates were also carried out with homogenates from the livers of mice, guinea pigs, hamsters and rabbits and of 7-OHMBA with these homogenates and with liver homogenates from pretreated rats.

(b) *Comparative experiments with liver and adrenal homogenates.* In the qualitative experiments, homogenates from rat-liver and adrenals from normal animals were prepared so that each incubation mixture contained 500 mg wet wt. of tissue in 0·1

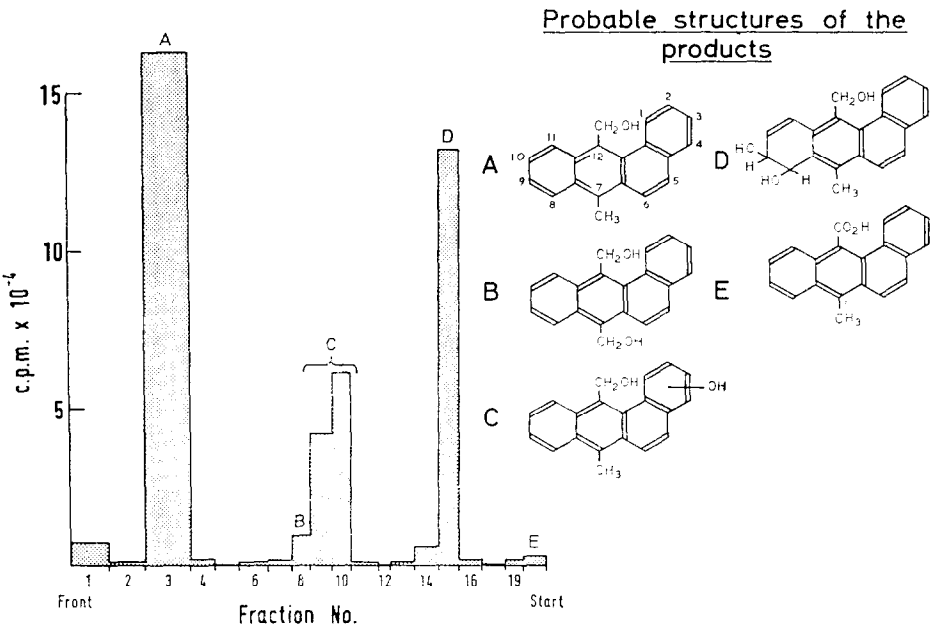

FIG. 2. The metabolism of 12-hydroxymethyl-7-methylbenz[a]anthracene in homogenates of the livers of rats pretreated with 3-methylcholanthrene.

M phosphate buffer (10 ml), together with the appropriate amounts of co-factors. Tritiated DMBA, 7-OHM-12-MBA, 12-OHM-7-MBA or 7-MBA (100 μmoles in 0·1 ml of acetone) was added to each incubation mixture. The mixtures were incubated for 30 min and extracted with ethyl acetate (10 ml). Portions (5 ml) of the extracts were evaporated in the presence of the appropriate mixtures of unlabelled metabolites obtained above, together with 7-OHM-12-MBA, 12-OHM-7-MBA, 7,12-DiOHMBA and 4-OHDMBA when DMBA was used as substrate, 7,12-DiOHMBA when 7-OHM-12-MBA and 12-OHM-7-MBA were used as substrates and 7-OHMBA when 7-MBA was used as substrate. Fluorescent and intermediate bands were removed from the chromatograms and the radioactivity on the silica gel counted as previously described.[8] The results are shown in Figs. 3–6.

Two series of quantitative experiments were carried out using homogenates prepared from (a) livers and adrenals from normal rats and (b) livers and adrenals from

rats that had been pretreated with 3-methylcholanthrene. Each series of experiments was carried out in duplicate using portions of homogenates from the same preparations and at the same time. Each incubation mixture contained homogenate from 250 mg wet wt. of tissue suspended in 0·1 M phosphate buffer to a total volume of 10 ml. Tritiated DMBA, 7-OHM-12-MBA or 12-OHM-7-MBA, (100 mμmole in 0·1 ml of acetone) was added to the incubation mixtures at 37° in a metabolic shaker (H. Mickle,

