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# K-REGION EPOXIDES OF POLYCYCLIC HYDROCARBONS: FORMATION AND FURTHER METABOLISM BY RAT-LUNG PREPARATIONS

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**Abstract**—Epoxides of 7-methylbenz[a]anthracene and of benzo[a]pyrene that have been identified as the K-region epoxides, 7-methylbenz[a]anthracene 5,6-oxide and benzo-[a]pyrene 4,5-oxide, have been detected as microsomal metabolites using preparations from the lungs of rats that had been pretreated with the microsomal mixed function oxidase inducer, 3-methylcholanthrene. It was also possible, using lung microsomal preparations from uninduced animals, to demonstrate the formation of an epoxide identified as the K-region derivative, benz[a]anthracene 5,6-oxide, as a microsomal metabolite of benz[a]-anthracene. The K-region epoxides of 7-methylbenz[a]anthracene and of benzo[a]pyrene could not always be detected as metabolites when lung microsomal preparations from uninduced rats were used. The activities of two other enzymes present in pulmonary tissue fractions that are involved in the further metabolism of polycyclic hydrocarbon epoxides have also been measured and the values compared with those obtained with rat-liver. When benz[a]anthracene 5,6-oxide was used as substrate, much lower levels of microsomal epoxide hydrase activity were found in lung than in liver, but soluble-supernatant fractions of rat-lung appeared to possess higher levels of glutathione *S*-epoxide transferase activity than were present in rat-liver.

The significance of these results in relation to the metabolic activation of polycyclic hydrocarbons by epoxide formation and to the induction of tumours of the respiratory tract by members of this class of chemical carcinogens is discussed.

IN A PRECEDING publication it was shown that polycyclic hydrocarbons are metabolized by rat-lung preparations to a variety of ring-hydroxylated products.[1] These metabolites, which were identified as dihydrodiols and phenols, are known, as a result of work with liver preparations,[2–4] to arise from epoxides that are formed by the action of the NADPH-dependent microsomal mixed-function oxidase on the aromatic double bonds of the hydrocarbons.[5–7] The previous results obtained with rat-lung[1] imply therefore that epoxides are also formed from hydrocarbons in this tissue. Since hydrocarbon epoxides are active in several biological systems[8–13] and also because polycyclic hydrocarbons are carcinogenic in rat-lung[14,15] and may well contribute to the incidence of tumours of the respiratory tract in man, it was thought worthwhile to attempt to detect the formation of epoxides as metabolites using lung preparations.

This paper describes the detection of K-region epoxides as metabolites of benz-[a]anthracene, 7-methylbenz[a]anthracene and benzo[a]pyrene using rat-lung microsomal preparations. Also included are results obtained in other experiments where the activities of the enzymes present in rat-lung that catalyse the conversion of epoxides (a) into dihydrodiols[16] and (b) into glutathione conjugates[17] have been measured and compared with the activities in rat-liver.

## MATERIALS AND METHODS

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Boehringer, Mannheim, Germany, cyclohexene oxide from R. N. Emmanuel Ltd., Wembley, Middx., and basic aluminium oxide from M. Woelm, Eschwege, Germany. $^3$H-Labelled polycyclic hydrocarbons (specific activities, benz[a]anthracene 510 mCi/m-mole; 7-methylbenz[a]anthracene 250 mCi/m-mole and benzo[a]pyrene 437 mCi/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks; $^3$H-labelled benz[a]anthracene 5,6-oxide (sp. act. 36·17 mCi/m-mole) and non-radioactive samples of benz[a]anthracene 5,6-oxide and 7-methylbenz[a]anthracene 5,6-oxide were prepared from the parent hydrocarbons as previously described.[18,19] Benzo[a]pyrene 4,5-oxide[20] was a gift from Dr. P. Sims of this Institute. *S*-(5,6-Dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione was prepared from benz[a]anthracene 5,6-oxide by reaction with GSH.[21]

*Chromatography of hydrocarbon metabolites*

(a) *Column chromatography.* The columns (1·3 × 4 cm) used in the present investigations were prepared from basic alumina deactivated to Woelm grade III by the addition of water (6%, w/w). Concentrated solutions containing microsomal metabolites of benz[a]anthracene, 7-methylbenz[a]anthracene or benzo[a]pyrene were chromatographed on these columns as previously described.[5–7]

(b) *Thin-layer chromatography (TLC).* Thin-layer chromatograms were prepared as described previously[22] and developed with either solvent (a) benzene–ethanol (19:1, v/v, solvent (b) benzene–ethanol (9:1, v/v) or solvent (c) butan-1-ol–propan-1-ol–aq. 2M-$NH_4OH$ (2:1:1, v/v). The products were usually located by inspecting the wet plates in u.v. light. Fluorescent and intermediate bands were marked off and the Silica gel removed from the chromatograms and the radioactivity present determined by liquid scintillation counting. GSH conjugates were located by spraying the plates with a solution of ninhydrin (0·5%) in butan-1-ol and heating with a hair-dryer for 2 min.

