Biochemical Pharmacology, Vol. 22, pp. 2853–2867 Pergamon Press, 1973. Printed in Great Britain.

# THE FORMATION OF "K-REGION" EPOXIDES AS HEPATIC MICROSOMAL METABOLITES OF 7-METHYLBENZ[a]ANTHRACENE AND 7,12-DIMETHYLBENZ[a]ANTHRACENE AND THEIR 7-HYDROXYMETHYL DERIVATIVES

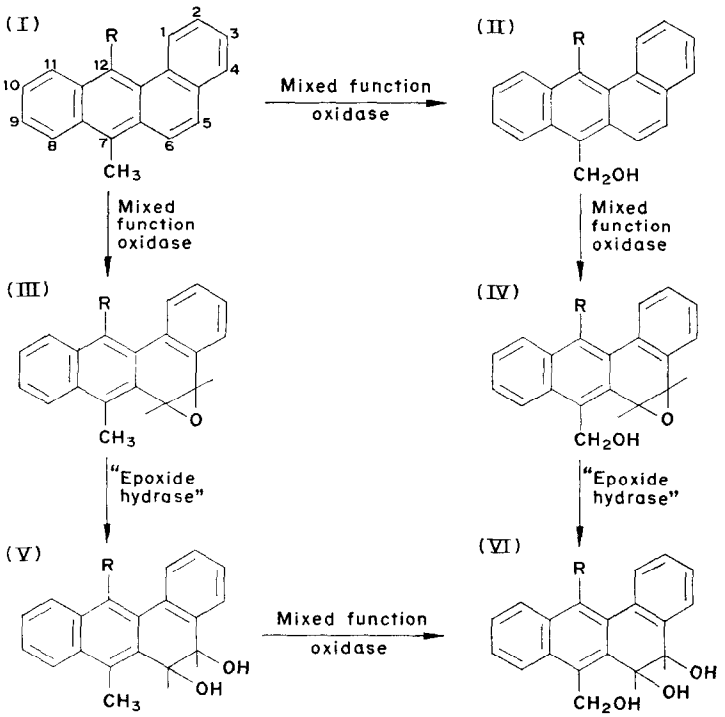
GERALD R. KEYSELL, JOAN BOOTH, PHILIP L. GROVER, ALAN HEWER and PETER SIMS

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, SW3 6JB, England

(*Received* 19 *February* 1973; *accepted* 9 *May* 1973)

**Abstract**—Epoxides have been detected as metabolites of 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene and their 7-hydroxymethyl derivatives using rat-liver microsomal systems. The metabolites, which are formed by the microsomal NADPH-dependent mixed-function oxidase, were detected in extracts of incubation mixtures that contained an inhibitor of the enzyme "epoxide hydrase". The epoxide metabolites were converted (a) by "epoxide hydrase" into products that were chromatographically identical with the related "K-region" *trans*-dihydrodiols, (b) by acid into products with the chromatographic properties of the related "K-region" phenols and (c) by reaction with GSH into products with the chromatographic properties of GSH conjugates. The identification of three metabolites as "K-region" epoxides was confirmed by recrystallization of the radioactive metabolites to constant specific activity in the presence of the appropriate unlabelled "K-region" epoxide. In common with other "K-region" epoxides, the epoxide metabolites were found to react with polyguanylic acid. When tested in Sprague–Dawley rats, the "K-region" epoxide of 7-hydroxymethyl-12-methylbenz[a]anthracene did not induce adrenal necrosis. *cis*-5,6-Dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene and *cis*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene were each metabolized by rat liver homogenates to the related 7-hydroxymethyl derivative.

INVESTIGATIONS[1–4] of the metabolism of 7-methylbenz[a]anthracene (Scheme 1, I; R = H) and 7,12-dimethylbenz[a]anthracene (I; R = Me) by liver homogenate prepared from normal rats showed that these carcinogenic hydrocarbons are converted into their respective hydroxymethyl derivatives, 7-hydroxymethylbenz[a]anthracene (II; R = H), 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) and 12-hydroxymethyl-7-methylbenz[a]anthracene, all of which are less active as carcinogens than their parent hydrocarbons.[5] In the metabolism of the hydrocarbons (I; R = H and Me) and the hydroxymethyl derivatives (II; R = H and Me) phenols, dihydrodiols and GSH conjugates are also formed.[1–4,6] When rats that had been pretreated with 3-methylcholanthrene were used, the overall rate of metabolism was increased and resulted in the formation of more polar metabolites such as the dihydrodiols of the hydroxymethyl derivatives.[7] The suggestion[8] that the dihydrodiols, phenols and glutathione conjugates that arise from hydrocarbons result from the metabolic formation of epoxide intermediates has been confirmed, and intermediates




SCHEME 1. Formation of "K-region" metabolites of 7-methylbenz[a]anthracene (I; R = H) and 7,12-dimethylbenz[a]anthracene (I; R = Me) and their 7-hydroxymethyl derivatives by rat-liver preparations.