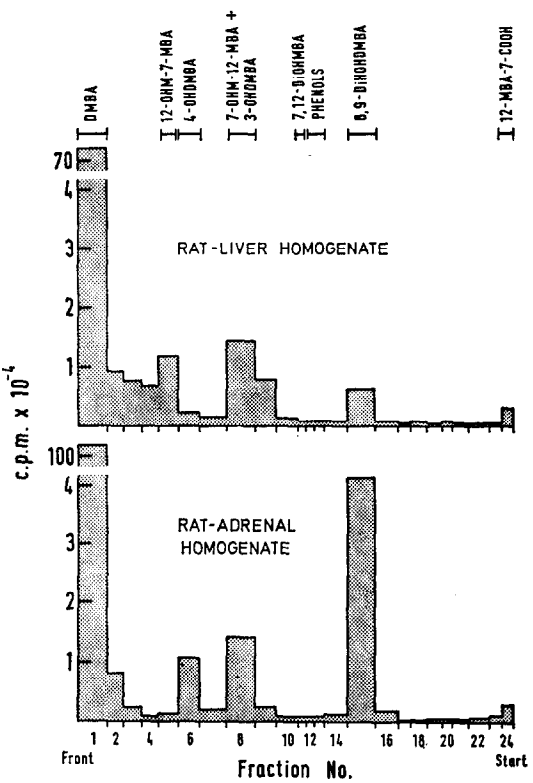

FIG. 3. The metabolism of 7,12-dimethylbenz[a]anthracene in homogenates of the livers and adrena of normal rats. The results shown were obtained as described in the text: the substrate (100 mμmoles; 5·5 × 10^6 cpm) was incubated with homogenate (equivalent to 500 mg of tissue) and the mixture extracted with ethyl acetate (10 ml). The products present in 5 ml of this extract were chromatographed on TLCs and the radioactivity in bands of silica gel determined. The positions of metabolites on the chromatograms are shown thus:———. Abbreviations are those used in the text.

Gomshall, Surrey) and the mixtures incubated for 15 min. They were extracted with ethyl acetate (10 ml) and 2 ml portions of the dried (Na_2SO_4) extracts evaporated in the presence of the marker compounds as indicated above. The residues were chromatographed on TLCs in solvent (b) and the radioactivity associated with metabolic products was determined as described above. The results, shown in Table 1, were calculated on the assumption that no tritium was lost during the metabolic conversions: protein was estimated as described.[15]

TABLE 1. THE COMPARATIVE METABOLISM OF 7,12-DIMETHYLBENZ[a]ANTHRACENE AND ITS HYDROXYMETHYL DERIVATIVES IN LIVER AND ADRENAL HOMOGENATES FROM NORMAL AND 3-METHYLCHOLANTHRENE-TREATED RATS

Substrate	Metabolite	Amounts ($\mu\mu$mole/mg protein) of ethyl acetate-soluble products formed from the substrate in 15 min by			
		Liver homogenate from		Adrenal homogenate from	
		Normal rats	3-Methyl-cholanthrene-treated rats	Normal rats	3-Methyl-cholanthrene-treated rats
7,12-Dimethyl-benz[a]-anthracene	4-Hydroxy-derivative	5	17	201	290
	7-Hydroxymethyl derivative*	22	29	320	330
	12-Hydroxymethyl derivative	31	21	15	15
	7,12-Dihydroxymethyl derivative	—	6	—	—
	Phenolic metabolites of the hydroxymethyl derivatives	—	21	—	—
	8,9-Dihydro-8,9-dihydroxy-derivative	23	640	1100	2310
	8,9-Dihydro-8,9-dihydroxy metabolites of the hydroxymethyl derivatives	—	11	—	—
	10,11-Dihydro-10,11-dihydroxy-7-hydroxy-methyl derivative	—	14	—	—
	7-Carboxy-12-methyl derivative	13	15	14	15
7-Hydroxymethyl-12-methylbenz[a]an-thracene	7,12-Dihydroxymethyl derivative	<2	22	14	17
	Phenolic metabolites	19	43	380	640
	8,9-Dihydro-8,9-dihydroxy derivative	6	280	420	950
	11,12-Dihydro-11,12-dihydroxy derivative	8	315	510	1105
	7-Carboxy derivative	13	18	18	22
12-Hydroxymethyl-7-methylbenz[a] an-thracene	7,12-Dihydroxymethyl derivative	<2	28	6	9
	Phenolic metabolites	15	63	410	725
	8,9-Dihydro-8,9-dihydroxy derivative	21	590	940	1860
	12-Carboxy derivative	12	15	14	18

Incubations and estimations were carried out as described in the text. The values represent means of duplicate determinations. The entry — indicates that no significant radioactivity was present in the fraction containing these reference compounds.

