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METABOLISM OF POLYCYCLIC HYDROCARBONS BY RAT-LUNG PREPARATIONS

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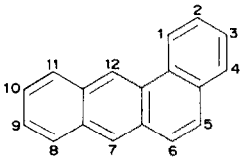
Abstract—The metabolism of benz[a]anthracene, 7-methylbenz[a]anthracene and benzo[a]pyrene has been studied in homogenates and microsomal fractions prepared from rat-lung. The results have been compared with those obtained in parallel experiments where rat-liver preparations were used and they show that, qualitatively, the hydrocarbons are metabolized to similar ethylacetate soluble products by both tissues. Quantitative estimations of the metabolism of the hydrocarbons indicated that rat-liver was more active than rat-lung when compared on the basis of the weight of tissue involved. Comparisons made on the basis of the protein content of the tissue preparations showed, however, that rat-lung was at least as active as rat-liver in the metabolism of polycyclic hydrocarbons. The metabolites found are mainly ring-hydroxylated products; this implies, therefore, that epoxides are formed from polycyclic hydrocarbons by the enzymes present in rat-lung.

THE BIOLOGICAL effects produced by polycyclic hydrocarbons, which include carcinogenesis in animals[1] and malignant transformation[2,3] and mutagenesis[4] in cells in culture, are now thought to result from the metabolic activation of these relatively inert compounds. Evidence is accumulating that the active metabolites which cause these effects are epoxides[5–10] formed by the action of the NADPH-dependent microsomal mixed function oxidase.[11–14] It has been found that the epoxides formed are subsequently either, (a) hydrated enzymically by the microsomal "epoxide hydrase" to yield the corresponding dihydrodiols;[15,16] (b) react with glutathione to give conjugates,[17] a reaction catalysed by an enzyme present in the soluble supernatant fraction that has been called "glutathione *S*-epoxide transferase,[18] or; (c) rearrange to give the corresponding phenols.[19] These conversions are regarded principally as detoxication reactions, but in addition the epoxides, which are alkylating agents,[20,21] can also react with nucleic acids and proteins in biological systems.[22,23]

Most of the detailed investigations of polycyclic hydrocarbon metabolism that have been carried out have used rat-liver preparations,[24–26] although the liver is not normally regarded as a target organ for hydrocarbon carcinogenesis. Since polycyclic hydrocarbons are active in the induction of tumours in the pulmonary tissues of the rat[27,28] and of other species[29,30] and may be active in man, our investigations of polycyclic hydrocarbons are being extended to include their metabolism by lung.

This paper describes quantitative studies of the metabolism of three hydrocarbons, benz[a]anthracene (I), 7-methylbenz[a]anthracene (II) and benzo[a]pyrene (III) by rat-lung preparations and also gives the comparative results obtained in parallel experiments where rat-liver preparations were used. Of the hydrocarbons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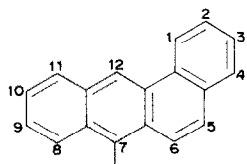
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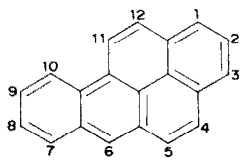
(III)

studied here, both benz[a]anthracene and benzo[a]pyrene are known to be atmospheric pollutants in urban areas[31] and to be constituents of tobacco smoke[32] and may, following metabolism, contribute to the induction of cancer of the respiratory tract in man.

MATERIALS AND METHODS

Materials. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and $NADP^+$ were purchased from Boehringer, Mannheim, Germany. ^{3}H-Labelled polycyclic hydrocarbons (sp. act., benz[a]anthracene 510 mCi/mmole; 7-methylbenz-[a]anthracene 250 mCi/mmole and benzo[a]pyrene 437 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks.

Tissue preparations. Male rats of the Wistar strain (body wt. approx. 200 g) were used in the investigations.

(a) *Rat liver.* Freshly removed liver (40 g) was homogenized in ice-cold phosphate buffer (0·1 M, pH 7·4, 4 vol.) using a Potter–Elvejhem type of homogenizer fitted with a Teflon pestle. The mixture was centrifuged at 4000 *g* for 15 min and the supernatant, designated "homogenate", decanted. For the preparation of microsomal and soluble supernatant fractions, homogenate was centrifuged at 20,000 *g* for 20 min, to remove mitochondria and the supernatant centrifuged again at 100,000 *g* for 1 hr in a refrigerated ultracentrifuge.