of this type have now been detected in the metabolism of naphthalene,[9] phenanthrene and benz[a]anthracene,[10] dibenz[a,h]anthracene[10,11] and benzo[a]pyrene and pyrene.[12] A preliminary report that 7,12-dimethylbenz[a]anthracene (I; R = Me) is also metabolized to a "K-region" epoxide has been published.[13] Since "K-region" epoxides of polycyclic hydrocarbons have been found to be alkylating agents that react with nucleic acids and protein *in vitro*[14] and in cells in culture,[15,16] they are currently suspected of being responsible for the biological effects attributed to the parent hydrocarbon. Furthermore, some are mutagens in bacteria,[17] in bacteriophage[18] and in mammalian cells[19] and some are more active than the parent hydrocarbon in inducing malignant transformations of cells in culture.[20–22]

Both 7,12-dimethylbenz[a]anthracene (I; R = Me)[23] and 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me)[5] induce adrenal necrosis when injected into rats and there is evidence that the hydrocarbon (I; R = Me) is active because of its metabolic conversion into the hydroxymethyl derivative (II; R = Me).[5,24] It is not known, however, whether further metabolism of the hydroxymethyl derivative (II; R = Me) is necessary for the induction of adrenal necrosis.

This paper described the detection of $^3$H-labelled "K-region" epoxides formed in the microsomal metabolism of the $^3$H-labelled methylated polycyclic hydrocarbons, 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene, and their $^3$H-labelled

7-hydroxymethyl derivatives. The alkylation of polyguanylic acid by these radioactive epoxides is described. The effect of 7-hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide (IV; R = Me) on the adrenal glands of rats was also investigated.

## EXPERIMENTAL

*Materials.* Glucose 6-phosphate (G 6-P), G 6-P dehydrogenase, $NADP^+$ and NADPH were purchased from Boehringer (London) Ltd., Ealing, London, U.K., polyguanylic acid (poly[G]) from Miles–Seravac Ltd., Maidenhead, Berks, U.K., and cyclohexene oxide from R. N. Emanuel, Wembley, Middlesex, U.K. Basic aluminium oxide (from M. Woelm, Eschevege, Germany) was mixed with distilled water (6% w/v) and the mixture kept for 2 hr to give alumina of activity grade III (Woelm scale). 7-Methylbenz[a]anthracene (I; R = H) (sp.act. 250 mCi/m-mole) and 7,12-dimethylbenz[a]anthracene (I; R = Me) (sp.act. 81·5 mCi/m-mole), generally labelled with tritium, were prepared by diluting more highly labelled samples (tritiated at the Radiochemical Centre, Amersham, Bucks, U.K.) with the appropriate unlabelled hydrocarbon and purifying the mixture on alumina as previously described.[15] 7-Hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) (sp.act. 81·5 mCi/m-mole), generally labelled with tritium, was prepared by oxidizing a $^3H$-labelled sample of the hydrocarbon (I; R = Me) with lead tetra-acetate.[1] The oxides, 7-methylbenz[a]anthracene 5,6-oxide (III; R = H),[25,26] 7,12-dimethylbenz[a]anthracene 5,6-oxide (III; R = Me),[27] 7-hydroxymethylbenz[a]anthracene 5,6-oxide (IV; R = H)[28] and 7-hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide (IV; R = Me)[27] together with phenols and dihydrodiols, were prepared as described[25,29] and were used as chromatographic standards with previously published $R_f$ values.[26,27]

*Preparation of* $^3H$*-labelled 7-hydroxymethylbenz*[a]*anthracene (II; R = H).* A sample of the $^3H$-labelled hydrocarbon (I; R = H) (1 g), *N*-bromo succinimide (1·4 g), and $\alpha\alpha'$-azoisobutyrodinitrile (50 mg) in $CCl_4$ (30 ml) were heated under reflux for 30 min in sunlight. The solution was cooled, succinimide removed by filtration and the filtrate evaporated to dryness under reduced pressure to yield $^3H$-labelled 7-bromomethylbenz[a]anthracene (0·5 g). The bromo-compound (0·5 g) and anhydrous potassium acetate (0·5 g), in acetic acid (50 ml), were heated under reflux for 2 hr. The mixture was poured into water and the solid that separated recrystallized from methanol to yield $^3H$-labelled 7-acetoxymethylbenz[a]anthracene (0·2 g). The acetoxy derivative (0·2 g) was heated under reflux with methanol (20 ml) containing 5% (w/v) KOH for 30 min. The mixture was diluted with water and the solid filtered off and recrystallized from benzene to yield $^3H$-labelled 7-hydroxymethylbenz[a]anthracene (65 mg) (sp.act. 300 mCi/m-mole) in plates, m.p. 166°, undepressed in admixture with unlabelled 7-hydroxymethylbenz[a]anthracene.[26]

*Chromatography:* (a) *Thin-layer chromatography.* This was carried out on glass plates that had been coated with layers of silica gel G (0·25 mm thick), and dried at 120°. The chromatograms were developed with either solvent (a), benzene; solvent (b), benzene–ethanol (9:1, v/v); solvent (c), benzene–ethanol (4:1, v/v); or solvent (d), butan-1-ol–propan-1-ol–aq.2 M-$NH_4OH$, (2:1:1, by vol.). The products were usually located by inspecting the wet plates in u.v. light; fluorescent and intermediate bands were marked off and removed from the plates and the radioactivity present on the silica gel estimated by liquid scintillation counting using a Packard Tricarb Spectrometer with a counting efficiency of about 35 per cent. The 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.