* Some of the radioactivity measured in these fractions may be due to the presence of 3-hydroxy-7,12-dimethylbenz[a]anthracene (see text).

RESULTS

(1) *Qualitative experiments*

The results of the large-scale incubation of tritiated 7-OHM-12-MBA and 12-OHM-7-MBA with rat-liver homogenate are shown in Figs. 1 and 2. Similar experiments with 7-MBA and DMBA have been described.[14]

Metabolism of 7-OHM-12-MBA by liver homogenates. The major metabolic product

formed when the hydroxymethyl derivative was incubated with liver homogenates from pretreated rats or from mice, hamster, guinea pigs and rabbits was a dihydrodiol that has not previously been recognised. On the two-dimensional, acid-treated TLC described above, it yielded a phenol that was detected as a violet fluorescent spot when the chromatograms were inspected in u.v. light that changed to a green fluorescence when the plates were exposed to ammonia. The metabolite was also detecetd

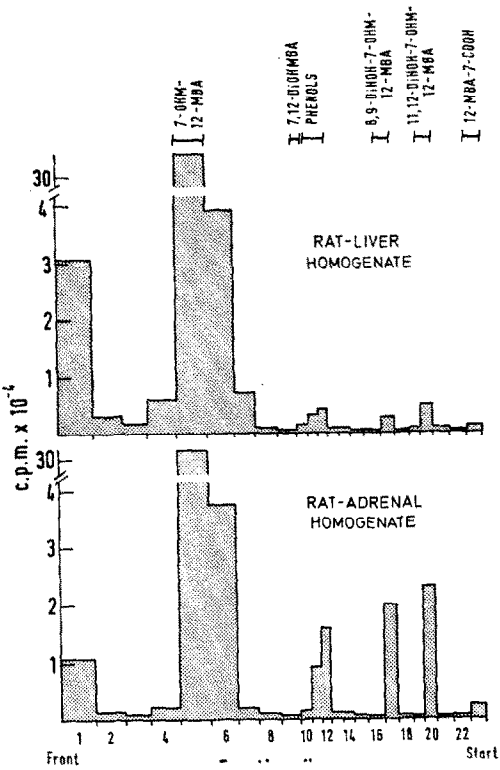

FIG. 4. The metabolism of 7-hydroxymethyl-12-methylbenz[a]anthracene in homogenates of the livers and adrenals of normal rats. The results shown were obtained as described in the text: the substrate (100 mμmoles; $2{\cdot}9 \times 10^6$ cpm) was incubated with homogenate (equivalent to 500 mg of tissue) and the mixture extracted with ethyl acetate (10 ml). The products present in 5 ml of this extract were chromatographed on TLCs and the radioactivity in bands of silica gel determined. The positions of metabolites on the chromatograms are shown thus:———. Abbreviations are those used in the text.

when DMBA was incubated with liver homogenates from mice, guinea pigs, hamsters and rabbits: the formation from DMBA of a chromatographically-similar metabolite by liver homogenates from pretreated rats has been reported.[14] It was not detected when DMBA was incubated with liver homogenate from normal rats and it was detected only with difficulty when 7-OHM-12-MBA was similarly incubated. When the metabolite was present in the incubation products, it was characterised both by its chromatographic properties and by its u.v. spectrum. The spectrum is similar to that of 10,11-dihydrobenz[a]anthracene except that some of the maxima are shifted to longer wavelengths in the metabolite (see Fig. 7). For this reason the metabolite is

formulated as 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene (10,11-diHOH-7-OHM-12-MBA) (Fig. 1; formula E). A closely related metabolite of both 7-MBA and 7-OHMBA is described below.

