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QUALITATIVE AND QUANTITATIVE STUDIES ON THE METABOLISM OF A SERIES OF AROMATIC HYDROCARBONS BY RAT-LIVER PREPARATIONS

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Abstract—Qualitative and quantitative investigations on the metabolism in rat-liver preparations of eleven tritiated hydrocarbons, phenanthrene, pyrene, chrysene, benz[a]anthracene, dibenz[a,h]anthracene, dibenz[a,c]anthracene, benzo[a]pyrene, benzo[e]pyrene, 3-methylcholanthrene, 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene were carried out. The qualitative studies, using homogenates of the livers of rats that had been pretreated with 3-methylcholanthrene, showed that all the major ethyl acetate-soluble metabolites of these hydrocarbons have been recognised: in most cases the structure of the metabolites have been confirmed. Quantitative studies using microsomes or homogenates from the livers of normal or 3-methylcholanthrene-treated rats showed increases in the amounts of metabolites formed from the liver preparations of the treated animals as compared with those from the normal animals. The amounts of dihydrodiols formed on the so-called 'K-region' varied among the hydrocarbons: with benzo[e]pyrene, the K-region dihydrodiol was the major metabolite, whereas with chrysene and benzo[a]pyrene no products of this type were detected. Intermediate amounts of K-region products were formed with other hydrocarbons. Large differences in the rates at which the individual hydrocarbons were metabolised were also found.

QUALITATIVE studies on the metabolism of benz[a]anthracene,[1] dibenz[a,h]anthracene,[1] benzo[a]pyrene,[2, 3] benzo[e]pyrene,[4] 3-methylcholanthrene,[5] 7-methylbenz[a]anthracene[6] and 7,12-dimethylbenz[a]anthracene[7–11] by rat-liver homogenates have been reported. The metabolism by rat-liver microsomes of phenanthrene, pyrene and benz[a]anthracene has also been investigated.[12] The many investigations of the metabolism of aromatic hydrocarbons in whole animals include qualitative studies on the metabolism of phenanthrene,[13–16] pyrene,[17] chrysene,[18, 19] benz[a]anthracene,[20] dibenz[a,h]anthracene[21, 22] and benzo[a]pyrene.[23, 24] Quantitative studies have been made[25] on the metabolism of 7,12-dimethylbenz[a]anthracene by rat-liver homogenates from rats of different ages.

In the work now described, qualitative investigations of the metabolism, by homogenates of the livers of rats pretreated with 3-methylcholanthrene, of phenanthrene (Fig. 1; formula A), pyrene (Fig. 2; A), chrysene (Fig. 3; A), benz[a]anthracene (Fig. 4; A), dibenz[a,h]anthracene (Fig. 5; A), dibenz[a,c]anthracene (Fig. 6; A), benzo[a]pyrene (Fig. 7; A), benzo[e]pyrene (Fig. 8; A), 3-methylcholanthrene (Fig. 9; A), 7-methylbenz[a]anthracene (Fig. 10; A) and 7,12-dimethylbenz[a]anthracene (Fig. 11;

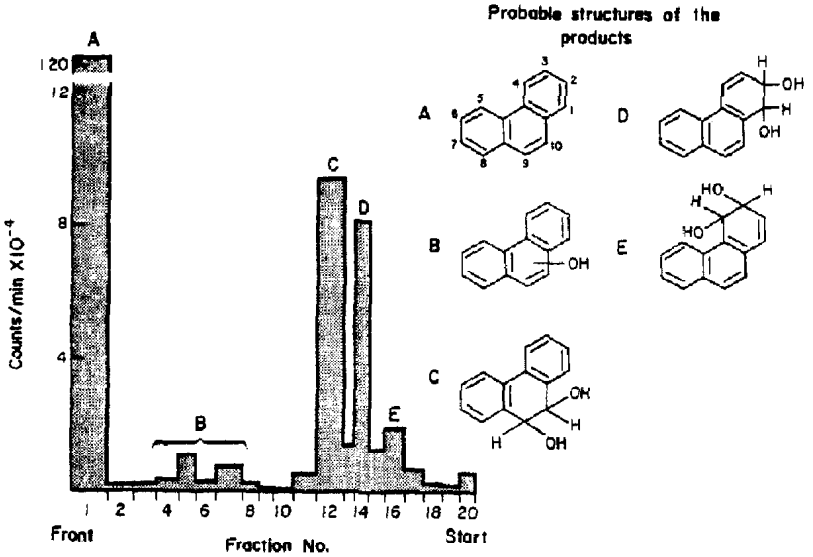

FIG. 1. Metabolism of phenanthrene.

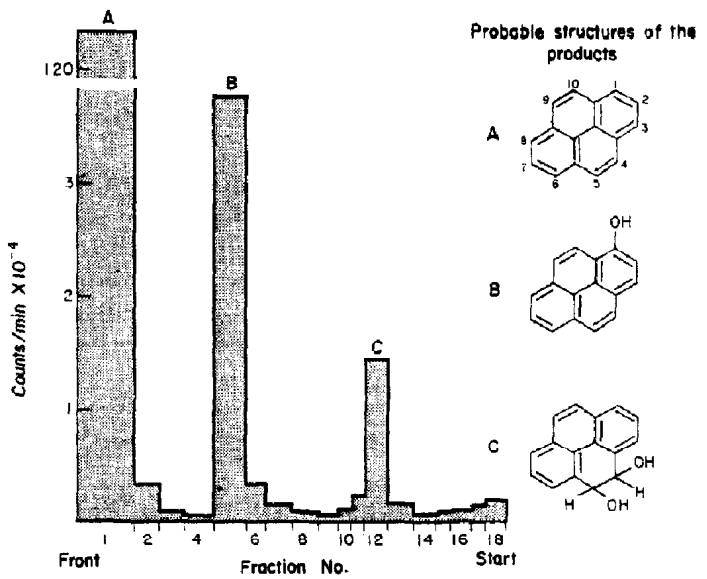

FIG. 2. Metabolism of pyrene.

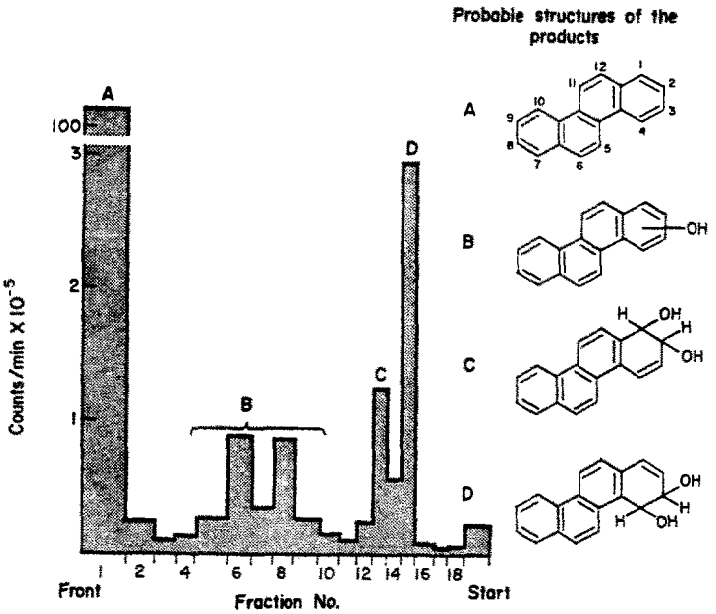

FIG. 3. Metabolism of chrysene.

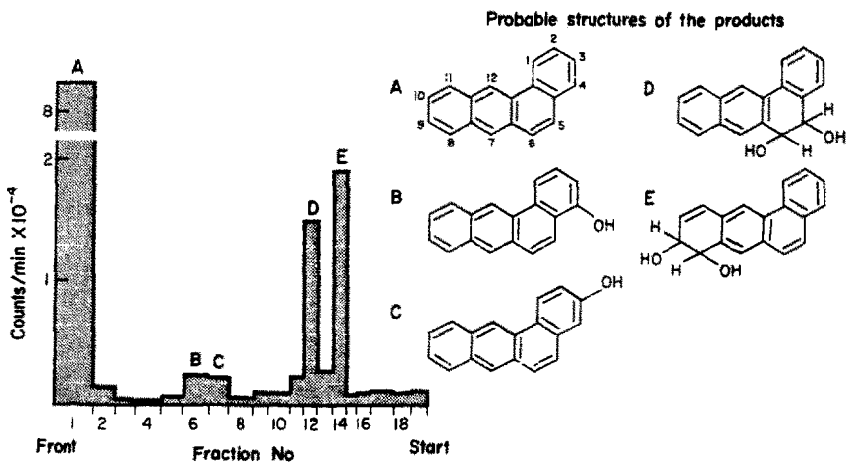

FIG. 4. Metabolism of benz[a]anthracene.