(b) *Column chromatography.* This was carried out on columns of 0·9 cm dia. packed with alumina to a height of 4·0 cm. The solvents used to develop the columns varied according to the material under examination and they are described in detail in Figs. 1 and 2. In each case, fractions (120 drops) were collected using a LKB 7000 fraction collector. In preliminary experiments, each of the synthetic "K-region" epoxides (III; R = H and Me and IV; R = H and Me) were chromatographed, together with the hydrocarbons and relevant hydroxylated derivatives, and the fractions in which each was eluted were determined by measuring (a) the u.v. absorbance of fractions at the appropriate wavelengths and (b) their u.v. spectra.

In the metabolic experiments, the mixtures of $^3$H-labelled metabolites were each chromatographed using the solvents described in Figs. 1 and 2 and the radioactivity

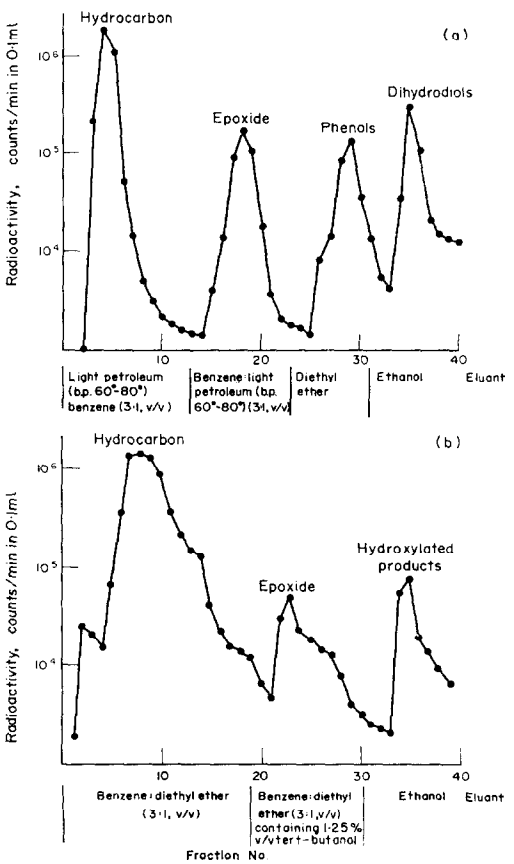



FIG. 1. Alumina column elution profiles of (a) $^3$H-labelled 7-methylbenz[a]anthracene (I; R = H) and (b) $^3$H-labelled 7-hydroxymethylbenz[a]anthracene (II; R = H) metabolites. The concentrated extract obtained from a rat-liver microsomal incubation, carried out as described in the text, was applied to a column (0·9 × 4 cm) of basic alumina (Woelm, activity grade III) and eluted with solvent. Fractions (120 drops) were collected and the radioactivity present in these fractions measured as described.

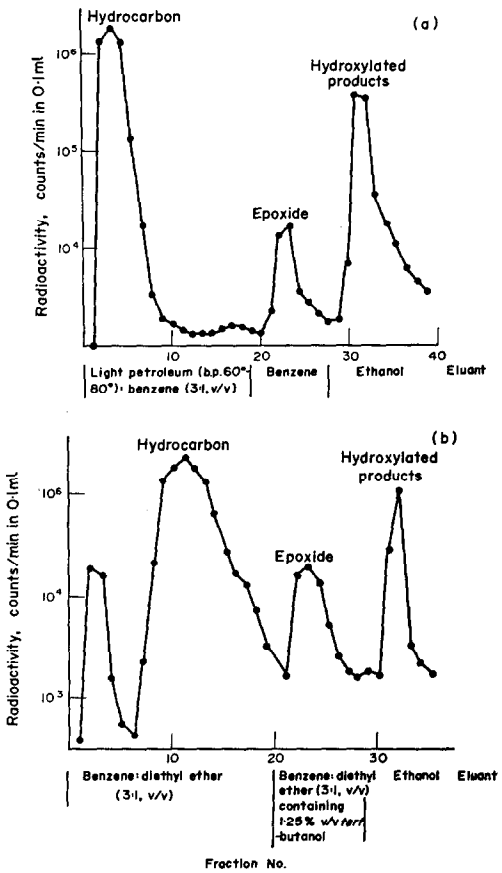



FIG. 2. Alumina column elution profiles of (a) $^{3}$H-labelled 7,12-dimethylbenz[a]anthracene (I; R = Me) and (b) $^{3}$H-labelled 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) metabolites. The concentrated extract obtained from a rat-liver microsomal incubation, carried out as described in the text, was applied to a column (0·9 × 4 cm) of basic alumina (Woelm, activity grade III) and eluted with solvent. Fractions (120 drops) were collected and the radioactivity present in these fractions measured as described.

present in each fraction determined by liquid scintillation counting as described above. From these measurements it was possible to identify the fractions thought to contain epoxide metabolites and in each experiment these were pooled and examined as described below.