*Tissue preparations.* Microsomal and soluble supernatant fractions were prepared from rat-lung and from rat-liver by the procedures described in the preceding paper.[1] In some cases, rat-lung microsomal fractions were prepared from animals that had been pretreated with 3-methylcholanthrene.[22]

*Detection of epoxides as microsomal metabolites.* Microsomal incubations were carried out using $^3$H-labelled hydrocarbons (400 μg) and rat-lung microsomal fraction ( ≡ 40 g rat lung) as previously described[7] except that the 3,4-dihydronaphthalene 1,2-oxide previously used as an epoxide hydrase inhibitor was replaced by cyclohexene oxide (30 μmoles). After being incubated at 30° for 10 min, the mixtures were extracted with an ether solution (80 ml) of the appropriate unlabelled K-region epoxide (2 mg), the ether extracts were dried ($Na_2SO_4$) and evaporated and the residues examined by chromatography on alumina columns as described above.

*Characterization of epoxides formed as microsomal metabolites.* Fractions eluted from alumina columns that were shown by their u.v. spectral characteristics to contain the unlabelled carrier epoxide and which, therefore, might also contain radioactive epoxide metabolites of either benz[a]anthracene, 7-methylbenz[a]anthracene or benzo[a]pyrene were pooled and the materials present subjected to procedures known to convert K-region epoxides enzymically into dihydrodiols or chemically

into phenols or into glutathione conjugates.[7] The chromatographic characteristics of these radioactive products were then compared by TLC with those of the appropriate authentic dihydrodiols, phenols or glutathione conjugates derived from benz[a]anthracene, from 7-methylbenz[a]anthracene or from benzo[a]pyrene as described.[5–7]

*Estimation of microsomal "epoxide hydrase" activity.* Mixtures containing a rat-lung or rat-liver microsomal fraction (≡ 1 g tissue) suspended in phosphate buffer (0·1 M, pH 7·4, 5 ml) and $^3$H-labelled benz[a]anthracene 5,6-oxide (1 μmole), added in acetone (0·2 ml), were incubated at 37°. Control mixtures containing microsomal fractions that had previously been heated at 100° for 5 min were also incubated with epoxide. After incubation, the mixtures were extracted with ethyl acetate (2 ml). Portions (0·1 ml) of the extract were co-chromatographed with *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene on thin-layer chromatograms developed in solvent (a). The Silica gel in the area of the chromatogram occupied by the reference diol was removed and the radioactivity present determined.

*Estimation of "glutathione S-epoxide transferase" activity.* Incubation mixtures contained soluble supernatant fraction (1 ml) prepared from rat-lung or from rat-liver, phosphate buffer (0·1 M, pH 7·4, 4 ml), glutathione (50 μmole) and $^3$H-labelled benz[a]anthracene 5,6-oxide, added in ethanol (0·1 ml). After incubation at 37° for 1 hr, acetone (5 ml) was added and the mixture centrifuged. Portions (0·2 ml) of the supernatant were co-chromatographed with authentic *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl) glutathione on thin-layer chromatograms developed in solvent (c). The developed chromatograms were treated with ninhydrin and the areas of silica gel occupied by the ninhydrin-positive reference conjugate removed and the radioactivity present determined by liquid scintillation counting.

*Protein estimations.* A modification[23] of the biuret reaction was used to estimate protein in tissue preparations. Casein was used as a standard protein.

## RESULTS

### *Formation of epoxides by rat-lung microsomal fractions*

(a) *From benz[a]anthracene.* When $^3$H-benz[a]anthracene was incubated with the NADPH-dependent microsomal mixed-function oxidase of rat-lung in the presence of an epoxide hydrase inhibitor and the ether-soluble products chromatographed on an alumina column, a radioactive metabolite was detected that behaved in a similar manner to the K-region epoxide previously detected as a metabolite of this hydrocarbon in rat-liver preparations.[5] The metabolite eluted from the column after the peakof unchanged hydrocarbon but before the fractions that contained hydroxylated metabolites (Fig. 1a). The further studies described below confirmed that this radioactive metabolite was benz[a]anthracene 5,6-oxide.