The u.v. spectrum of the metabolite formed from the hydroxymethyl derivative by liver homogenates from pretreated rats and formulated[7] as 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene (8,9-diHOH-7-OHM-12-

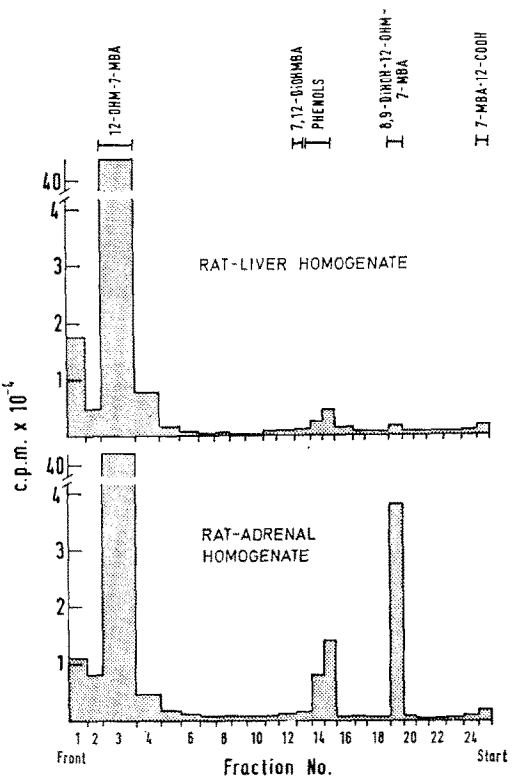

FIG. 5. The metabolism of 12-hydroxymethyl-7-methylbenz[a]anthracene in homogenates of the livers and adrenals of normal rats. The results shown were obtained as described in the text: the substrate (100 mμmoles; 2·35 × 10^6 cpm) was incubated with homogenate (equivalent to 500 mg of tissue) and the mixture extracted with ethyl/acetate (10 ml). The products present in 5 ml of this extract were chromatographed on TLCs and the radioactivity in bands of silica gel determined. The positions of metabolites on the chromatograms are shown thus:———Abbreviations are those used in the text.

MBA) (Fig. 1; D) is shown in Fig. 8. The spectrum is similar to that of 8,9-DiHOHDMBA.[14] The dihydrodiol (D) was also detected in the products from the incubation of 7-OHM-12-MBA with liver homogenates from animals of the other species mentioned above.

7,12-DiOHMBA (Fig. 1; B) was a comparatively minor metabolite in these experiments. The phenolic metabolites were previously tentatively identified as the 3- and 4-hydroxy derivatives of 7-OHM-12-MBA:[7] a product with the chromatographic

properties of the 4-hydroxy compound was formed when 4-OHDMBA was incubated with rat-liver homogenate.[8] 12-MBA-7-COOH (Fig. 1; F) has also been previously identified in the metabolic products of the hydroxymethyl compound.[7]

Metabolism of 12-OHM-7-MBA *in liver homogenates.* The major product from the incubation of the hydroxymethyl derivative with liver homogenates from pretreated rats and from mice, guinea pigs, hamsters and rabbits was that previously formulated as 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]-anthracene (8,9-DiHOH-12-OHM-7-MBA) (Fig. 2; D): the u.v. spectrum shown in Fig. 8 is similar

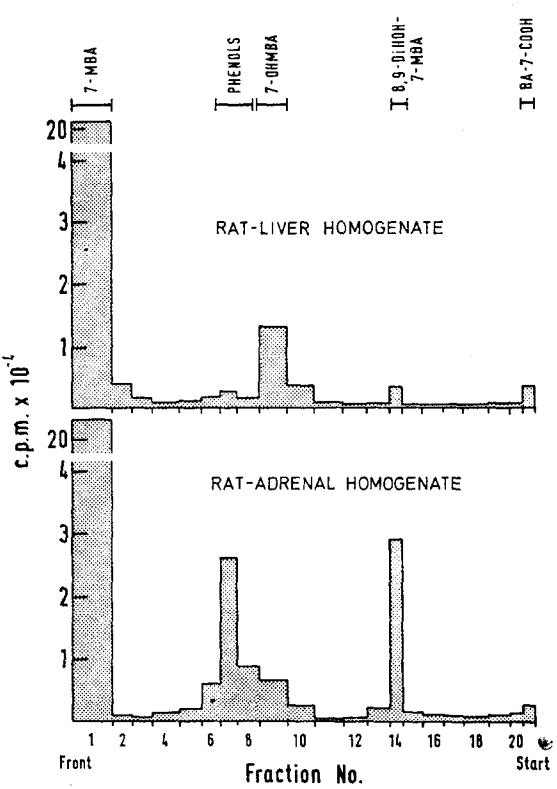

FIG. 6. The metabolism of 7-methylbenz[a]anthracene in homogenates of the livers and adrenals of normal rats. The results shown were obtained as described in the text: the substrate (100 mμmole; $1{\cdot}2 \times 10^6$ cpm) was incubated with homogenate (equivalent to 500 mg of tissue) and the mixture extracted with ethyl acetate (10 ml). The products present in 5 ml of this extract were chromatographed on TLCs and the radioactivity in bands of silica gel determined. The positions of metabolites on the chromatograms are shown thus:———Abbreviations are those used in the text.