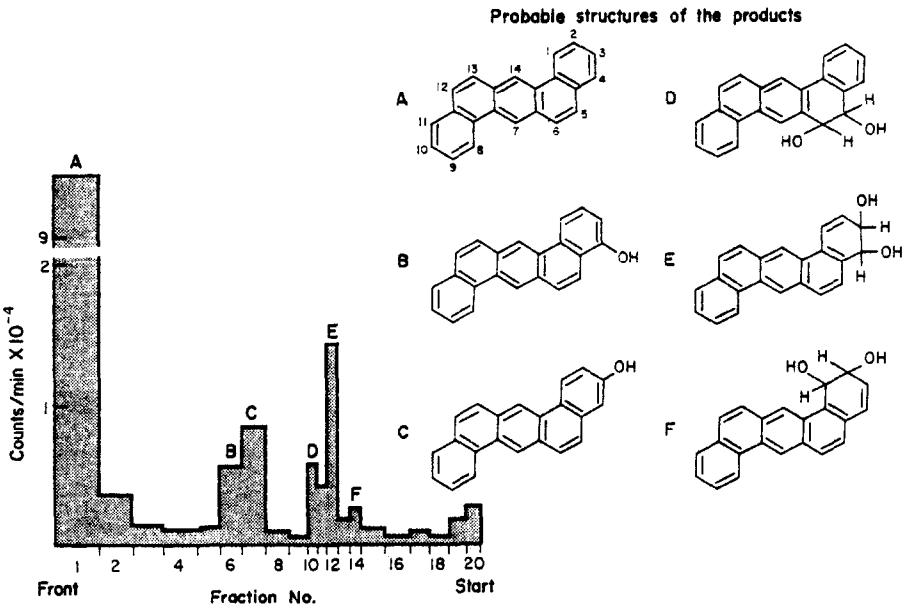

FIG. 5. Metabolism of dibenz[a,h]anthracene.

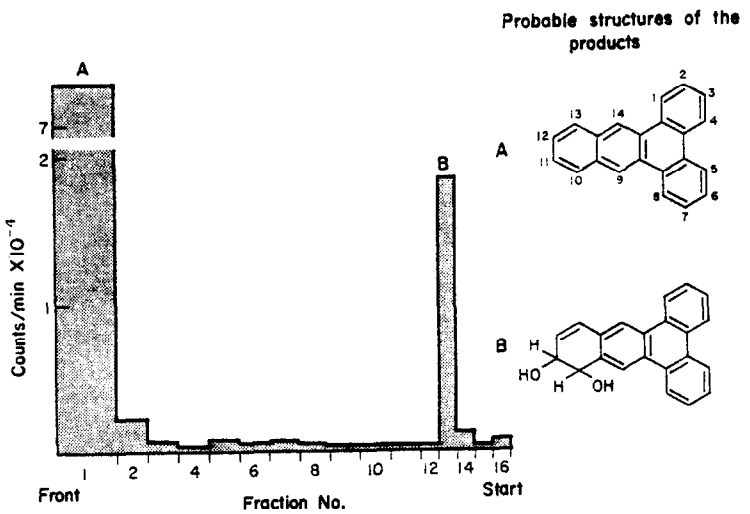

FIG. 6. Metabolism of dibenz[a,c]anthracene.

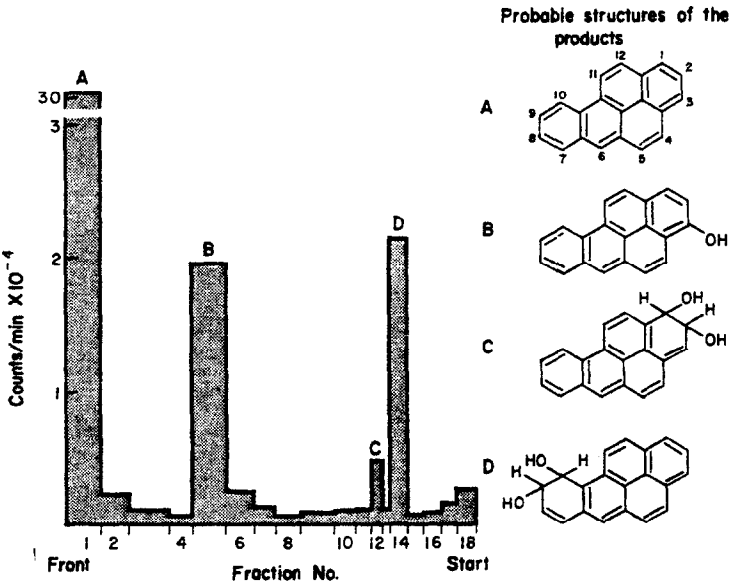

FIG. 7. Metabolism of benzo[a]pyrene.

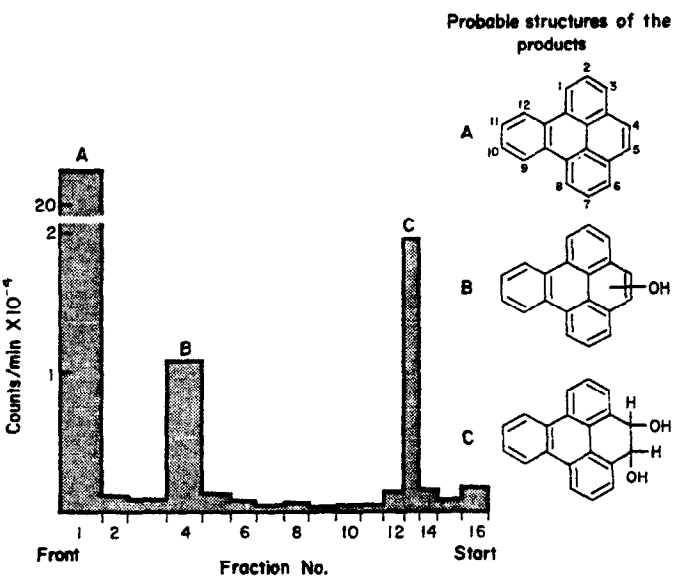

FIG. 8. Metabolism of benzo[e]pyrene.

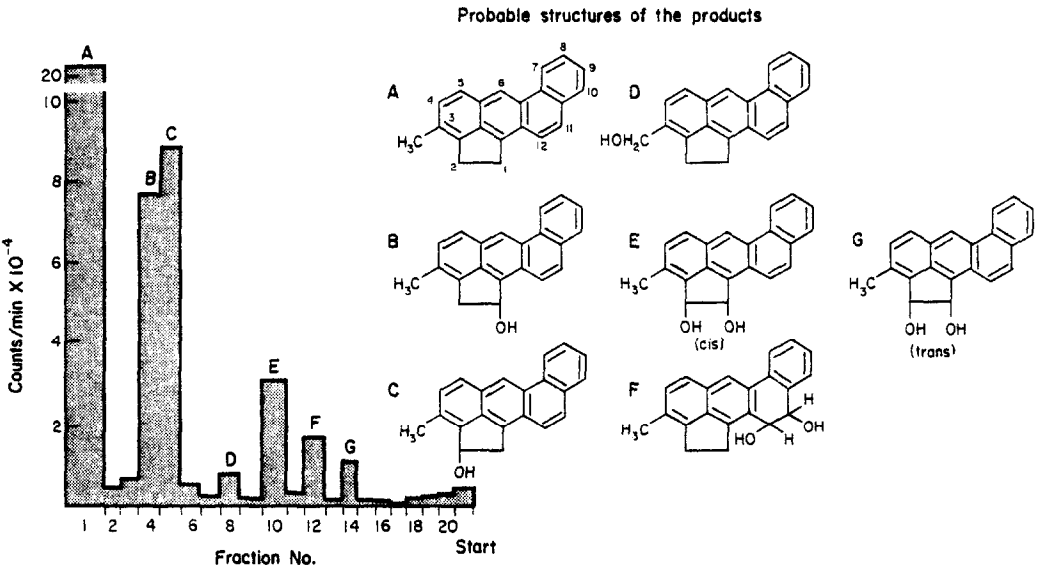

FIG. 9. Metabolism of 3-methylcholanthrene.