(b) *From 7-methylbenz[a]anthracene and benzo[a]pyrene.* When 7-methylbenz[a]anthracene and benzo[a]pyrene were metabolized by a microsomal system that contained rat-lung microsomal fractions prepared from untreated animals and the metabolites were chromatographed on alumina columns, radioactive peaks coincident with the peaks of u.v. absorption of the unlabelled carrier epoxides, 7-methylbenz[a]anthracene 5,6-oxide or benzo[a]pyrene 4,5-oxide, were not always present when the radioactivity in these fractions was measured.

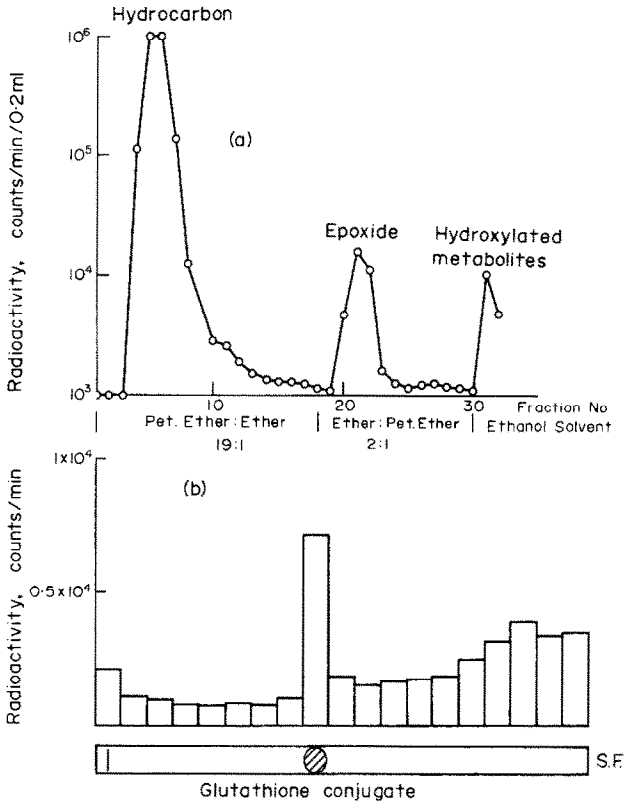


FIG. 1. (a) Alumina column chromatography of the metabolites formed by a rat-lung microsomal preparation from $^3$H-benz[a]anthracene. The concentrated ether extract from a microsomal incubation (see text), which also contained unlabelled benz[a]anthracene 5,6-oxide, was applied to a column (1·3 × 4 cm) of alumina (Woelm basic alumina of activity grade III) and eluted with solvent. 100-Drop fractions were collected and radioactivity measured. (b) Formation of acid-labile glutathione conjugate of benz[a]anthracene epoxide. Fractions containing the radioactive epoxide peak were pooled, the materials present reacted with glutathione as previously described[21] and the radioactivity associated with the products measured after separation by TLC in solvent (c). S.F. indicates solvent front.

Similar experiments were therefore carried out with 7-methylbenz[a]anthracene and with benzo[a]pyrene using rat-lung microsomal fractions prepared from animals that had been pretreated with 3-methylcholanthracene. Under these conditions radioactive metabolites that were subsequently identified as the K-region epoxides 7-methylbenz[a]anthracene 5,6-oxide and benzo[a]pyrene 4,5-oxide were found to be present in the eluate fractions from alumina columns that contained the appropriate unlabelled carrier epoxides (Figs. 2a and 3a).