to those of the isomeric 8,9-DiHOH-7-OHM-12-MBA and the DMBA metabolite, 8,9-DiHOHDMBA.[14] There was no evidence in any of these experiments for the formation of a product with the u.v. spectrum expected for an 10,11-dihydro-10,11-dihydroxy compound. The formation of DiOHMBA and the phenolic metabolites has been discussed previously:[7] the latter are probably the 3- and 4-hydroxy derivatives of 12-OHM-7-MBA. A synthetic sample of 7-MBA-12-COOH (Fig. 2; E) was

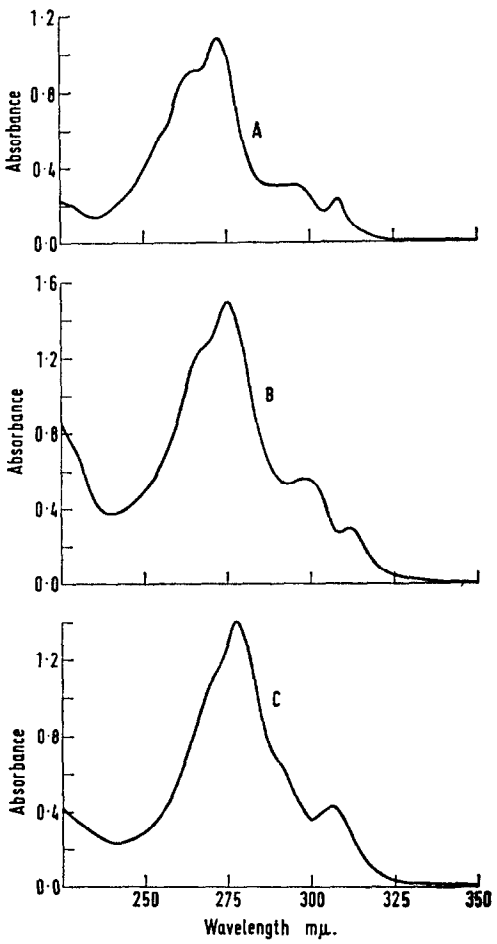

FIG. 7. Ultra-violet spectra of A, 10,11-dihydrobenz[a]anthracene; B, 10,11-dihydro-10,11-dihydroxy-7-hydroxymethylbenz[a]anthracene and C, 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene. Spectra were obtained as described in the text.

not available for direct comparison, but the chromatographic properties of this acid should be similar to those of 12-MBA-7-COOH.

Metabolism of 7-MBA *and* 7-OHMBA *in liver homogenates.* 7-MBA was converted by liver homogenates from pretreated rats and from those of mice, guinea pigs, hamsters and rabbits into 8,9-DiHOH-7-MBA and small amounts of 8,9-DiHOH-7-OHMBA and a metabolite believed to be 10,11-DiHOH-7-OHMBA. Neither of the two dihydrodiols of the hydroxymethyl derivative was detected when liver homogenates from normal rats were used. 7-OHMBA was converted into 8,9-DiHOH-7-OHMBA and 10,11-DiHOH-7-OHMBA by liver homogenates from animals of all the species investigated.

The presence of 10,11-DiHOH-7-OHMBA was detected in the products from these incubations by its behaviour on two-dimensional, acid-treated TLCs, when it gave rise