*Metabolism of polycyclic hydrocarbons by microsomal fractions.* Microsomal fractions were prepared as described[30] from the livers of male Wistar rats (35–45 days old) that had been pretreated with 3-methylcholanthrene (5 mg in 0·5 ml arachis oil), administered by intraperitoneal injection 48 hr before death. Incubations were carried out in 0·1 M-pyrophosphate buffer (pH 8·0) prepared from 0·1 M-tetrasodium pyrophosphate and 0·1 M-disodium dihydrogen pyrophosphate. Standard incubation mixtures contained a liver microsomal fraction (equivalent to 10 g liver), G 6-P (1 m-mole), G 6-P dehydrogenase (96 units), NADPH (48 μmole), nicotinamide (2

m-mole), $MgCl_2$ (1 m-mole) and cyclohexene oxide (30 μmole) in 80 ml of reaction mixture. The mixtures were incubated for 10 min at 30°, the substrate (10 μmole in 0·5 ml ethanol) added and the incubations continued for a further 10 min. Each mixture was extracted with diethyl ether (80 ml) containing the appropriate unlabelled "K-region" epoxide (2 mg). The ether extracts were dried over anhydrous $Na_2SO_4$ and concentrated to 1 ml under reduced pressure. The solvent (20 ml) used in the initial development of the alumina column (see Figs. 1 and 2) was added to each extract, the mixtures were evaporated to 2 ml under reduced pressure and then chromatographed on alumina as described above.

*The metabolism of* cis-5,6-*dihydro*-5,6-*dihydroxy*-7-*methylbenz*[a]*anthracene* (*V; R = H*) *and* cis-5,6-*dihydro*-5,6-*dihydroxy*-7,12-*dimethylbenz*[a]*anthracene* (*V; R = Me*). Each dihydrodiol (0·5 μmole) in ethanol (0·2 ml) was added to a homogenate prepared[6] from 1 g of liver in 10 ml KCl (1·15% w/v), using rats that had been pretreated with 3-methylcholanthrene as described above, together with 10 ml of phosphate buffer (pH 7·4) containing $NADP^+$ (6 mg) and G 6-P (30 mg). Each mixture was shaken in a water bath at 37° for 30 min and extracted with ethyl acetate (40 ml). The extracts were dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The residues were applied to the base lines of thin-layer chromatograms that were developed with solvent (a). The metabolites were located as described above.

*Acid-catalysed rearrangement of epoxides to phenols.* Column chromatography fractions containing the epoxides obtained as described above, were each mixed with acetic acid (1 ml) containing 1 drop of conc. HCl and the solution concentrated under reduced pressure. Methanol (5 ml) containing 5% (v/v) aq. $NH_3$ (s.g. 0·88) was added to each of the mixtures and portions (0·5 ml) of the solutions were chromatographed, together with the appropriate reference phenols, in either solvent (a) or solvent (b). The chromatograms were divided into bands as described above and the radioactivity present in each was determined.

*Enzyme-catalysed conversion of epoxides into dihydrodiols.* The same fractions containing the epoxides were concentrated under reduced pressure and the concentrates each diluted with ethanol (1 ml). The solutions were each added to a microsomal fraction obtained from rat liver (10 g) suspended in phosphate buffer (100 ml, pH 7·4), and the mixture incubated at 37° for 17 hr. The mixtures were extracted with ethyl acetate (100 ml) and the products present in the extracts examined by TLC in either solvent (b) or solvent (c) using the appropriate *trans*-dihydrodiols as chromatographic markers. The radioactivity present in bands of silica gel removed from the chromatogens was estimated as described.

*Formation of glutathione derivatives of epoxides.* Fractions containing the epoxides obtained as described above were evaporated to dryness under reduced pressure. The residues were each redissolved in acetone (5 ml) in the presence of the appropriate synthetic "K-region" epoxide (5 mg) and the solutions heated under reflux in the dark for 4 hr with a solution of GSH (2·5 mg) and $NaHCO_3$ (5 mg) in water (5 ml). Water (20 ml) and activated charcoal (0·4 g) were added and the mixtures adjusted to pH 4·0 with acetic acid. The charcoal was filtered off, washed twice with water (10 ml, pH 4·0) and the absorbed material eluted from the charcoal with three volumes of methanol (20 m) containing 5% (v/v) aq. $NH_3$ (s.g. 0·88). The methanol extracts were combined and concentrated under reduced pressure and the solution analysed by TLC with solvent (d). The chromatograms were treated with ninhydrin

as described above and ninhydrin positive and other bands were removed and the radioactivity present in each was determined.