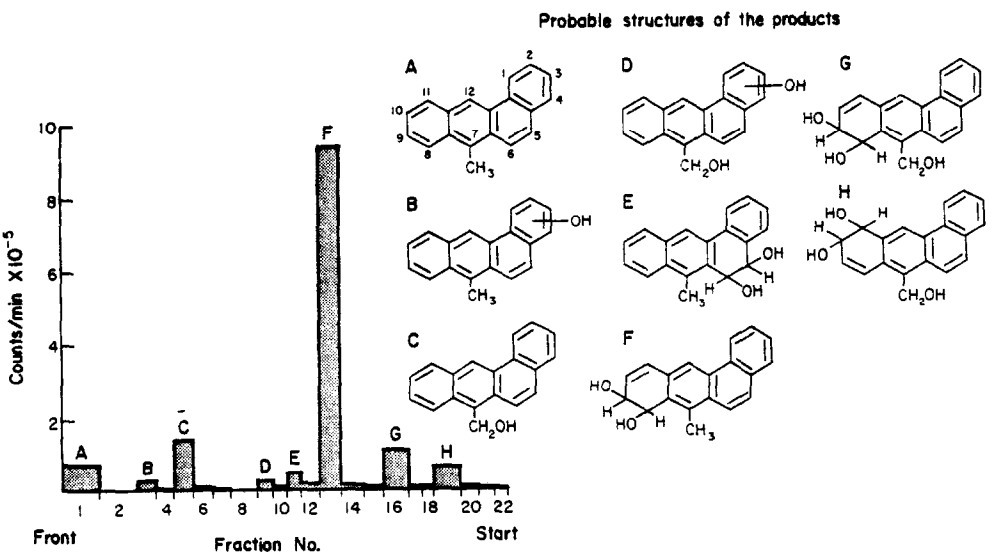

FIG. 10. Metabolism of 7-methylbenz[a]anthracene.

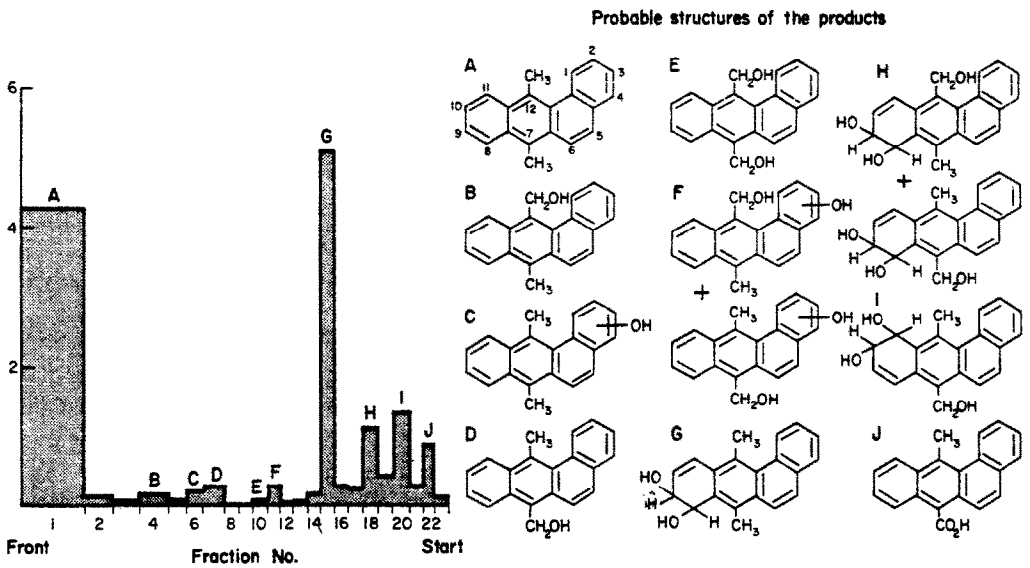

FIG. 11. Metabolism of 7,12-dimethylbenz[a]anthracene.

A) were carried out. Quantitative experiments on the metabolism of these hydrocarbons by liver microsomes and homogenates from normal and pretreated rats were also performed.

EXPERIMENTAL

Materials. The aromatic hydrocarbons, benz[a]anthracene (sp. act. 750 mc/mM), dibenz[a,h]anthracene (sp. act. 400 mc/mM), dibenz[a,c]anthracene (sp. act. 300 mc/mM), benzo[a]pyrene (sp. act. 500 mc/mM), benzo[e]pyrene (sp. act. 450 mc/mM), 3-methylcholanthrene (sp. act. 400 mc/mM) and 7,12-dimethylbenz[a]anthracene (sp. act. 400 mc/mM), generally labelled with tritium, were stock samples obtained from the Radiochemical Centre, Amersham, Bucks. Phenanthrene (sp. act. 169 mc/mM), pyrene (sp. act. 145 mc/mM) and chrysene (sp. act. 540 mc/mM) that had been generally labelled with tritium at the Radiochemical Centre, were purified by passing a solution in light petroleum (b.p. 60–80°) through an alumina-packed column. Tritiated 7-methylbenz[a]anthracene (sp. act. 1·78 c/mM) was the gift of Dr. P. Brookes of this Institute. Unlabelled hydrocarbons were obtained from commercial sources except 7-methylbenz[a]anthracene, which was synthesised as described.[6]

The reference compounds, including the 'K-region' dihydrodiols, related to phenanthrene,[14–16] pyrene,[17] chrysene,[26] benz[a]anthracene,[20] dibenz[a,h]anthracene,[1] benzo[a]pyrene,[3, 27] benzo[e]pyrene,[4] 3-methylcholanthrene,[5] 7-methylbenz[a]anthracene[5] and 7,12-dimethylbenz[a]anthracene[7] were prepared or obtained as indicated.

Thin-layer chromatography. Thin-layer chromatograms (TLCs) were prepared by coating glass plates (20 × 20 cm) with silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25 mm thickness. The chromatograms were developed for 15 cm in (a) benzene or (b) benzene–ethanol (19:1, v/v) or (c) benzene–ethanol (9:1, v/v) and were inspected in u.v. light whilst still wet, both before and after exposure to ammonia.

The 2,6-dichloroquinonechloroimide-Na_2CO_3 spray reagent used as indicated below has been described.[15]

The two-dimensional acid-treated TLCs referred to below were developed in the first direction with solvent (b) or (c), sprayed with concentrated hydrochloric acid and heated in an oven to 105° for 5 min. The chromatograms were developed in the second direction in solvent (a).

Ultra-violet spectra were measured in ethanol on a Unicam SP 800 recording spectrophotometer. Solutions of compounds separated on TLCs were obtained by removing the appropriate bands from the chromatograms, shaking the silica gel with ethanol and centrifuging to obtain a clear solution.

Experiments with rat-liver preparations. Male rats of the Chester Beatty strain (body wt. approx. 180 g) were used throughout these investigations. The pretreated animals were injected intraperitoneally with 3-methylcholanthrene (5 mg) in arachis oil (0·5 ml) 48 hr before they were killed. Liver homogenates and microsomal preparations containing the appropriate co-factors were prepared as described.[25, 28]

(a) *Large-scale incubations.* These were carried out using homogenates prepared from rat-liver (40 g), obtained from animals that had been pretreated with 3-methylcholanthrene, in 0·1 M phosphate buffer (pH 7·4, prepared from NaH_2PO_4 and Na_2HPO_4) (250 ml). The homogenates were heated to 37° and solutions of the aromatic hydrocarbons (5 μmoles) in acetone (5 ml) added. The hydrocarbon solutions were prepared from the unlabelled materials together with suitable amounts of the tritium-labelled compounds. The mixtures were incubated at 37° for 30 min and were shaken vigorously from time to time. At the end of the incubation period the mixtures were each extracted once with ethyl acetate (250 ml). The extracts were dried over anhydrous sodium sulphate and evaporated and the residues applied to the base lines of TLCs, which were developed with solvent (b) or (c) as indicated below. The positions of the major bands of metabolites were found by examination of the chromatograms in u.v. light. These, and intermediate bands, were marked off and the silica gel removed from the TLC and extracted with ethanol (3 ml). The solutions were centrifuged to remove silica gel and duplicate 0·1 ml portions of each fraction removed and the radioactivity present determined by liquid scintillation counting as described.[25] The results are shown in Figs. 1–11. The u.v. spectra of solutions giving high counts of radioactivity were measured as described above. The tests carried out on the metabolites present in these solutions to confirm their structures are described below.

Incubations were also carried out with homogenates from rat-liver (40 g) in which unlabelled hydrocarbons (2 mg) were used. The mixtures of metabolites thus obtained were used as chromatographic standards in the quantitative experiments described below.