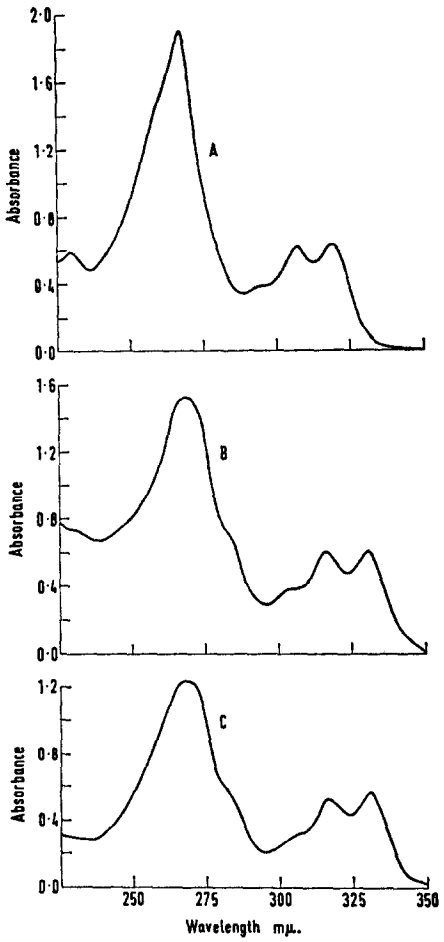

FIG. 8. Ultra-violet spectra of A, 8,9-dihydro-8,9-dihydroxy-7-hydroxymethylbenz[a]anthracene; B, 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene and C, 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]anthracene. Spectra were obtained as described in the text.

to a product, detected in u.v. light, with a violet fluorescence that turned green with ammonia. The metabolite was also characterised by its u.v. spectrum: that of the product obtained by the incubation of 7-OHMBA with liver homogenate from pre-treated rats is shown in Fig. 7. The spectrum of 8,9-DiHOH-7-OHMBA similarly obtained is shown in Fig. 8.

The incubation products of 7-MBA with homogenates of the livers of mice, guinea pigs, hamsters and rabbbits all contained a metabolite with the chromatographic properties of 7-OHMBA: the formation of this compound as a metabolite of 7-MBA in rat-liver homogenates has been reported.[13]

Comparative experiments with liver and adrenal homogenates from normal rats. The results of four series of experiments in which (a) DMBA, (b) 7-OHM-12-MBA, (c) 12-OHM-7-MBA or (d) 7-MBA were incubated with either liver or adrenal homogenates are shown in Figs. 3–6. The four series cannot be directly compared since

different preparations were used for each. The results from each series of experiments are discussed individually below.

(a) *Metabolism of DMBA.* Figure 3 shows that in the liver homogenate experiment DMBA was metabolized essentially as described previously.[1,7] The major products were the hydroxymethyl derivatives, 7-OHM-12-MBA and 12-OHM-7-MBA. Small amounts of 4-OHDMBA and 8,9-DiHOHDMBA were also formed and possibly the carboxylic acids, 12-MBA-7-COOH and 7-MBA-12-COOH, arising from further oxidation of the hydroxymethyl groups.

The metabolism of DMBA by adrenal homogenate was quite different. The major product was 8,9-DiHOHDMBA and the products believed to be 3- and 4-OHDMBA were also present. The absolute identification of the phenols proved difficult, but high counts of radioactivity were associated with the 4-OHDMBA spot when the incubation products were chromatographed in solvent (a), (b) or (c) on TLCs together with this phenol. The product believed to be 3-OHDMBA is difficult to separate chromatographically from 7-OHM-12-MBA.[1] It is possible, therefore, that the counts of radioactivity associated with this region of the chromatogram are due to the phenol rather than to the hydroxymethyl derivative. The fact that very little 12-OHM-7-MBA appears to be formed from DMBA by adrenal homogenate supports this view and indicates that hydroxylation of the methyl groups of DMBA by this system is not an important metabolic pathway.

Confirmatory evidence for the presence of 8,9-DiHOHDMBA in the metabolic products was obtained on a two-dimensional, acid-treated TLC described above: when the products, together with the products from an incubation of unlabelled DMBA with liver homogenate from pretreated rats were chromatographed in this system, much of the radioactivity was associated with the phenolic acid-decomposition product of the dihydrodiol.

(b) *Metabolism of 7-OHM-12-MBA.* Figure 4 shows that 7-OHM-12-MBA is converted by rat-liver homogenate into small amounts of 7,12-DiHOH, phenolic metabolites, 8,9-DiHOH-7-OHM-12-MBA and 10,11-DiHOH-7-OHM-12-MBA. Rat-adrenal homogenates, on the other hand, converts the hydroxymethyl derivative mainly into products with the chromatographic properties of the phenolic derivatives described above and of 8,9-DiHOH-7-OHM-12-MBA and 10,11-DiHOH-7-OHM-12-MBA. The identities of the two dihydrodiols were confirmed on two-dimensional acid-treated TLCs in a manner similar to that used for the adrenal metabolite of DMBA, 8,9-DiHOHDMBA, except that the marker compounds were obtained from the incubation of unlabelled 7-OHM-12-MBA with rat-liver homogenate. With both dihydrodiols radioactivity was associated with the phenolic decomposition products.