The 100,000 *g* supernatant, designated "soluble supernatant fraction" was decanted. A "washed microsomal fraction" was prepared by resuspending the microsomal pellet in phosphate buffer (0·1 M, pH 7·4, 20 vol.) and recentrifuging at 100,000 *g* for 1 hr.

(b) *Rat lung.* Freshly removed tissue (20 g) was homogenized in ice-cold phosphate buffer (0·1 M, pH 7·4, 4 vol.) for 1·5 min in an atomix blender (M.S.E. Ltd., Manor Royal, Crawley, Sussex). The mixture was strained through cotton gauze to remove fragments of fibrous connective tissue and "homogenate", a "soluble supernatant fraction" and a "washed microsomal fraction" prepared by methods identical to those described above for rat-liver.

Metabolism of polycyclic hydrocarbons by tissue preparations. Quantitative investigations of the metabolism of benz[a]anthracene, 7-methylbenz[a]anthracene and benzo[a]pyrene were carried out using both homogenate and microsomal fractions prepared from rat-lung. For comparison, similar experiments were performed with homogenate and microsomal fractions prepared from the livers of the same rats that were used as a source of rat-lung. Each incubation mixture contained microsomes or homogenate equivalent to 1 g tissue, suspended in phosphate buffer (0·1 M, pH 7·4, 10 ml) containing $NADP^+$ (8·5 mg), glucose 6-phosphate (63 mg), $MgCl_2$ (25 mg) and glucose 6-phosphate dehydrogenase (0·7 units). ^{3}H-Labelled hydrocarbon (20 nmoles) was added in acetone (0·1 ml) and the mixture

was incubated at 37° for 15 min, extracted with ethyl acetate and portions of the extracts examined by TLC as previously described[26] using appropriate mixtures of unlabelled reference compounds prepared as described below.

Thin-layer chromatography. Thin-layer chromatograms were prepared as described previously[26] and developed with either solvent (a) benzene–ethanol (19:1, v/v) or solvent (b) benzene–ethanol (9:1, v/v). The products were usually located by inspecting the wet plates in u.v. light. Fluorescent and intermediate bands were marked off, the silica gel removed from the chromatograms and the radioactivity present determined by liquid scintillation counting.

Compounds used as chromatographic standards. Non-radioactive dihydrodiols and phenols that were used as chromatographic reference compounds in the separation of radioactive metabolites formed from benz[a]anthracene, 7-methylbenz[a]-anthracene and benzo[a]pyrene were obtained from large-scale incubations of the unlabelled hydrocarbons with rat-liver homogenate.[26] They were identified by their thin-layer chromatographic and by their u.v. spectral characteristics.[33–35]

The reference compounds obtained in this way were (a) from benz[a]anthracene, 5,6-dihydro-5,6-dihydroxybenz[a]anthracene and 8,9-dihydro-8,9-dihydroxbenz[a]-anthracene; (b) from 7-methylbenz[a]anthracene, 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene, 5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene, 8,9-dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene and 7-hydroxymethylbenz-[a]anthracene and; (c) from benzo[a]pyrene, 4,5-dihydro-4,5-dihydroxybenzo[a]-pyrene, 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene, 9,10-dihydro-9,10-dihydroxy-benzo[a]pyrene and 3-hydroxybenzo[a]pyrene. All of these products, except for the 3,4-dihydrodiol of 7-methylbenz[a]anthracene, have previously been identified as metabolites of their respective parent hydrocarbons.[33–35]