(b) *Quantitative experiments.* Four series of experiments were carried out using (a) microsomes prepared from the livers of normal rats, (b) microsomes from the livers of rats pretreated with 3-methylcholanthrene, (c) homogenate from the livers of normal rats and (d) homogenates from the livers of rats pretreated with 3-methylcholanthrene. Each incubation mixture contained microsomes or homogenate equivalent to 1 g of liver together with the cofactors in the amounts previously described,[25, 28] in 0·1 M phosphate buffer (10 ml). Duplicate incubations were heated to 37° in a metabolic shaker (H. Mickle, Gomshall, Surrey) and 20 mμmoles of one of the tritiated hydrocarbons in acetone (0·1 ml) added. The mixtures were shaken at 37° for 15 min and

extracted with ethyl acetate (10 ml). The extracts were dried (Na_2SO_4) and portions (5 ml) evaporated to dryness and the residues chromatographed on TLCs. Portions of the appropriate metabolic products obtained from the incubations of the unlabelled hydrocarbons and other markers, such as the 'K-region' dihydrodiols (where the large-scale incubations indicated that these were not formed in appreciable amounts by metabolism) were added to the residues before chromatography. The chromatograms were developed in solvent (c) and examined in u.v. light. Fluorescent bands and intermediate bands, corresponding to those obtained in the large-scale experiments (see Figs. 1–11), were marked off and the silica gel removed. The radioactivity present was determined by liquid scintillation counting as previously described.[25] The results shown in Table 1 were calculated on the assumption that no tritium was lost during the metabolic transformations.

RESULTS

1. *Qualitative experiments*

The results of the examination of the products of the metabolism of each of the aromatic hydrocarbons by rat-liver homogenates from animals that have been pretreated with 3-methylcholanthrene are discussed separately. In general, they show that all the major ethyl acetate-soluble metabolic products of the hydrocarbons examined can be accounted for and that the structures assigned to the metabolites estimated in the quantitative experiments are probably correct. Because tritiated hydrocarbons of widely different specific activities were used in these experiments, the results from the individual hydrocarbon incubations cannot be directly compared.

The metabolism of phenanthrene. The results of the large-scale (Fig. 1) experiments show that the major metabolic products of phenanthrene in rat-liver preparations are dihydrodiols. This is in agreement with earlier experiments in whole animals,[14, 16] when the metabolic formation of mercapturic acids was also noted.[15] The phenanthrols formed in the large-scale incubation were further characterised: fractions 4–7 were combined and evaporated and the major portion of the residue examined on TLCs developed with solvent (a). The phenanthrols were detected both by their violet fluorescence in u.v. light and their colours with the 2,6-dichloroquinonechloroimide reagent (see Table 2): 1-, 2-, 3- and 4-phenanthrol were present but 9-phenanthrol was not detected. The relative proportions of the phenanthrols present were determined by chromatographing the remainder of the combined fractions, together with small amounts of the synthetic phenols, on a TLC developed in solvent (a). The fluorescent spots were removed and the radioactivity present in each was counted as before. The results in Table 2 show that 1- and 2-phenanthrol are the major products.

The identities of the dihydrodiols were established on two-dimensional acid-treated TLCs: 1,2-dihydro-1,2-dihydroxyphenanthrene (Fig. 1; D) yielded products indistinguishable from 1- and 2-phenanthrol, 3,4-dihydro-3,4-dihydroxyphenanthrene (Fig. 1; E) yielded 3- and 4-phenanthrol and 9,10-dihydro-9,10-dihydroxyphenanthrene (Fig. 1; C) yielded 9-phenanthrol. The relative amounts of the dihydrodiols formed in these experiments are similar to the relative amounts of the dihydrodiols and their conjugates formed when animals are treated with phenanthrene.[16]

The metabolism of pyrene. Only two major metabolic products were formed from pyrene by rat-liver preparations. 1-Hydroxypyrene (Fig. 2; B) was characterised in the extracts of the large-scale incubation by direct comparison with the authentic phenol

TABLE 1. AMOUNTS OF ETHYL ACETATE-SOLUBLE PRODUCTS FORMED FROM TRITIATED AROMATIC HYDROCARBONS BY RAT-LIVER PREPARATIONS

Hydrocarbon	Metabolite	Amount of ethyl acetate soluble product formed ($\mu\mu$moles/g wet wt of liver) from hydrocarbons per min by			
		Liver microsomes from		Liver homogenate from	
		Normal rats	3-Methylcholanthrene-treated rats	Normal rats	3-Methylcholanthrene-treated rats
Phenanthrene	Phenanthrols	8	42	6	23
	1,2-Dihydro-1,2-dihydroxy	42	241	36	182
	3,4-Dihydro-3,4-dihydroxy	8	44	5	21
	9,10-Dihydro-9,10-dihydroxy*	45	280	37	190
Pyrene	1-Hydroxy	2	41	3	38
	4,5-Dihydro-4,5-dihydroxy*	3	45	3	16
Chrysene	Chrysenols	3	44	2	34
	1,2-Dihydro-1,2-dihydroxy	2	22	1	15
	3,4-Dihydro-3,4-dihydroxy	4	58	3	38
	5,6-Dihydro-5,6-dihydroxy*	—	—	—	—
Benz[a]anthracene	3-Hydroxy	5	26	3	19
	4-Hydroxy	7	28	5	22
	5,6-Dihydro-5,6-dihydroxy*	34	282	22	190
	7,8-Dihydro-7,8-dihydroxy	26	227	25	210
Dibenz[a,h]anthracene	3-Hydroxy	21	81	12	63
	4-Hydroxy	15	52	8	57
	1,2-Dihydro-1,2-dihydroxy	4	29	3	19
	3,4-Dihydro-3,4-dihydroxy	28	152	21	110
	5,6-Dihydro-5,6-dihydroxy*	17	71	9	36
Dibenz[a,c]anthracene	Dihydrodihydroxy metabolite	41	242	16	109
Benzo[a]pyrene	3-Hydroxy	11	63	5	33
	1,2-Dihydro-1,2-dihydroxy	2	13	1	7
	4,5-Dihydro-4,5-dihydroxy*	—	—	—	—
	9,10-Dihydro-9,10-dihydroxy	13	68	7	39
	11,12-Dihydro-11,12-dihydroxy*	—	—	—	—
Benzo[e]pyrene	Phenolic metabolite	19	105	15	95
	4,5-Dihydro-4,5-dihydroxy*	36	188	27	158
3-Methylcholanthrene	1-Hydroxy	37	180	26	161
	2-Hydroxy	40	210	33	201
	3-Hydroxymethyl derivative	4	22	2	15
	cis-1,2-Dihydroxy	12	75	10	68

TABLE 1—*continued*

	trans-1,2-Dihydroxy	6	32	3	22
	11,12-Dihydro-11,12-dihydroxy*	11	62	5	36
7-Methylbenz[a]anthracene	Phenolic metabolites	2	13	2	9
	7-Hydroxymethyl derivative	58	112	40	62
	Phenolic metabolites of 7-hydroxymethyl derivative	—	12	—	4
	5,6-Dihydro-5,6-dihydroxy*	9	25	3	5
	8,9-Dihydro-8,9-dihydroxy	22	410	15	345
	8,9-Dihydro-8,9-dihydroxy-7-hydroxymethyl derivative	—	48	—	36
	10,11-Dihydro-10,11-dihydroxy 7-hydroxymethyl derivative	—	21	—	15
7,12-Dimethylbenz[a]-anthracene	Phenolic metabolites	10	34	7	19
	7-Hydroxymethyl derivative	32	38	36	26
	12-Hydroxymethyl derivative	42	32	23	22
	7,12-Dihydroxymethyl derivative	—	12	—	8
	Phenolic metabolites of the hydroxymethyl derivatives	—	34	—	22
	5,6-Dihydro-5,6-dihydroxy*	—	10	—	—
	8,9-Dihydro-8,9-dihydroxy	32	565	21	400
	8,9-Dihydro-8,9-dihydroxy metabolites of the hydroxy-methyl derivatives	—	101	—	82
	10,11-Dihydro-10,11-dihydroxy-7-hydroxy-methyl derivative	—	125	—	85
	7-Carboxy-12-methyl derivative	—	—	—	12

Incubations and estimations were carried out as described in the text. The values represent the means of duplicate determinations. The entry—indicates that no significant counts were obtained from the fractions containing these compounds.

* 'K-region' dihydrodiols.