(c) *Metabolism of 12-OHM-7-MBA.* The results shown in Fig. 5 show that 12-OHM-7-MBA is converted by rat-liver homogenate into small amounts of 7,12-DiHOH, phenolic metabolites and 8,9-Di-HOH-12-OHM-7-MBA. With rat-adrenal homogenates, the products were mainly those with the chromatographic properties of the ring-hydroxylated metabolites, 8,9-DiHOH-12-OHM-7-MBA and the phenols. The identity of the dihydrodiol was confirmed on a two-dimensional acid-treated TLC as described above using the products of the incubation of 12-OHM-7-MBA with rat-liver homogenate as a source of marked compound.

(d) *Metabolism of 7-MBA.* The results shown in Fig. 6 indicate that the metabolism of 7-MBA in rat-liver homogenate was essentially that described previously:[13] small

amounts of phenols, 7-OHMBA and 8,9-DiHOH-7-MBA were formed. The products from the incubation of 7-MBA with rat-adrenal homogenate had the chromatographic properties of the phenols (presumably a mixture of the 3- and 4-hydroxy derivatives of 7-MBA) and 8,9-DiHOH-7-MBA. The dihydrodiol was characterised on 9 two-dimensional, acid-treated TLC as before using the metabolite formed from 7-MBA by rat-liver homogenate as a marker.

(2) *Quantitative experiments*

The results of these experiments given in Table 1 show that the relative proportions of the metabolites of DMBA and its hydroxymethyl derivatives formed by liver homogenates from normal rats are similar to those reported elsewhere.[8] With livers from rats that were pretreated with 3-methylcholanthrene, however, there were large increases in the proportions of the ring-hydroxylation products (phenols and dihydrodiols) formed. The conditions used in these experiments are not necessarily optimal: time course studies and studies on the effects of alterations in substrate concentrations on the yields of products have not been carried out.

A comparison of the results of the incubations of rat-liver and rat-adrenal homogenates both with DMBA and its hydroxymethyl derivatives showed that, on a milligram of protein basis, the latter homogenates were more efficient than the former in converting the substrates into ring-hydroxylated products. Moreover, there was, at the most, only two-fold increases in the amounts of the products formed by adrenal homogenates from pre-treated rats compared with those formed by adrenal-homogenates from normal animals.

In the experiments with DMBA, the dihydrodiols, 8,9-DiHOH-7-OHM-12-MBA, 8,9-DiHOH-12-OHM-7-MBA and 10,11-DiHOH-7-OHM-12-MBA, whose formation requires both ring- and methyl group-hydroxylation of the hydrocarbon, were formed in the experiments with the liver homogenates from treated rats, but not in those with adrenal homogenates. The dihydrodiols were formed, however, when the hydroxymethyl derivatives themselves were used as substrates in the experiments with adrenal homogenates. This provides further evidence that little or no hydroxylation of the methyl groups of DMBA occurs with these homogenates.

DISCUSSION

The earlier work on the relationship of the metabolism of DMBA to its ability to induce adrenal necrosis in rats suggested that the metabolic hydroxylation of the methyl group at C-7 by the liver was an essential prerequisite. This seemed likely because 7-OHM-12-MBA was more active as an inducer of adrenal necrosis than the parent hydrocarbon.[5] Moreover, if the liver function was impaired, either by treating the rat with carbon tetrachloride or by partial hepatectomy, then the effect of DMBA on the adrenals was reduced, whereas that of 7-OHM-12-MBA was unaffected.[16] Rats can be protected from the DMBA- or 7-OHM-12-MBA-induced adrenal damage by pretreatment with any one of a number of compounds, most of which are known to be inducers of hepatic microsomal enzymes:[5,10,17] the effect of the protectors could be abolished by the administration of ethionine.[18] It seemed likely, therefore, that the effect of the "protectors" was both to increase the amounts of the ring-hydroxylated products at the expense of hydroxylation of the methyl group and to deactivate the hydroxymethyl compound by further ring hydroxylation.[6,7] The results given here provide

further evidence that these enzyme-induction processes occur when liver homogenate from rats pretreated with 3-methylcholanthrene are used. Studies on the rates of metabolism of the substrate *in vivo* have not been reported so that some caution is necessary in applying the results of the *in vitro* studies to the situation in the whole animal.