The 3,4-dihydrodiol of 7 methylbenz[a]anthracene appears to be formed as a metabolite of the hydrocarbon when liver homogenates are used that have been prepared from rats which have not previously been treated with compounds, such as 3-methylcholanthrene, that induce microsomal drug-metabolizing enzymes. The metabolite appeared to be a dihydrodiol from its position on chromatograms developed in solvent (b) and its u.v. spectrum, which is shown in Fig. 1, is distinct from those of the corresponding 5,6-, 8,9- and 10,11-dihydrodiols of 7-methyl-benz[a]anthracene. In addition, when the metabolite was decomposed by treatment with acid and the products re-examined by TLC, two fluorescent spots were seen under u.v. light in that region of the chromatograms to which phenols derived from polycyclic hydrocarbons are known to migrate. Both decomposition products had a violet fluorescence in u.v. light but on exposure to NH_3, the fluorescence of the faster-moving product changed to pink whilst that of the slower-moving component changed to green. These two products arising from the acid-decomposition of the dihydrodiol were also chromatographically indistinguishable from two derivatives of 7-methylbenz[a]anthracene that had previously been tentatively identified as 3-hydroxy-7-methylbenz[a]anthracene and 4-hydroxy-7-methylbenz[a]anthra-cene.[34] The u.v. spectrum of the faster moving phenol was also similar to that of 4-hydroxybenz[a]anthracene except that the peaks were shifted to slightly longer wavelengths. Taken together this evidence appears to be sufficient to justify the identification of this metabolite of 7-methylbenz[a]anthracene as the dihydrodiol, 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene. Mixtures of the 3,4-dihydro-

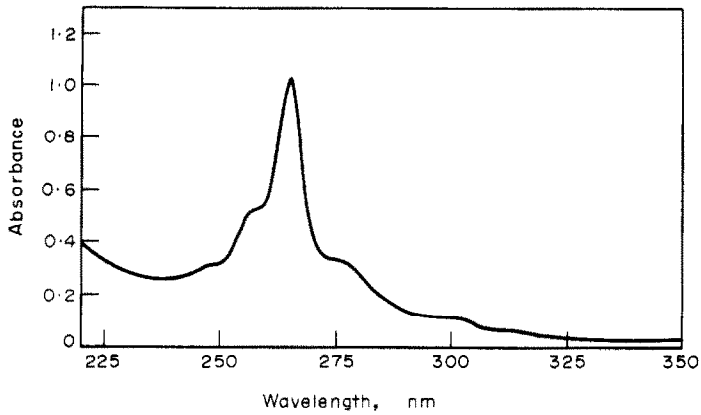

FIG. 1. Ultraviolet spectrum of metabolite of 7-methylbenz[a]anthracene identified as 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene.

diol with an authentic sample of *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz-[a]anthracene were not resolved on chromatograms developed with solvents (a) or (b).

Protein estimations. A modification[36] of the biuret reaction was used to estimate protein in tissue preparations: casein was used as a standard protein.

RESULTS

The radioactive metabolites that were formed when the ^{3}H-labelled hydrocarbons benz[a]anthracene, 7-methylbenz[a]anthracene or benzo[a]pyrene were incubated with rat-lung microsomal fractions were separated by TLC. Figures 2–4 show the distribution of radioactive products on these chromatograms. For comparison, each figure also shows the distribution of radioactive metabolites obtained when a rat-liver microsomal fraction, prepared from the livers of the same rats that were used as a source of lung tissue, was utilized. The positions on the chromatograms occupied by the relevant authentic reference compounds that were employed in the identification of the radioactive metabolites are also indicated in each of these figures. Results that were, in most cases, qualitatively very similar to those shown in Figs. 2–4 were obtained when tissue homogenates were used in place of microsomal preparations.

The rates of formation of each of the identified metabolites of the hydrocarbons in lung and in liver incubation mixtures have been calculated from the specific activities of the ^{3}H-labelled hydrocarbons and are given in Table 1. In this Table the results have been expressed on the basis both of the weights of tissue used and of the protein contents of the respective tissue preparations.

Benz[a]anthracene. Figure 2 shows that the main metabolites formed from benz[a]anthracene by both lung and liver microsomal preparations were two dihydrodiols, 5,6-dihydro-5,6-dihydroxybenz[a]anthracene and 8,9-dihydro-8,9-dihydroxybenz[a]anthracene. These results, which are expressed in terms of the weight of tissue used, indicate that liver microsomal fractions are considerably more active in the metabolism of benz[a]anthracene than those prepared from rat-lung. Reference to Table 1 shows, however, that the two tissues metabolize benz[a]anthracene at