TABLE 2. METABOLIC FORMATION OF PHENANTHROLS FROM PHENANTHRENE BY LIVER HOMOGENATE FROM 3-METHYLCHOLANTHRENE-TREATED RATS

Compound	R_f	Colour with 2,6-dichloroquinone-chloroimide and Na_2CO_3	Radioactivity associated with phenolic spot (cpm × 10^{-3})
1-Phenanthrol	0·32	Blue	20·2
2-Phenanthrol	0·22	Purple-red	10·4
3-Phenanthrol	0·28	Grey	4·1
4-Phenanthrol	0·45	Blue-green	6·5
9-Phenanthrol	0·35	Violet	0·1

Chromatograms were developed with solvent 1. Radioactivity was determined as described in the text. All compounds formed violet fluorescent spots when the chromatograms were examined in u.v. light.

on TLCs developed with solvents (a) and (b) and by its u.v. spectrum. 4,5-Dihydro-4,5-dihydroxypyrene (Fig. 2; C) was characterised on a two-dimensional, acid-treated TLC, when a phenol indistinguishable from 4-pyrenol was formed, and by its u.v. spectrum.

In whole animals, pyrene is converted into these metabolites[17] and into 1,3- and 1,6-dihydroxypyrenes,[29] which presumably arise by the further hydroxylation of 1-pyrenol.

The metabolism of chrysene. In the large-scale experiment, chrysene yielded a mixture of four phenols and two dihydrodiols (see Fig. 3). The two classes of compounds were interrelated since, with acid, each dihydrodiol yielded a pair of phenols that were chromatographically identical with phenols produced metabolically. The authentic chrysenols were not available for direct comparison, but their structures have been deduced by comparison of their chromatographic properties (see Table 3)

TABLE 3. METABOLIC FORMATION OF CHRYSENOLS FROM CHRYSENE BY LIVER HOMOGENATE FROM 3-METHYLCHOLANTHRENE-TREATED RATS

Probable identity of compound	R_f	Fluorescence		Colour with 2,6-2,6-dichloroquinone chloroimide and Na_2CO_3	Radioactivity associated with phenolic spot (cpm × 10^{-4})
		Immediate	After exposure to NH_3		
1-Chrysenol	0·36	Violet	Yellow	Blue	15·5
2-Chrysenol	0·21	Violet	Blue	Grey	3·2
3-Chrysenol	0·27	Violet	Blue-green	Brown	2·2
4-Chrysenol	0·55	Violet	Pink	Violet	8·3
5-Chrysenol	0·40	Violet	Blue	Blue-green	0·4

Chromatograms were developed with solvent 1. Radioactivity was determined as described in the text.

with those of the phenanthrols shown in Table 2 and, where possible, from a comparison with published u.v. spectra. The spectra of the chrysenols obtained by the elution of material from TLCs were ill-defined, but, for example, that of the product believed to be 1-chrysenol was very similar to that of the metabolite obtained from the faeces of mice injected with chrysene.[19] An estimate of the relative amounts of the chrysenols formed was obtained by combining fractions 5–9 from the large-scale

incubation and chromatographing a portion on a TLC developed with solvent (a). A portion of the phenol, presumably 5-chrysenol, obtained when synthetic *trans*-5,6-dihydro-5,6-dihydroxychrysene was heated with hydrochloric acid, was added to the combined fractions. The areas on the chromatograms containing the phenolic metabolites were detected by means of their fluorescence when the chromatograms were examined in u.v. light. These areas were removed from the chromatograms and the radioactivity present on the silica gel measured as before. The results shown in Table 3 indicate that 1-chrysenol, which was formed as a metabolite by rats treated with the hydrocarbon,[18] is the major, but not the only phenol formed by rat-liver homogenates.

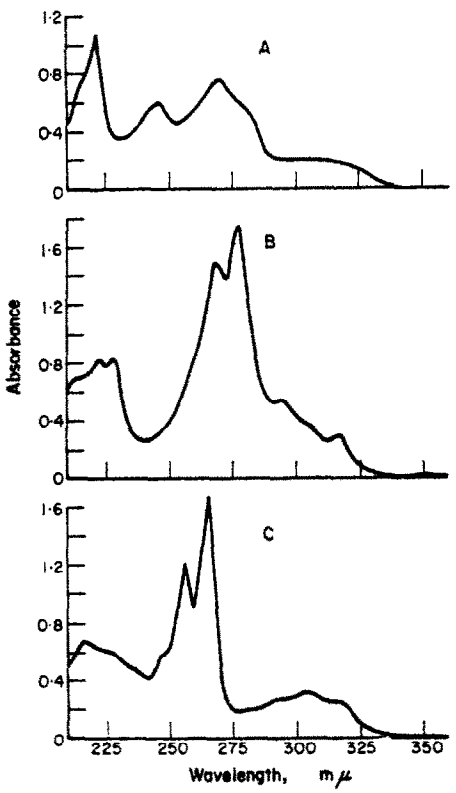

FIG. 12. Ultra-violet spectra: A, metabolite, probably 1,2-dihydro-1,2-dihydroxychrysene; B, metabolite, probably 3,4-dihydro-3,4-dihydroxychrysene; C, 5,6-dihydro-5,6-dihydroxychrysene.

On TLCs developed with either solvent (b) or (c), the faster-moving of the two dihydrodiols formed from chrysene by rat-liver homogenates formed a pink fluorescent spot in u.v. light, and, on two-dimensional, acid-treated TLCs, yielded phenols believed to be 1- and 2-chrysenols. The slower-moving compound similarly gave a violet fluorescent spot in u.v. light and yielded phenols believed to be 3- and 4-chrysenols. The dihydrodiols have therefore been formulated as 1,2-dihydro-1,2-dihydroxychrysene (Fig. 3; C) and 3,4-dihydro-3,4-dihydroxychrysene (Fig. 3; D)

respectively. Neither of the two dihydrodiols was identical with the synthetic *trans*-5,6-dihydro-5,6-dihydroxychrysene: no significant radioactivity was detected in the fraction in which the 5,6-compound was expected (see Fig. 3). The u.v. spectra of the three dihydrodihydroxy compounds are shown in Fig. 12.

The metabolism of benz[a]anthracene. Four metabolic products resulted from the metabolism of benz[a]anthracene in the large-scale incubation. Of the two phenols, the 4-hydroxy compound (Fig. 4; B) was characterised by direct comparison with the authentic compound on TLCs developed in solvent (a). The second phenol was identical in its chromatographic properties with the product excreted by rats treated with benz[a]anthracene that was previously[20] tentatively identified as 3-hydroxybenz-[a]anthracene (Fig. 4; C).

5,6-Dihydro-5,6-dihydroxybenz[a]anthracene (Fig. 4; D) was identified by direct comparison with the authentic dihydrodiol on TLCs and by the fact that it yielded a product indistinguishable from 5-hydroxybenz[a]anthracene on two-dimensional, acid-treated TLCs. The u.v. spectra of the metabolite and the synthetic dihydrodiol were identical.

The second dihydrodiol formed in the metabolism was identical on TLCs with the product formed by rats treated with benz[a]anthracene that was previously tentatively identified as 8,9-dihydro-8,9-dihydroxybenz[a]anthracene (Fig. 4; E).[20] Further evidence for its structure was obtained on two-dimensional acid-treated TLCs. The chromatographic properties and the u.v. spectra of the phenols produced were identical with synthetic 8- and 9-hydroxybenz[a]anthracene. The u.v. spectra of the phenols and the dihydrodiol from which they were derived are shown in Fig. 13.

These results are essentially in agreement with those from earlier experiments[1] when benz[a]anthracene was incubated with liver-homogenates from normal rats, except that the formation of small amounts of 1,2-dihydro-1,2-dihydroxybenz[a]anthracene and 12-hydroxybenz[a]anthracene was also detected in the earlier work. These products were not found in the work now reported, perhaps because of the different chromatographic conditions employed.

In previous experiments with whole animals it was found[20] that all the above metabolites were formed together with 1-hydroxybenz[a]anthracene and small amounts of a dihydrodiol believed to be 10,11-dihydro-10,11-dihydroxybenz[a]-anthracene: these metabolites were not identified in the work now described.