*Conversion of epoxide metabolites to dihydrodiols, phenols and glutathione conjugates.* The fractions eluted from alumina columns that contained unlabelled K-region epoxides of either benz[a]anthracene, 7-methylbenz[a]anthracene or benzo[a]-pyrene were pooled and evaporated and the residue treated by procedures known to convert K-region epoxides into dihydrodiols, into phenols or into glutathione conjugates.[7] Some of the results obtained when radioactive epoxide metabolites were subjected to these procedures and the products that were formed examined

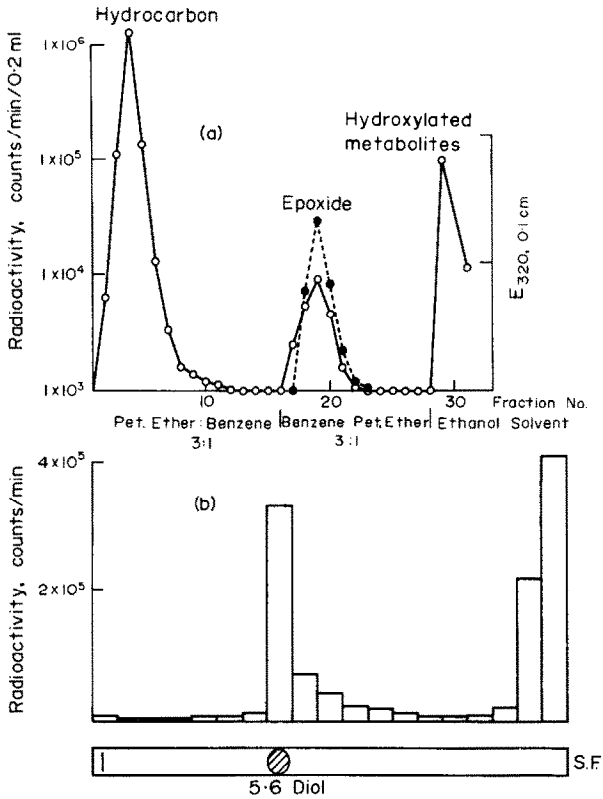


FIG. 2. (a) Alumina column chromatography of the metabolites formed by a 3-methylcholamthrene treated rat-lung microsomal preparation from $^{3}$H-7-methylbenz[a]anthracene. 100-Drop fractions were collected and radioactivity and u.v. absorption (●) measured. (For details, see text and legend to Fig. 1a). (b) Enzyme-catalysed conversion of the radioactive epoxide metabolite of 7-methylbenz-[a]anthracene into the corresponding dihydrodiol. Fractions containing the epoxide were pooled, the materials present incubated with rat-liver microsomal "epoxide hydrase" as previously described[7] and the radioactivity associated with the products measured after separation by TLC in solvent (a). S.F. indicates solvent front.

by TLC are shown in Figs. 1–3. Figure 2b shows that the radioactive epoxide formed as a metabolite of 7-methylbenz[a]anthracene by the rat-lung microsomal mixed-function oxidase can be converted by incubation with rat-liver microsomal "epoxide hydrase" in the absence of cofactors, into a product with the chromatographic characteristics of the K-region dihydrodiol, *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene. Figures 1b and 3b show that the radioactive epoxide metabolites formed from benz[a]anthracene and from benzo[a]pyrene can be converted chemically into products with the chromatographic characteristics of *S*-(5,6-dihydro 6-hydroxybenz[a]anthracen-5-yl) glutathione and 4-hydroxybenzo-[a]pyrene respectively.

*Microsomal "epoxide hydrase" activity.* The results obtained when the "epoxide hydrase" activity present in rat-lung and in rat-liver microsomal preparations was measured, using $^{3}$H-benz[a]anthracene 5,6-oxide as substrate, are shown in Fig. 4 and in Table 1, where the rates of formation of the corresponding dihydrodiol have