Evidence has been adduced that 7-OHM-12-MBA may not be the proximate agent responsible for adrenal necrosis: the hydroxylating enzyme inhibitor, SKF 525A (β-diethylaminoethyldiphenyl-*n*-propyl acetate), protects against DMBA and 7-OHM-12-MBA-induced adrenal necrosis.[19] Moreover, 3-methylcholanthrene will protect the adrenals against large doses of DMBA and 7-OHM-12-MBA injected intravenously:[20] it seemed unlikely that increases in hepatic enzymes could inactivate large amounts of these compounds before the adrenals are exposed to them. In the work now described, however, no evidence has been found that DMBA or 7-OHM-12-MBA are converted by liver or adrenal homogenates into products that might be active in inducing adrenal necrosis. All the products detected were of the types that are formed in the metabolism of many other aromatic hydrocarbons[14] which are not active in the induction of adrenal necrosis. The dihydrodiol, 10,11-DiHOH-7-OHM-12-MBA, has not previously been identified as a metabolite of DMBA or of 7-OHM-12-MBA: hydroxylation of the 10,11-bond of 12-OHM-7-MBA, a compound that is not an inducer of adrenal necrosis,[5] does not occur. It seems unlikely, however, that this difference in metabolism between the hydroxymethyl derivatives is related to the difference in their ability to induce adrenal necrosis since 7-MBA and 7-OHMBA, both of which are inactive as inducers of adrenal necrosis, are metabolised on the 10,11-bond to yield 10,11-DiHOH-7-OHMBA.

The fact that pretreatment with 3-methylcholanthrene, which is both a hepatic enzyme "inducer" and a "protector" against DMBA and 7-OHM-12-MBA-induced adrenal necrosis, has little effect on the levels of the hydroxylating enzymes in the adrenal suggests that the metabolism of DMBA and 7-OHM-12-MBA in the adrenal is not important in the induction of adrenal necrosis: if the action of the "protectors" is to alter the metabolic pathways of the producers of adrenal necrosis, then this must take place at some site in the body other than in the adrenals.

The results of the metabolism of 7-OHM-12-MBA by rat adrenal homogenate differ from those reported earlier[10] where no metabolism of the hydroxymethyl derivative by adrenal homogenate was detected. The reason for this difference is not known: in the earlier experiments,[10] metabolism of 7-OHM-12-MBA by rat-liver homogenate was found.

In agreement with the results now reported, metabolism of DMBA by adrenal homogenate from rats that were pretreated with 3-methylcholanthrene was similar to that in adrenal homogenate from normal animals.[9] The levels of "benzopyrene hydroxylase" are not raised in the adrenals of similarly pretreated rats.[21] Rat-adrenal homogenates were unable to demethylate *p*-chloro-*N*-methylaniline,[22] a reaction that presumably involves hydroxylation of the *N*-methyl group: *N*-demethylase activity was found in the adrenal of guinea pigs, but the levels were not increased by enzyme inducers. It thus appears that in adrenals of adult rats the levels of the enzyme that hydroxylate methyl groups are either low or completely absent, whereas the levels of the enzymes that carry out the ring-hydroxylations, at least of aromatic hydrocarbons, are relatively high.

With many hydrocarbons little or no metabolism occurs at the so-called "K-

regions",[14] which are the most active bonds in the molecules. In the work now described "K-region" dihydrodiols were not encountered. The experiments with rat-liver homogenate confirm earlier work[7] with DMBA and its hydroxymethyl derivatives, where the "K-region" dihydrodiols could not be detected. The experiments with rat-adrenal homogenates show that these preparations are similarly unable to metabolise the hydrocarbon or the hydroxymethyl derivatives at these bonds.

The experiments with liver homogenates from animals of species other than the rat indicate that they metabolise the hydrocarbons and the hydroxymethyl derivatives by metabolic routes similar to those of rat-liver homogenate. Although quantitative experiments were not carried out, the heights of the peaks in the u.v. spectra of the dihydrodiols show that guinea pig-, hamster- and rabbit-liver homogenates metabolise DMBA to dihydrodiols more readily than those from rats and mice.

It will be seen from Figs. 1, 2, 4 and 5, that homogenates of both rat liver and adrenal converted the hydroxymethyl derivatives of DMBA into products less polar than the substrates themselves. The nature of these metabolites is being investigated.

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