TABLE 1. RATES OF FORMATION OF HYDROCARBON METABOLITES BY RAT-LUNG AND RAT-LIVER PREPARATIONS

		Amount of ethylacetate-soluble product formed							
		pmoles g wet wt tissue min				pmoles mg protein min			
		Homogenate		Microsomes		Homogenate		Microsomes	
Hydrocarbon	Metabolite	Liver	Lung	Liver	Lung	Liver	Lung	Liver	Lung
Benz[a]anthracene	5,6-Dihydro-5,6-dihydroxy	18·3	2·80	157	24·5	0·09	0·04	3·53	2·70
	8,9-Dihydro-8,9-dihydroxy	201	28·0	228	65·0	1·18	0·35	5·12	7·20
7-Methylbenz[a]anthracene	3,4-Dihydro-3,4-dihydroxy and 5,6-dihydro-5,6-dihydroxy	112	41·0	257	45·6	0·62	0·33	5·59	3·65
	8,9-Dihydro-8,9-dihydroxy	205	84·7	297	52·8	1·13	0·68	6·47	4·23
	7-Hydroxymethyl	8·41	33·3	21·6	43·2	0·05	0·27	0·47	3·46
Benzo[a]pyrene	4,5-Dihydro-4,5-dihydroxy	3·40	4·24	19·4	8·90	0·02	0·05	0·44	0·99
	7,8-Dihydro-7,8-dihydroxy	6·90	4·95	12·3	3·58	0·04	0·06	0·28	0·40
	9,10-Dihydro-9,10-dihydroxy	11·2	5·00	33·9	5·90	0·07	0·06	0·76	0·66
	3-Hydroxy	23·2	13·6	27·2	8·77	0·14	0·17	0·61	0·98

Incubations and estimations were carried out as described in the text. The values represent the means of duplicate determinations.

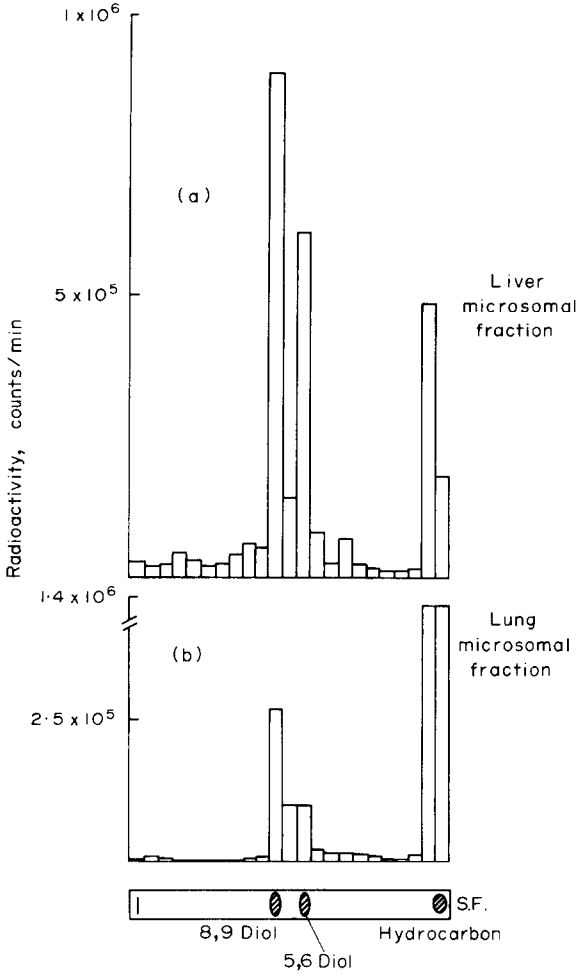

FIG. 2. Metabolism of ^{3}H-benz[a]anthracene by rat-lung and rat-liver microsomal preparations. Mixtures incubated as described in the text were extracted with ethylacetate and portions of the extracts (≡0·5 g tissue) together with relevant non-radioactive reference compounds were examined using Silica gel TLC developed in solvent (b) as previously described.[26] S.F. indicates solvent front.

much more similar rates when the difference in the protein contents of the two microsomal preparations is taken into account.

7-Methylbenz[a]anthracene. The metabolites formed from 7-methylbenz[a]-anthracene by rat-lung and liver are again mainly dihydrodiols. As Fig. 3 shows, the most prominent radioactive bands are coincident with the reference dihydrodiols, 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene and 8,9-dihydro-8,9-dihydroxy-7-methylbenz[a]anthracene. In addition, a radioactive band coincident with 7-hydroxymethylbenz[a]anthracene was also present and proportionally more of this metabolite appeared to be formed by rat-lung than by rat-liver preparations; this is also borne out by the data on the metabolism of 7-methylbenz[a]anthracene given in Table 1.