The metabolism of dibenz[a,h]anthracene. The major phenolic metabolites were chromatographically identical with products previously identified in experiments with rat-liver homogenates[1] as 4- and 3-hydroxybenz[a,h]anthracene (Fig. 5; B, C). Three dihydrodiols were also identified in these earlier experiments. In the present work, the identities of the dihydrodiols were confirmed on two-dimensional acid-treated thin-layer chromatograms. The product believed to be 1,2-dihydro-1,2-dihydroxybenz[a,h]-anthracene (Fig. 5; F) yielded 1- and 2-hydroxydibenz[a,h]anthracene, that believed to be 3,4-dihydro-3,4-dihydroxydibenz[a,h]anthracene (Fig. 5; E) yielded 3- and 4-hydroxydibenz[a,h]anthracene, chromatographically identical with the phenols B and C above and that believed to be 5,6-dihydrodihydroxydibenz[a,h]anthracene (Fig. 5; D) yielded 5-hydroxybenz[a,h]anthracene. The last dihydrodiol was also characterised by direct comparison on TLCs, developed with solvents (b) and (c), with the authentic *trans*-isomer, synthesized as previously described.[1]

Earlier work on the metabolism of dibenz[a,h]anthracene has been reviewed[30] and

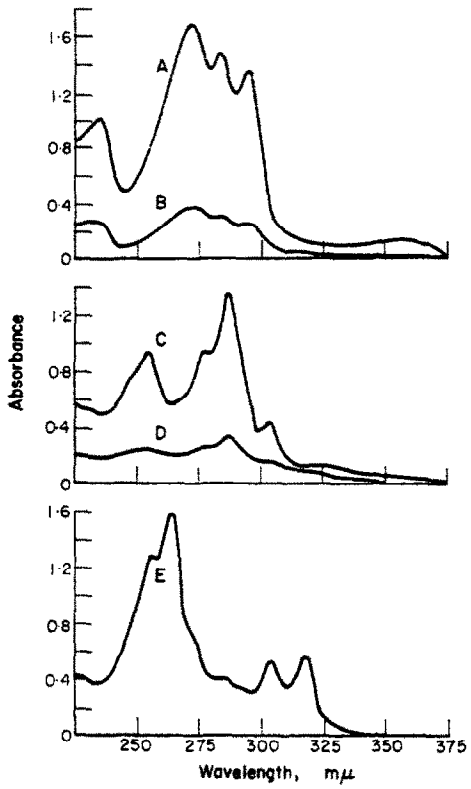

FIG. 13. Ultra-violet spectra: A, 8 hydroxybenz[a]anthracene; B, phenol obtained from acid decomposition of metabolite. C, 9-hydroxybenz[a]anthracene: D, phenol obtained from decomposition of metabolite; E metabolite, probably 8,9-dihydro-8,9-dihydroxybenz]a]anthracene.

shows that this hydrocarbon is converted by rabbits into 2,9-dihydroxydibenz[a,h]-anthracene and 2-hydroxydibenz[a,h]anthracene and by rats and mice into 4,10-dihydroxydibenz[a,h]anthracene.

The metabolism of dibenz[a,c]anthracene. Only one metabolic product was formed by rat-liver homogenates from this hydrocarbon. It appeared to be a dihydrodiol since it was decomposed by acid to yield two phenols which on TLCs developed in solvent (a) had R_f 0·33 and R_f 0·21. The faster-moving phenol was detected as a blue fluorescent spot when the chromatograms were examined in u.v. light, which turned pink on exposure to ammonia. Under similar conditions the slower-moving phenol formed a violet fluorescent spot that turned bright green with ammonia. The u.v. spectra of the phenols and the parent dihydrodiol are shown in Fig. 14. The dihydrodiol is tentatively formulated as 10,11-dihydro-10,11-dihydroxydibenz[a,c]anthracene (Fig. 6; B), by analogy with the metabolism of hydrocarbons such as anthracene[31] and benz[a]-anthracene.[1] The metabolism of dibenz[a,c]anthracene does not appear to have been studied previously.

The metabolism of benzo[a]pyrene. Three major products were formed in the metabolism of benzo[a]pyrene by rat-liver homogenates. The identity of 3-hydroxybenzo[a]-

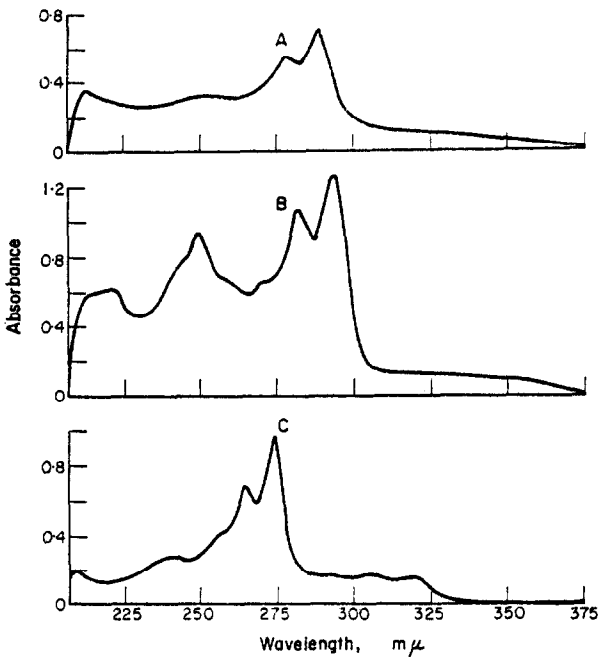

FIG. 14. Ultra-violet spectra: A and B, phenols obtained from acid-decomposition of metabolite; C, metabolite, possibly 10,11-dihydro-10,11-dihydroxydibenz[a,c]anthracene.

pyrene (Fig. 7; B) was confirmed by a comparison of its u.v. spectrum (Fig. 15) with published data.[2]

Evidence that the two dihydrodiols are 1,2-dihydro-1,2-dihydroxybenzo[a]pyrene (Fig. 9; C) and 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene (Fig. 7; D) has been presented.[3, 27] The u.v. spectra of the two metabolites are shown in Fig. 15: the spectrum of 9,10-dihydrobenzo[a]pyrene (see Fig. 15) is identical with that of the related dihydrodiol and provides confirmatory evidence for the proposed structure of this metabolite. The dihydrodiols were also examined on two-dimensional, acid-treated TLCs: the 1,2-dihydrodiol yielded a phenol previously shown[3] to be 1-hydroxybenzo[a]pyrene, whilst the 9,10-dihydrodiol yielded a phenol identical with authentic 9-hydroxybenzo[a]pyrene, prepared as previously described.[27]

The two 'K-region' dihydrodiols, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene and 11,12-dihydro-11,12-dihydroxybenzo[a]pyrene, were not detected in these experiments, thus confirming earlier[3] observations. Recent experiments[4] in which benzo[a]pyrene was incubated with mouse embryo cells also failed to yield the 'K-region' dihydrodiols, although the presence of the three metabolites B, C and D above was detected. The metabolism of benzo[a]pyrene in whole animals has been reviewed.[30]

The metabolism of benzo[e]pyrene. The products (see Fig. 8) obtained from the action of rat-liver homogenate on benzo[e]pyrene were identical with those previously reported.[4] The phenolic metabolite was previously tentatively identified as 3-hydroxybenzo[e]pyrene, but no further evidence for its structure was obtained in the present work. The dihydrodiol was characterised as 4,5-dihydro-4,5-dihydroxybenzo[e]pyrene

(Fig. 8; C) by a direct comparison with the synthetic compound on TLCs and by its u.v. spectrum. The u.v. spectra of the metabolites have been recorded elsewhere.[4]

The metabolism of benzo[e]pyrene in whole animals has not been studied, but mouse embryo cells grown in culture convert the hydrocarbon into the phenol and dihydrodiol described above.[4]

The metabolism of 3-methylcholanthrene. The metabolic products of 3-methylcholanthrene are shown in Fig. 9. The major metabolites were the 1- and 2-hydroxy derivatives (Fig. 9; B and C). Previous work[5] has shown that in rat-liver homogenates

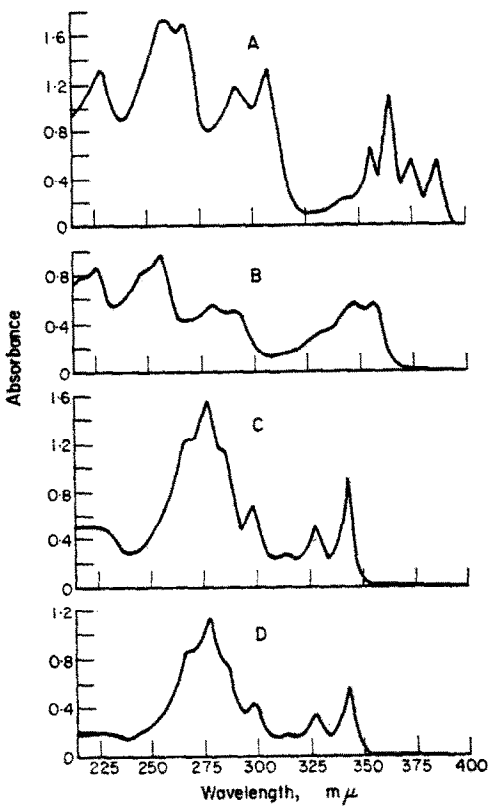

FIG. 15. Ultra-violet spectra: A, metabolite, probably 3-hydroxybenzo[a]pyrene; B, metabolite, probably 1,2-dihydro-1,2-dihydroxybenzo[a]pyrene; C, metabolite, probably 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene; D, 9, 10-dihydrobenzo[a]pyrene.

some non-enzymic oxidations of these products occur to yield the corresponding keto compounds. These were not detected in the present work because, in the solvent system used, they have the same mobility on TLCs as the parent hydrocarbon. The hydroxy compounds were identified by direct comparison with the synthetic compounds and by their u.v. spectra.