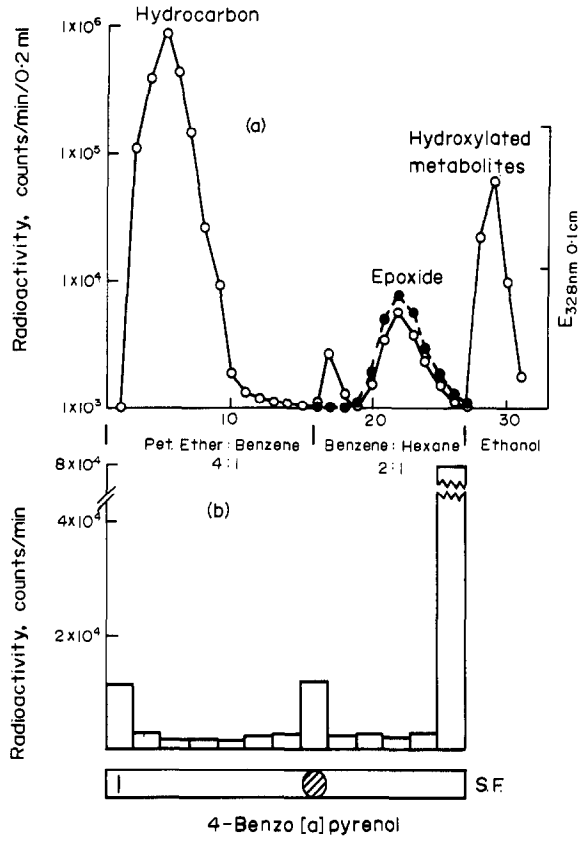


FIG. 3. (a) Alumina column chromatography of the metabolites formed from $^{3}$H-benzo[a]pyrene by a 3-methylcholanthrene-treated rat-lung microsomal preparation. 100-Drop fractions were collected and radioactivity (○) and u.v. absorption (●) measured. (For details, see text and legend to Fig. 1a.) (b) Acid-catalysed rearrangement of radioactive epoxide metabolite of $^{3}$H-benzo[a]pyrene into the corresponding phenol. Fractions containing the epoxide were pooled, the materials treated with acid as previously described[21] and the radioactivity associated with the products measured after separation by TLC in solvent (b). S.F. indicates solvent front.

been calculated on the basis of both the wet weight of tissue used and on the protein content of the tissue preparation. The formation of *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene from the corresponding epoxide was not detected in control experiments when microsomal preparations that had been heated at 100° for 5 min were employed. The data in Fig. 4 and in Table 1 clearly shows that lower levels of "epoxide hydrase" activity are present in rat-lung microsomal preparations than occur in similar preparations from rat-liver when benz[a]-anthracene 5,6-oxide is used as substrate for this enzyme.

"*Glutathione* S-*epoxide transferase*" *activity.* The rates of conversion of benz[a]-anthracene 5,6-oxide into the corresponding glutathione conjugate, *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl) glutathione, catalysed by the "glutathione *S*-epoxide transferase" present in soluble supernatant fractions of rat-lung and of rat-liver, are shown in Table 2 where they have been expressed on the basis of both the

TABLE 1. RATES OF FORMATION OF *trans*-5,6-DIHYDRO-5,6-DIHYDROXYBENZ[a]ANTHRACENE FROM BENZ[a]ANTHRACENE 5,6-OXIDE CATALYSED BY MICROSOMAL "EPOXIDE HYDRASE" PREPARATIONS

| | Amount of *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene formed | |
|---|---|---|
| Rat tissue preparation | nmoles/gm wet wt tissue/min | nmoles/mg protein/min |
| Lung microsomal fraction | 1·16 | 0·12 |
| Liver microsomal fraction | 30·12 | 0·99 |

Incubations and estimations were carried out as described in the text. The values given represent the mean of two determinations made after incubation at 37° for 20 min.

TABLE 2. RATES OF FORMATION OF *S*-(5,6-DIHYDRO-6-HYDROXYBENZ[a]ANTHRACEN-5-YL) GLUTATHIONE FROM BENZ[a]ANTHRACENE 5,6-OXIDE, CATALYSED BY "GLUTATHIONE *S*-EPOXIDE TRANSFERASE"

| | Amount of *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl) glutathione formed | |
|---|---|---|
| Rat tissue preparation | nmoles/g wet wt tissue/min | nmoles/mg protein/min |
| Lung soluble supernatant fraction | 2·83 | 0·034 |
| Liver soluble supernatant fraction | 1·86 | 0·012 |

Incubations and estimations were carried out as described in the text. The values given represent the mean of two determinations made at a benz[a]anthracene 5,6-oxide concentration of 0·1 mM.