*Reaction of epoxides with polyguanylic acid.* Fractions containing the epoxides obtained as described above were concentrated under reduced pressure and acetone (10 ml) added. The solutions were evaporated to small volumes, adjusted to 1 ml with acetone and incubated for 2 hr at 37° with polyguanylic acid (2 mg) in water (2 ml) previously adjusted to pH 7·4 with 0·1 M-phosphate buffer. The mixtures were extracted with diethyl ether (3 × 3 ml) and the aqueous phases chromatographed on Sephadex G25 columns (0·9 × 60 cm) as previously described.[31] Fractions (12 drops) were collected and the absorbance at 260 nm and the radioactivity present in each fraction estimated.

*Recrystallization of radioactive epoxides to constant specific activity.* Samples (100–200 mg) of the appropriate unlabelled "K-region" epoxide were added to the pooled fractions containing radioactive epoxide obtained in the metabolic experiments described above, in which the hydrocarbons (I; R = H and Me) and the hydroxymethyl derivative (II; R = H) were used as substrates. The mixtures to which the "K-region" synthetic epoxides (III; R = H or Me) were added were recrystallized repeatedly from cyclohexane–benzene (9:1, v/v) and that to which the synthetic "K-region" epoxide (IV; R = H) was added was recrystallized repeatedly from cyclohexane–benzene (3:1, v/v). The "K-region" epoxide (IV; R = Me), which was the expected product when the hydroxymethyl derivative (II; R = Me) was used as substrate, has only been obtained as a gum,[27] so that recrystallization to constant specific activity of this epoxide was not possible.

*Induction of adrenal necrosis in rats.* Female Sprague–Dawley rats (51-days old) were anaesthetized with ether and injected intravenously via the caudal vein with either (a) an emulsion (0·3 ml), prepared from dimethyl sulphoxide (1 ml), arachis oil (2 ml) and 4% (v/v) Tween 80 in isotonic saline (2 ml), (b) an emulsion (0·5 ml) prepared as before but also containing 7,12-dimethylbenz[a]anthracene (I; R = Me) (5 mg) or (c) an emulsion (0·3 ml) prepared as before but also containing 7-hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide (IV; R = Me) (3 mg). The rats were killed 3 days later and the adrenals weighed and examined for haemorrhagic necrosis. The adrenals were each homogenized in isotonic saline (3 ml) and haemoglobin estimated as described.[32]

## RESULTS

The elution profiles shown in Figs. 1 and 2 were obtained by chromatography on alumina columns of the diethyl ether extracts of incubation mixtures in which either 7-methylbenz[a]anthracene (I; R = H), 7,12-dimethylbenz[a]anthracene (I; R = Me), 7-hydroxymethylbenz[a]anthracene (II; R = H) or 7-hydroxymethyl-12-methylbenz-[a]anthracene (II; R = Me) had been incubated with rat liver microsomes.

Similar profiles were obtained with each substrate, but with the hydroxymethyl compounds (II; R = H and Me) it was necessary to use more polar eluting solvents. Under the standard conditions used, the unmetabolized substrates were eluted from the columns in the early fractions and these were followed by fractions that were believed to contain the "K-region" epoxide. In the control experiments the synthetic "K-region" epoxides were found to elute from the columns in these fractions. Later

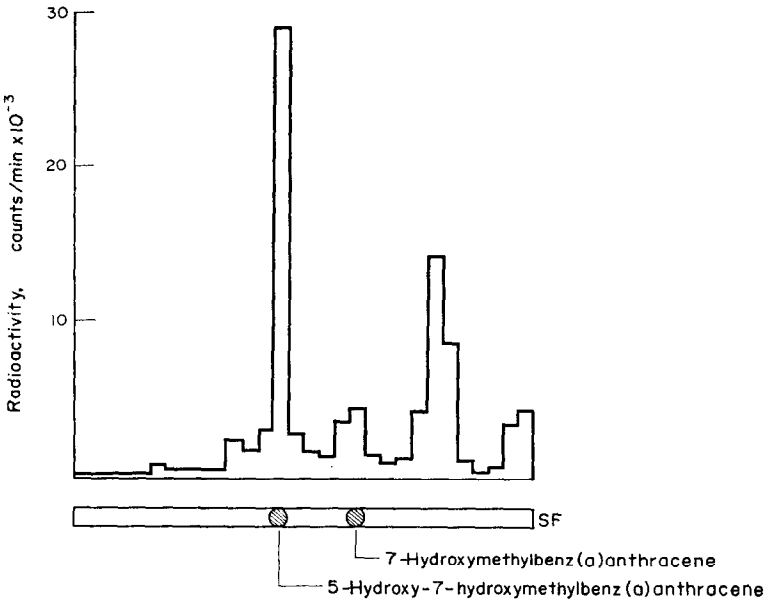



FIG. 3. Acid catalysed rearrangement of the radioactive epoxide of $^3$H-labelled 7-hydroxymethylbenz[a]anthracene (II; R = H) to the corresponding phenol. The fractions eluted from the alumina column that contained the epoxide (Fig. 1) were pooled and acetic acid (0·5 ml) and 1 drop of conc. HCl added. The solution was reduced in volume by evaporation and methanol (5 ml) containing ammonia (5% v/v) added and a portion (0·5 ml) of the solution examined by TLC on silica gel in benzene–ethanol (9:1, v/v).

fractions from the columns contained more polar products such as phenols and dihydrodiols but these were not examined in detail.