The third metabolite was identical in its chromatographic properties with a product previously tentatively identified as 3-hydroxymethylcholanthrene (Fig. 9; D): no

further evidence of its structure has been obtained. Evidence has been obtained[19] that 3-methylcholanthrene is converted by mice into cholanthrene 3-carboxylic acid, indicating metabolic attack on the 3-methyl group.

Products with the chromatographic properties of *cis*- and *trans*-1,2-dihydroxy-3-methylcholanthrene (Fig. 9; E and G) were also formed: these were further characterised on two-dimensional, acid-treated TLCs, when each yielded a product indistinguishable from 3-methylcholanthrene-2-one. Of the two products, the *cis*-isomer was present in the greater amount. The interconversion of the two isomers by liver homogenates has been discussed.[5] The K-region dihydrodiol, 11,12-dihydro-11,12-dihydroxy-3-methylcholanthrene (Fig. 9; F), was characterised by direct comparison of its properties on TLCs with the synthetic *trans*-isomer and by the formation of 11-hydroxy-3-methylcholanthrene when the product was chromatographed on two-dimensional, acid-treated TLCs.

The metabolism of 7-methylbenz[a]anthracene. The metabolism of this hydrocarbon in rat-liver homogenates was essentially the same as that previously described.[6] The phenolic metabolites (Fig. 10; B) were then tentatively identified as 3- and 4-hydroxy-7-methylbenz[a]anthracene: no further evidence of their structure has been obtained. The identity of 7-hydroxymethylbenz[a]anthracene (Fig. 10; C) was confirmed by a direct comparison with the synthetic compound on TLCs and by its u.v. spectrum.

The phenolic metabolites related to the 7-hydroxymethyl derivative were previously tentatively identified as 1-, 3- and 4-hydroxy-7-hydroxymethylbenz[a]anthracene (Fig. 10; D):[6] they were also formed when the hydroxymethyl derivative was itself incubated with rat-liver homogenate. Table 1 shows that they are only formed when livers from animals that had been pretreated with 3-methylcholanthrene are used.

Of the dihydrodiols formed, the K-region product, 5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene (Fig. 10; E) was a comparatively minor metabolite, especially when livers from animals that had been pretreated were used. Its structure was confirmed by direct comparison on TLCs with the synthetic compound and by its u.v. spectrum.

The major metabolic product from the livers of pretreated animals was a dihydrodiol believed to be 8,9-dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene (Fig. 10; F). The u.v. spectrum of this metabolite is shown in Fig. 16 and is essentially the same as that of the corresponding metabolite of benz[a]anthracene, 8,9-dihydro-8,9-dihydroxybenz[a]anthracene (Fig. 4; D), whose u.v. spectrum is shown in Fig. 13. The corresponding metabolite of 7,12-dimethylbenz[a]anthracene, 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene (Fig. 11; G) (described below) also gives a similar u.v. spectrum (see Fig. 16): with both the methyl derivatives, however, the peaks are shifted to longer wavelengths.

The dihydrodiols derived from the 7-hydroxymethyl compound were tentatively assigned the structures 8,9-dihydro-8,9-dihydroxy-7-hydroxymethylbenz[a]anthracene (Fig. 10; G) and 10,11-dihydro-10,11-dihydroxy-7-hydroxymethylbenz[a]anthracene (Fig. 10; H). The evidence for this will be described in a later paper.

The metabolism of 7-methylbenz[a]anthracene in whole animals has not been described. The hydrocarbon is converted by mouse embryo cells into the hydroxymethyl derivative and into 8,9-dihydrodiols.[4]

The metabolism of 7,12-dimethylbenz[a]anthracene. The metabolism of 7,12-dimethylbenz[a]anthracene in the large-scale rat-liver homogenate gave results similar

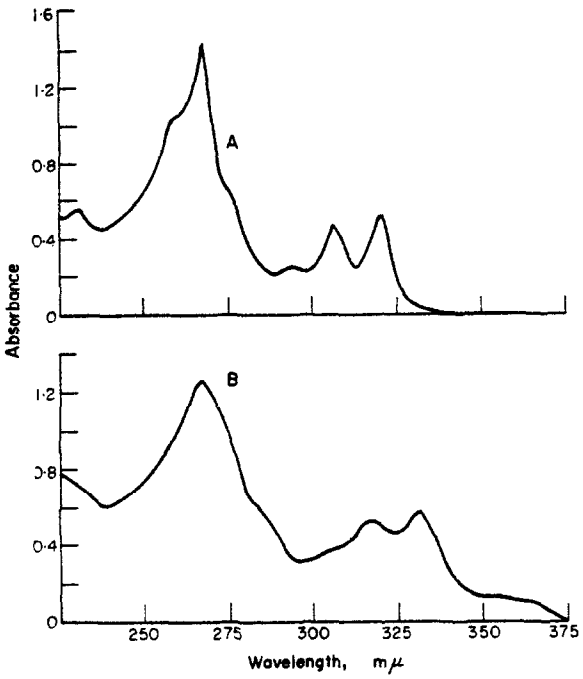

FIG. 16. Ultra-violet spectra: A, metabolite, probably 8,9-dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene; B, metabolite, probably 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene.

to those previously described,[7, 8, 25] except that the amounts of the hydroxymethyl derivatives (Fig. 11; B and D) were much reduced. This is presumably because in the highly active preparation used, the derivatives were further metabolised into the 8,9-dihydro-8,9-dihydroxy derivatives of the 7- and 12-hydroxymethyl compounds (Fig. 11; H) and into 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene (Fig. 11; I): the formation and characterization of these metabolites will be discussed in a later paper.

The formation of the dihydrodiol, 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene (Fig. 11; G), has been discussed previously:[8] its u.v. spectrum is recorded in Fig. 16. The relationship of this compound to the corresponding metabolites of benz[a]anthracene and 7-methylbenz[a]anthracene has been described above.

The phenolic metabolites were previously tentatively identified as 3- and 4-hydroxy-7,12-dimethylbenz[a]anthracene.[7] No further evidence of their structures was found in this work, but it has been shown[11] that one of the metabolites of the hydrocarbon in rat-liver homogenates is chromatographically identical to synthetic 4-hydroxy-7,12-dimethylbenz[a]anthracene.

Other products seen when livers from animals pretreated with 3-methylcholanthrene were used were 7,12-dihydroxymethylbenz[a]anthracene (Fig. 11; E), a mixture of phenolic derivatives of the hydroxymethyl derivatives (Fig. 11; F) and 12-methylbenz[a]anthracene-7-carboxylic acid (Fig. 11; J): the presence of these metabolites in the products of the incubation of 7,12-dimethylbenz[a]anthracene with rat-liver homogenate has been described previously.[8]

The K-region dihydrodiol, 5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene was not detected in the products of large-scale experiments in which 7,12-dimethylbenz[a]anthracene was incubated with rat-liver homogenate. Its formation when the hydrocarbon is incubated with liver microsomes from rats pretreated with 3-methylcholanthrene has been demonstrated.[25]

2. *Quantitative experiments*

The results of the four series of experiments are shown in Table 1. Because the alteration of any one of a number of factors such as the method of tissue homogenization,[32] the rate of shaking and the availability of oxygen, the amount of substrate added to the incubation mixtures, and the age, sex and nutritional status of the animals[33] from which the livers were taken, affects the rate at which hydrocarbons are metabolized by liver preparations, the experiments in the series a–d were carried out at the same time on the same batch of liver preparation and under the same incubation conditions. Thus, whilst a meaningful comparison of the amounts of the metabolites formed from the individual hydrocarbons in any one series may be made, a comparison of the amounts of metabolites formed from any one hydrocarbon in the four series is probably less reliable. The differences between the amounts of metabolites formed by preparations from pretreated animals and the similar preparations from untreated animals are sufficiently great, however, to demonstrate the induction of hepatic enzymes in the animals treated with 3-methylcholanthrene. The increases in the amounts of metabolites formed varied with each hydrocarbon: increases of between 5- and 20-fold (or even more) were obtained, but for any one hydrocarbon, the relative increases in the amounts of each metabolite formed from the liver preparation from normal and pretreated animals were generally of the same order. Exceptions were found with the two methylbenz[a]anthracenes, where ring-hydroxylated products were formed by the preparations from pretreated animals that were not formed by the preparations from normal animals. This is probably because the hydroxymethyl derivatives are intermediates in the oxidations and are further metabolized by the more active preparations.