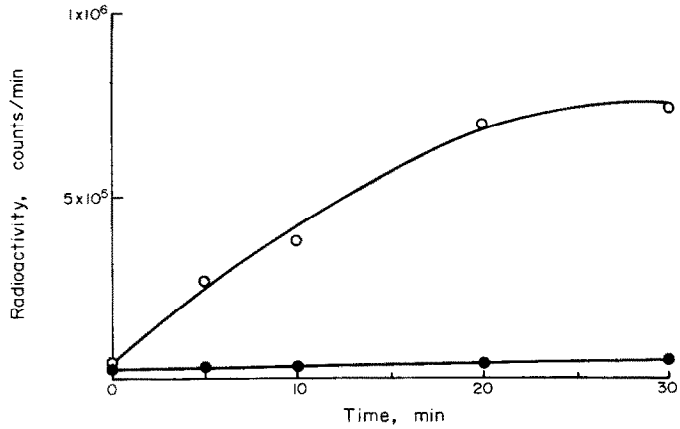


FIG. 4. "Epoxide hydrase" activity of rat-lung and of rat-liver microsomal fractions. Incubation mixtures containing $^3$H-benz[a]anthracene 5,6-oxide (1 μmole) and either a rat-lung or rat-liver microsomal fraction (≡ 1 g tissue) resuspended in phosphate buffer (0·1 M, pH 7·4) were set up as described in the text and incubated at 37°. After incubation the mixtures were extracted with ethyl acetate and the amounts of $^3$H-*trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene present in portions of these extracts from rat-lung (●) or from rat-liver (○) preparations was estimated as described in the text following TLC in solvent (a).

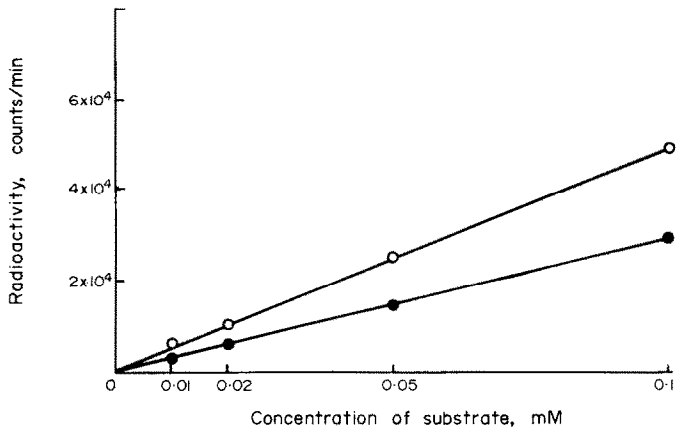


FIG. 5. "Glutathione *S*-epoxide transferase" activity present in the soluble supernatant fractions prepared from rat-lung and from rat-liver. Incubation mixtures containing $^3$H-benz[a]anthracene 5,6-oxide, glutathione (50 μmole) and soluble supernatant fraction (1 ml) prepared from either rat-lung or from rat-liver were set up as described in the text and incubated at 37° for 1 hr. After incubation, acetone (1 vol.) was added, the mixture centrifuged and the $^3$H-*S*-(5,6-dihydro-6-hydroxybenz-[a]anthracen-5-yl) glutathione present in portions of the clear supernatant from rat-ling (○) and from rat-liver (●) estimated as described in the text following TLC in solvent (c).

wet weight of tissue and the protein content of the preparations. The effect of increases in the concentration of the epoxide substrate within the range 0·01–0·10 mM on the rate of formation of the conjugate as catalysed by the enzyme present in rat-lung and in rat-liver soluble supernatant fractions is shown in Fig. 5. Together these results indicate that rat-lung possesses considerably higher levels of glutathione *S*-epoxide transferase activity than have been found to be present in rat-liver.

## DISCUSSION

Microsomal fractions prepared from the lungs of untreated rats have been found, in the presence of the appropriate cofactors for the mixed-function oxidase, to convert $^3$H-benz[a]anthracene into products that include one identified as the K-region epoxide, benz[a]anthracene 5,6-oxide (Fig. 1). This epoxide was also detected as a metabolite of benz[a]anthracene when liver preparations were used[5] and its formation no doubt leads to that of the K-region dihydrodiol of benz[a]-anthracene, *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene, which is a major metabolite of this hydrocarbon[22] and to the glutathione conjugate, *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione which has also been detected as a product of benz[a]anthracene metabolism.[21] It is very probable that ether extracts of rat-lung and of rat-liver microsomal incubation mixtures in which benz[a]-anthracene had been metabolized, also contained the non-K-region epoxide, benz[a]-anthracene 8,9-oxide, which in turn would be expected to be converted to the other main dihydrodiol metabolite, 8,9-dihydro-8,9-dihydroxybenz[a]anthracene.[22] Attempts to resolve non-K-region epoxides from other products by column chromatography on alumina have not succeeded to date, however, chiefly because epoxides of this type appear to decompose on chromatography much more readily than those formed on K-region bonds.[24,25]