In order to confirm the identities of the products in the epoxide-containing fractions, four types of reactions were carried out. In the first, the products were treated with acid as described above and the phenols thus produced examined by TLC. In each case the major peaks of radioactivity, presumably arising from the rearrangement of the epoxides formed by metabolism, moved to the same position on the plate as the corresponding synthetic "K-region" phenol. The chromatographic profile obtained when the metabolic product from $^3$H-labelled 7-hydroxymethylbenz[a]anthracene (II; R = H) was used is shown in Fig. 3. The major peak of radioactivity is coincident with the "K-region" phenol, 5-hydroxy-7-hydroxymethylbenz[a]anthracene.

In the second series of experiments the products present in the epoxide-containing peaks were incubated with the enzyme "epoxide hydrase" present in rat-liver microsomal fractions. Examination by TLC of the products from these reactions showed that with all four substrates, major peaks of radioactivity were present, coincident with the related "K-region" *trans*-dihydrodiols. The chromatographic profile obtained when the product of the metabolism of $^3$H-labelled 7-methylbenz[a]anthracene (I; R = H) was incubated with a rat-liver microsomal fraction is shown in Fig. 4. The main peak of radioactivity was coincident with the "K-region" dihydrodiol, *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene (V; R = H).

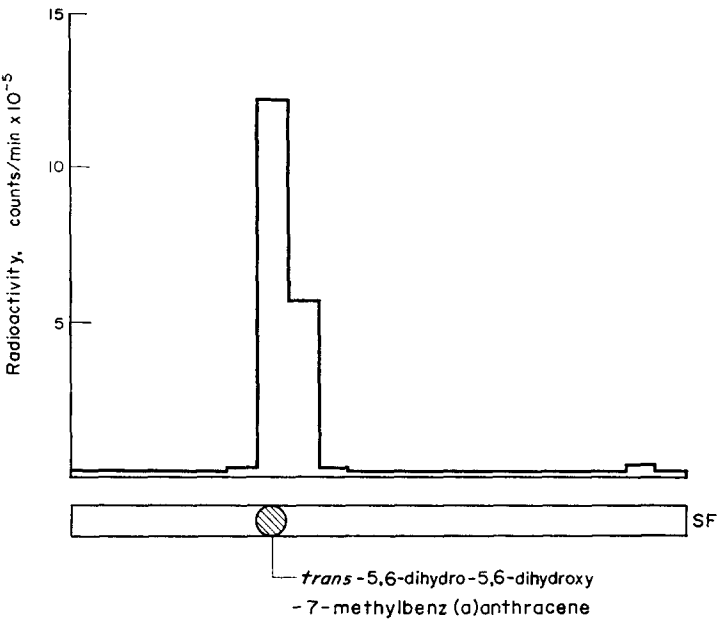



FIG. 4. Enzyme-catalysed conversion of a radioactive 7-methylbenz[a]anthracene epoxide to the corresponding dihydrodiol. Eluant fractions from an alumina column thought to contain a radioactive epoxide derived from 7-methylbenz[a]anthracene were pooled, concentrated and incubated with a rat-liver microsomal fraction as described in the text. The ether-soluble products formed were examined on TLC developed in solvent (a).

The products present in the epoxide-containing fractions were each converted chemically into GSH conjugates in the presence of the related unlabelled "K-region" epoxides. Examination by TLC of the conjugates thus formed showed that the regions containing ninhydrin-positive material were also regions of high radioactivity. A typical example of the chromatographic profiles obtained is shown in Fig. 5, where the products of the metabolism of $^{3}$H-labelled 7,12-dimethylbenz[a]anthracene (I; R = Me) were allowed to react with GSH in the presence of unlabelled 7,12-dimethylbenz-[a]anthracene 5,6-oxide (III; R = Me). Most of the radioactivity was present in the same region of the chromatogram as S-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]-anthracen-5-yl)glutathione.

In the final series of experiments, the products in the epoxide-containing fractions were incubated with Poly [G] and the mixtures passed through Sephadex G25. The elution profiles thus obtained showed that reaction of the epoxide metabolites of each of the $^{3}$H-labelled substrates with the polynucleotide had occurred. A typical example of the elution profiles obtained is shown in Fig. 6, where the epoxide metabolite (IV; R = Me) of $^{3}$H-labelled 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) was incubated with poly [G].