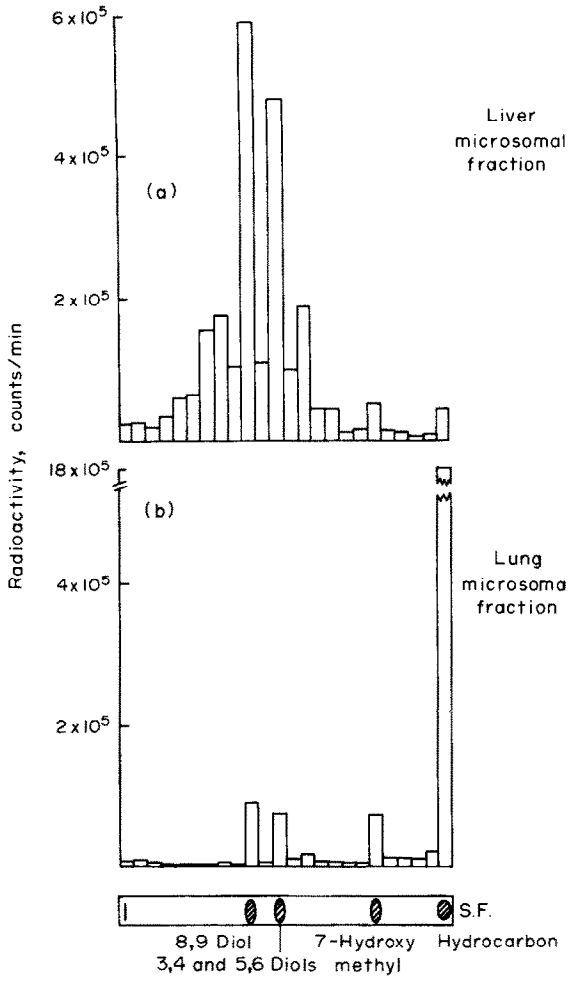

FIG. 3. Metabolism of ^{3}H-7-methylbenz[a]anthracene by rat-lung and rat-liver microsomal preparations. Mixtures incubated as described in the text were extracted and the products examined as described in the legend to Fig. 2, using solvent (b). S.F. indicates solvent front.

Benzo[a]pyrene. This hydrocarbon was less readily metabolized by tissue preparations than the other two substrates studied. Radioactive bands coincident with the reference dihydrodiols, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene, 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene and 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene and with 3-hydroxybenzo[a]pyrene were present on the chromatograms (Fig. 4). The pattern of metabolites formed by rat-lung preparations was qualitatively similar to those formed when liver preparations were used but, as Fig. 4 shows, the amounts of the individual products formed appear to be smaller with rat-lung than with rat-liver when the results are expressed in terms of tissue weight. When the results are calculated on the basis of the protein content of the tissue preparation, however, rat-lung appears to be at least as active as rat-liver in the metabolism of benzo[a]-pyrene (Table 1).