In experiments using rat-lung microsomal fractions prepared from unstimulated animals, attempts to detect radioactive metabolites of $^3$H-7-methylbenz[a]-anthracene and of $^3$H-benzo[a]pyrene that possessed the properties of the K-region epoxides derived from these hydrocarbons were not always successful but good evidence for the formation of 7-methylbenz[a]anthracene 5,6-oxide (Fig. 2) and of benzo[a]pyrene 4,5-oxide (Fig. 3) was obtained when the hydrocarbons were metabolized using lung microsomal fractions obtained from rats that had been pretreated with 3-methylcholanthrene. These epoxides have previously been identified as metabolites of 7-methylbenz[a]anthracene and of benzo[a]pyrene respectively following the incubation of these hydrocarbons with rat-liver microsomal fractions prepared from pretreated animals.[6,7]

The detection in rat-lung of the microsomal "epoxide hydrase" which catalyses the hydration of epoxides to the corresponding dihydrodiols[16] is in accord with the results obtained in the preceding paper.[1] Here the main metabolites formed from polycyclic hydrocarbons by rat-lung preparations were found to be dihydrodiols and phenols, the dihydrodiols presumably being formed in these experiments by the action of the "epoxide hydrase" on the epoxides that are the initial products of double-bond oxidation. When $^3$H-benz[a]anthracene 5,6-oxide was used as substrate, the levels of "epoxide hydrase" activity present in rat-lung preparations were obviously much lower than those found in corresponding liver preparations (Fig. 4, Table 1). This is in agreement with previous estimations of "epoxide hydrase" activity in guinea pigs in which $^3$H-styrene oxide was employed[26] and where lung was also found to be very low in hydrase activity compared to liver.

"Glutathione *S*-epoxide transferase", the enzyme catalysing the conjugation of epoxides with GSH,[17] has been shown to be present in rat-lung soluble supernatant fraction but, unlike the "epoxide hydrase", this enzyme appears to be more active in rat-lung than in rat-liver (Fig. 5, Table 2) when $^3$H-benz[a]anthracene 5,6-oxide is employed as substrate. In its distribution therefore, the epoxide transferase appears

to differ from that of "glutathione *S*-aryltransferase", which has been reported to be much less active in rat-lung than in rat-liver.[27]

The results reported in this and in the preceding paper[1] indicate that the pathways for the metabolism of polycyclic hydrocarbons that have been described for rat-liver[2–4,22] also exist in rat-lung and demonstrate that pulmonary tissue of this species is capable of metabolizing hydrocarbons via a range of epoxide intermediates to dihydrodiols, to phenols and to glutathione conjugates. Metabolic activation to biologically effective epoxides[8–13] may well be involved in the reported induction of tumours of the respiratory tract in rats by polycyclic hydrocarbons.[14,15] In this context two other points seem worth mentioning here. Firstly, that the K-region epoxides of 7-methylbenz[a]anthracene and benzo[a]pyrene were only routinely detectable in extracts of *in vitro* microsomal incubation mixtures when lung microsomal preparations from animals pretreated with 3-methylcholanthrene were used. In tests for carcinogenicity *in vivo*, the hydrocarbons remain in contact with lung tissue for relatively long periods and, since many polycyclic hydrocarbons are potent inducers of the microsomal mixed-function oxidases,[28] these substances can apparently induce their own metabolic activation in the target tissue. A similar situation appears likely to exist in man where long-term exposure of pulmonary tissues to the polycyclic hydrocarbons present in the urban atmosphere[29] and in tobacco smoke[30] may be implicated in lung-cancer induction. Secondly, comparable amounts of hydrocarbon metabolism appear to occur in *in vitro* systems when either rat-lung or rat-liver microsomal preparations are used and when the results are calculated as the basis of the protein contents of the preparations.[1] In contrast, estimation of "epoxide hydrase" activity has shown that in the rat, lung-tissue is relatively deficient in this enzyme compared to liver (Table 1). This could be interpreted as indicating that, in pulmonary tissue in this species, the rate of epoxide formation may exceed the rate of epoxide hydration to dihydrodiol, a situation which could in turn lead, in intact cells, to significant levels of reactive epoxides being released from the endoplasmic reticulum. Although there is at present no direct evidence to support this interpretation, considerations of this type probably merit further investigation. They may also help to explain the wide variations in carcinogenic potency and in tissue specificity which exist within a series of essentially similar compounds that are metabolized by relatively simple common pathways.

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