DISCUSSION

It is likely that the microsomal oxidation of aromatic hydrocarbons involves the addition of oxygen, by a mechanism not yet completely understood, to specific double bonds to form epoxides. These then either rearrange to yield phenols, react with water to yield dihydrodiols or react with glutathione to yield glutathione conjugates.[1, 16, 34, 35] The work now described is concerned with products formed by the first two reactions, which are catalysed by proteins and microsomal enzymes respectively,[36, 37] since any glutathione conjugates formed in the experiments with liver homogenates are not extractable from aqueous solutions by ethyl acetate. They would not be formed in the experiments with microsomes because both glutathione and the enzymes catalysing its addition to the intermediates formed in the metabolism of hydrocarbons are absent.[38]

Since the microsomal oxidations involve the addition of oxygen to aromatic double bonds, it might be expected that the bonds with the greatest double bond character would react most readily: this should be reflected in the amounts of phenols and dihydrodiols finally produced by the reactions described above. With phenanthrene

there is a direct relationship between the amounts of dihydrodiols formed and the values of the calculated bond *ortho*-localization energies,[39] but with the other hydrocarbons examined, the relationship between localization energy and the relative amounts of metabolic products is less obvious.

It has been realised for many years that the most active double bonds in the aromatic molecules are the phenanthrene type of bonds (the so-called K-regions) and the values of the *ortho*-localization energies of these have been related to the carcinogenic activities of the hydrocarbons.[40] Chemically the K-region bonds resemble olefinic double bonds and undergo addition reactions with, for example, osmium tetroxide. Of the hydrocarbons examined in the work now described only the dibenz[a,c]anthracene molecule does not possess a K-region. It will be seen from Figs. 1–11 and from Table 1, however, that only a few of the hydrocarbons with K-regions yielded products where hydroxylation of these bonds had occurred. Moreover, where hydroxylation did occur, the K-region dihydrodiol was not necessarily the major product.

The hydrocarbons were not all good substrates for the hydroxylating enzymes. Pyrene, chrysene and benzo[a]pyrene, for example, were less readily metabolized than many of the others. If, however, the amounts of 'K-region' dihydrodiol formed are expressed as percentages of the total metabolism, as shown in Table 4, then the carcinogenic hydrocarbons, dibenz[a,h]anthracene, benzo[a]pyrene, 3-methylcholanthrene, 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene yield relatively less K region products than the inactive or weakly active hydrocarbons, phenanthrene, pyrene, benz[a]anthracene and benzo[e]pyrene. Chrysene is an exception to this rule: it is virtually inactive as a carcinogen although it is an initiator of tumours in mouse skin when croton resin is used as a promoter.[41] Repeated attempts to show the metabolic formation from chrysene of the 'K-region' dihydrodiol, 5,6-dihydro-5,6-dihydroxychrysene, were unsuccessful: chrysene is oxidized chemically to the 'K-

TABLE 4. THE METABOLIC FORMATION OF 'K-REGION' DIHYDRODIOLS FROM AROMATIC HYDROCARBONS BY RAT-LIVER HOMOGENATES

'K-region' dihydrodiol derived from	'K-region' dihydrodiols formed in the metabolism of hydro-carbons (as percentages of the total ethyl acetate-soluble metabolites) by			
	Liver microsomes from		Liver homogenates from	
	Normal rats	3-Methylcholanthrene-treated rats	Normal rats	3-Methylcholanthrene treated rats
Phenanthrene	43	45	44	46
Pyrene	60	52	50	30
Chrysene	0	0	0	0
Benz[a]anthracene	47	50	40	43
Dibenz[a,h]anthracene	20	22	17	13
Benzo[a]pyrene*	0	0	0	0
Benzo[e]pyrene	64	64	64	62
3-Methylcholanthrene	10	11	6	7
7-Methylbenz[a]anthracene	10	4	5	1
7,12-Dimethylbenz[a]anthracene	0	1	0	0

These percentages were calculated from the yields of ethyl acetate-soluble products recorded in Table 1.

* The benzo[a]pyrene molecule possesses two non-equivalent 'K-regions': neither yielded dihydrodiols.

region' dihydrodiol by osmium tetroxide.[42] Chrysene 5,6-oxide, the expected intermediate of 'K-region' metabolism, is converted by rat-liver preparations into the related dihydrodiol and glutathione conjugate (P. Sims, unpublished observations).

Table 4 also shows that the relative amounts of the K-region dihydrodiols formed by rat-liver homogenates are less than those formed by rat-liver microsomes. It has been shown[28] that, with a series of aromatic hydrocarbons incubated with a microsomal hydroxylating system in the presence of added protein or DNA, binding to the macromolecules of intermediates produced by metabolism occurs. It is possible that in the liver preparations used in the present work, some of the 'K-region' metabolites or, more likely, their epoxide precursors, have reacted with cellular macromolecules, particularly protein. Binding through the 'K-regions' of dibenz[a,h]-anthracene and benzo[a]pyrene to the skin of mice painted with the hydrocarbons has been reported.[43, 44] It is likely that, if the intermediates are epoxides, reactions with glutathione also occurred in the experiments with rat-liver homogenates. Benz[a]-anthracene 5,6-oxide and dibenz[a,h]anthracene 5,6-oxide are converted into glutathione conjugates in this system,[1] but it is not yet known what proportions of the metabolic products of the hydrocarbons are represented by conjugates of this type.

It thus appears that the formation of phenols and dihydrodiols at a particular position or bond depends on one or several factors, including:—

(1) Variations in the relative stabilities of the intermediate epoxides in respect of the rearrangements that lead to phenols—there are gains in resonance energy in the formation of the fully aromatic compounds: presumably in cases where these gains are greatest, rearrangement of epoxide to phenol is most likely to occur. It has been noted[16] that phenols formed by hydroxylation at the 'K-regions' have not been detected in experiments carried out either *in vivo* or *in vitro*. There are marked variations in the relative amounts of phenols and dihydrodiols produced at sites on the molecules other than the 'K-region': phenols, or dihydrodiols and sometimes mixtures of both phenols and dihydrodiols are formed.

(2) Variations in the relative rates of reactions of epoxides with water, catalysed by the enzyme 'epoxide hydrase'[36]—marked differences between the rates at which a number of the 'K-region' epoxides react enzymically with water have been found (ref. 37, and P. Sims, unpublished observations): with the larger polycyclic hydrocarbons, epoxides on bonds other than those of the 'K-regions' have not yet been synthesized.

(3) Removal of the epoxides from the system by enzymic conjugations with glutathione—although a number of 'K-region' epoxides react with glutathione in the presence of the conjugating enzyme,[45] relative rates of these reactions are not available.

(4) Removal of the epoxides from the systems by reaction with proteins and nucleic acids—the reactions of the 'K-region' epoxides of phenanthrene and dibenz[a,h]anthracene with DNA, RNA and histones have been studied,[46] but comparative studies of reactions of these types with a longer series of epoxides have not yet been carried out.

There is also the possibility that the molecular geometry of the hydrocarbon will play some part in deciding whether or not a particular bond is available for enzymic oxidation. It is not yet known how important this factor is in the metabolism of the hydrocarbons.

It should be pointed out that only small proportions of the added hydrocarbons are

metabolised in these incubations, but that other experiments have shown that, in spite of this, reductions in the amounts of the hydrocarbons added lead to reductions in the amounts of the metabolites formed. Possibly the hydrocarbons are absorbed by proteins and are thus not available for hydroxylation.

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