The results described above indicate that not only are all four substrates converted by the microsomal system into epoxides but also that in each case these are the "K-region" epoxides. With three of the substrates, 7-methylbenz[a]anthracene (I; R = H), 7,12-dimethylbenz[a]anthracene (I; R = Me) and 7-hydroxymethylbenz[a]-

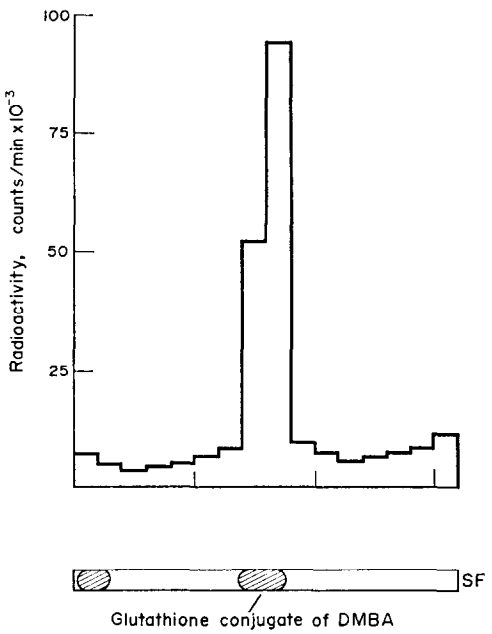



FIG. 5. Formation of a glutathione conjugate from a radioactive epoxide metabolite of 7,12-dimethylbenz[a]anthracene. Eluant fractions from an alumina column believed to contain a radioactive epoxide derived from 7,12-dimethylbenz[a]anthracene were pooled and unlabelled 7,12-dimethylbenz[a] anthracene 5,6-oxide (III; R = Me) (5 mg) added. The solution was evaporated to dryness under reduced pressure and the residue redissolved in acetone (5 ml). After the addition of a solution of GSH (2·5 mg) and $NaHCO_3$ (5 mg) in water (5·0 ml), the mixture was refluxed for 4 hr, the conjugate was isolated using activated charcoal as described in the text and was examined by TLC using solvent (d). The chromatogram was subsequently treated with ninhydrin.

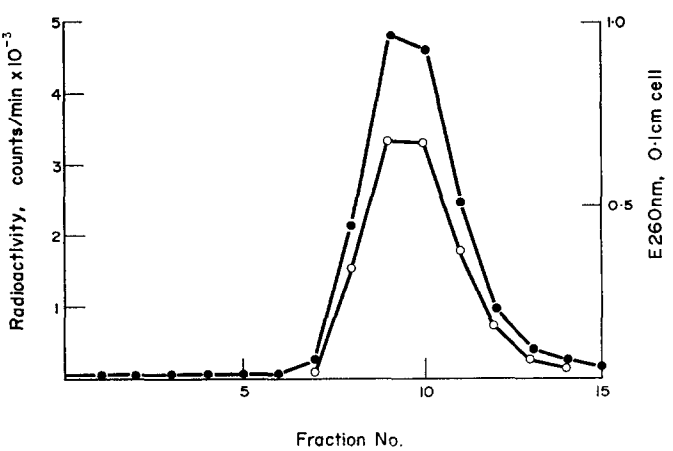



FIG. 6. Sephadex G25 elution profiles of the products of reaction of a radioactive epoxide metabolite of 7-hydroxymethyl-12-methylbenz[a]anthracene and polyguanylic acid. Fractions obtained from an alumina column were pooled, concentrated and the residues redissolved in acetone as described in the text. The material present in this solution was reacted with polyguanylic acid and passed through a G25 Sephadex column as previously described[31] and the radioactivity (○) and the u.v. absorption at 260 nm (●) measured.

TABLE 1. RECRYSTALLIZATION OF SOME "K-REGION" EPOXIDES CONTAINING RADIOACTIVE EPOXIDE FORMED IN THE METABOLISM OF THE CORRESPONDING $^3$H-LABELLED SUBSTRATE

| Crystallization no. | 7-Methylbenz[a]anthracene 5,6-oxide (III; R = H) | | 7,12-Dimethylbenz[a]anthracene 5,6-oxide (III; R = Me) | | 7-Hydroxymethylbenz[a]anthracene 5,6-oxide (IV; R = H) | |
|---|---|---|---|---|---|---|
| | Yield of epoxide (mg) | Dis/min per mg ($\times 10^{-3}$) | Yield of epoxide (mg) | Dis/min per mg ($\times 10^{-3}$) | Yield of epoxide (mg) | Dis/min per mg ($\times 10^{-3}$) |
| 1 | 193 | 562 | 130 | 18·7 | 68 | 1468 |
| 2 | 158 | 549 | 122 | 17·6 | 59 | 832 |
| 3 | 136 | 585 | 115 | 18·6 | 49 | 750 |
| 4 | 95 | 498 | 98 | 17·4 | 31 | 862 |
| 5 | 52 | 537 | 90 | 18·0 | 5 | 740 |

Incubations using $^3$H-labelled substrate were carried out as described in the text. Fractions containing $^3$H-labelled epoxides were obtained from the reaction products by chromatography on alumina as indicated in Figs. 1 and 2. These were mixed with the appropriate unlabelled "K-region" epoxide and the mixtures repeatedly recrystallized from cyclohexane–benzene mixtures as indicated in the text.