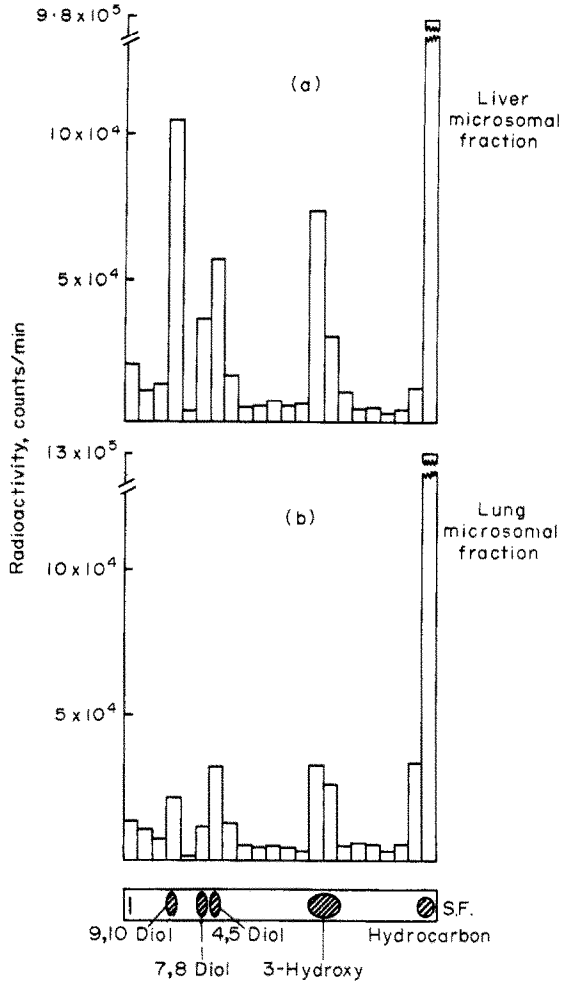

FIG. 4. Metabolism of ^{3}H-benzo[a]pyrene by rat-lung and rat-liver microsomal preparations. Mixtures incubated as described in the text were extracted and the products examined as described in the legend to Fig. 2, using solvent (a). S.F. indicates solvent front.

DISCUSSION

The metabolic conversion of the three polycyclic hydrocarbons studied in these experiments into hydroxylated derivatives appears to be qualitatively similar in both rat-lung and rat-liver preparations and the results obtained with rat-liver preparations are in general agreement with those reported earlier for this tissue.[26] Quantitative differences in the amounts of the various hydroxylated products formed were apparent when lung and liver were compared on the basis of the weight of tissue used (Figs. 2–4; Table 1); the liver preparations being appreciably more active and the differences being more marked with benz[a]anthracene than with benzo[a]pyrene. The metabolic activities of the two tissues were more comparable when they were considered on the basis of the protein contents of the tissue preparations and it may be significant that, on this basis, microsomal preparations from

rat-lung appeared to form the "K-region" dihydrodiol of benzo[a]pyrene, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene, twice as rapidly as similar preparations from rat-liver.

The only hydroxylated derivative detected in the present experiments, which has not previously been identified as a metabolite, was the 3,4-dihydrodiol of 7-methylbenz[a]anthracene, 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene. Liver preparations obtained from rats that had been pretreated with a microsomal enzyme inducer were used in most of the previous investigations of 7-methylbenz-[a]anthracene metabolism[26] but in the present work the tissues were both taken from unstimulated animals. Shifts in the proportions of metabolites formed at different sites of hydrocarbon molecules are known to occur as a result of pretreatment with enzyme inducers[37] and, therefore, the occurrence of a metabolite in extracts from normal tissue preparations that was not seen when tissue from pretreated animals was used is not altogether surprising.

Ring-hydroxylated metabolites of polycyclic hydrocarbons undoubtedly arise from epoxide precursors; the dihydrodiols resulting from the enzyme-catalysed hydration of epoxides that involves the microsomal epoxide hydrase[15,16] and the phenols resulting from epoxide rearrangements.[19] In the present experiments smaller amounts of dihydrodiols were found in extracts of incubation mixtures that contained either rat-lung or rat-liver homogenate in comparison with the amounts present in extracts where microsomal preparations were used (Table 1). The reasons for this are not known with certainty but may be associated with loss of epoxide (a) via the formation of GSH conjugates, a reaction catalysed by the enzyme "glutathione *S*-epoxide transferase", which was presumably not present in washed microsomal preparations since it is a soluble-supernatant enzyme,[18] and (b) through covalent interactions with other constituents of homogenate such as nucleic acids and proteins.[20–23]

Enzymes involved in the metabolism of a variety of foreign compounds are known to be present in rat-lung[38] and previous studies have included indirect measurements of the activity of the mixed-function oxidase responsible for the initial step in the oxidation of the double bonds of polycyclic hydrocarbons like benzo[a]pyrene.[39] This microsomal enzyme of rat-lung is probably involved in the metabolic activation of the hydrocarbons known to be carcinogenic to the respiratory tract in this species[27,28] and may well play a part in the induction of cancer in rat-lung that has been reported to result from the application of cigarette-smoke condensates that contain polycyclic hydrocarbons.[40]

The results obtained in the experiments described here indicate that, as in rat-liver, the main ethylacetate-soluble metabolites formed from the polycyclic hydrocarbons benz[a]anthracene, 7-methylbenz[a]anthracene and benzo[a]pyrene by rat-lung preparations are ring-hydroxylated products. These results imply therefore, that a variety of polycyclic hydrocarbon epoxides are formed by metabolism in rat-lung since dihydrodiols and phenols almost certainly arise from epoxide intermediates. A subsequent paper will describe experiments designed to detect the formation and further metabolism of polycyclic hydrocarbon epoxides by rat-lung preparations.

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