Table 2. Induction of adrenal necrosis in Sprague–Dawley rats

| Compound | Dose (mg) | No. of rats | Mean wt of adrenal glands (mg) | Visual appearance | No. of rats with adrenal necrosis/no. of rats examined | Haemoglobin estimation (units) |
|---|---|---|---|---|---|---|
| None | – | 8 | 22 | Normal | 0/8 | 0·43 |
| 7-Hydroxymethyl-12-methylbenz[a] anthracene 5,6-oxide (IV; R = Me) | 3 | 8 | 20·5 | Normal | 0/8 | 0·46 |
| 7,12-Dimethylbenz[a]anthracene (I; R = Me) | 5 | 8* | 34·1 | Haemorrhagic necrosis | 3/3 | 1·57 |

Sprague–Dawley rats were injected into the caudal vein with an arachis oil emulsion, prepared as described in the text, containing the compound under test. The adrenal glands were examined after 3 days. Haemoglobin was estimated as described.[32]

* Two rats died on day 1 and 3 rats died on day 2.

anthracene (II; R = H), it was possible to confirm this by mixing the products present in the epoxide-containing fractions with the corresponding unlabelled "K-region" epoxide and recrystallizing to constant specific activities. The results of these experiments are summarized in Table 1.

The results obtained in experiments in which either *cis*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene (V; R = H) or *cis*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene (V; R = Me) was incubated with rat-liver homogenates showed that products were formed that were chromatographically identical with the corresponding *cis*-dihydrodiols (VI; R = H or Me) of the hydroxymethyl derivative. Thus with both substrates, hydroxylation of the C-7 methyl group had occurred.

The results in Table 2 show that 7-hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide (IV; R = Me) does not induce adrenal necrosis in Sprague–Dawley rats.

## DISCUSSION

The results described above show that 7-methylbenz[a]anthracene (I; R = H), 7,12-dimethylbenz[a]anthracene (I; R = Me), 7-hydroxymethylbenz[a]anthracene (II; R = H) and 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) are all metabolized to epoxides by microsomal fractions from the livers of rats that were pretreated with 3-methylcholanthrene. Cyclohexene oxide was incorporated into the incubation mixtures to act as an inhibitor of the microsomal enzyme "epoxide hydrase" that would otherwise convert the epoxides into dihydrodiols. Since the metabolites were converted (a) chemically into compounds with the chromatographic characteristics of the corresponding "K-region" phenol by acid, (b) enzymically into compounds with the characteristics of the corresponding "K-region" *trans*-dihydrodiol by the "epoxide hydrase" of rat-liver microsomes and (c) chemically into compounds with the characteristics of the related GSH-conjugate by reaction with GSH, the metabolites are the "K-region" epoxides. In the case of the hydrocarbons (I; R = H and Me) and the hydroxymethyl derivative (II; R = H) the identities of the metabolites were confirmed by their recrystallization to constant specific activities in the presence of the corresponding unlabelled synthetic "K-region" epoxide. These results are in agreement with those[10,12] obtained with other polycyclic hydrocarbons, which are also metabolized by rat-liver microsomal fractions to the related "K-region" epoxides.

The hydrocarbons (I; R = H and Me) and the hydroxymethyl derivatives (II; R = H and Me) are all metabolized by rat-liver microsomes into dihydrodiols formed at bonds other than those of the "K-regions"[29,33,34] and presumably these metabolites arise through the intermediate formation of epoxides. No evidence was obtained, however, for the formation of non-"K-regions" epoxides of this type in the experiments now described. This may be because non-"K-region" epoxides of polycyclic hydrocarbons are less stable than "K-region" epoxides: benzo[a]pyrene 7,8-oxide and benzo[a]pyrene 9,10-oxide[35] and benz[a]anthracene 8,9-oxide[36] are decomposed into phenols when chromatographed on alumina columns of the type described above.

Earlier work[26] on the metabolism of 7-methylbenz[a]anthracene (I; R = H) by rat-liver homogenates and microsomal fractions showed that both preparations metabolized the hydrocarbon into the "K-region" dihydrodiol, *trans*-5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene (V; R = H). With 7,12-dimethylbenz[a]-anthracene, however, the "K-region" dihydrodiol, *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene was only detected when rat-liver microsomal frac-

tions were used.[37] Similar results were obtained when the hydroxymethyl derivatives (II; R = H)[26,28] or (II; R = Me)[27,29] were incubated with either liver homogenates or microsomal fractions. Thus in the microsomal systems, the "K-region" epoxides that are first formed are further metabolized, presumably by the enzyme "epoxide hydrase", to dihydrodiols. In homogenates, however, they probably react preferentially either with GSH,[6] a reaction presumably catalysed by the enzyme "glutathione S-epoxide transferase" present in the soluble fraction,[38] or with other cellular constituents. The metabolic studies carried out on the *cis*-dihydrodiols (V; R = H or Me) show that the metabolically formed *trans*-dihydrodiols (VI; R = H or Me) of the hydroxymethyl derivative can probably arise by one of two routes, depending on whether hydroxylation of the C-7 methyl groups occurs before or after metabolism at the bonds of the "K-regions".

In their reactions with poly [G], the epoxides (III and IV; R = H and Me) that are formed by metabolism resemble other synthetic "K-region" epoxides. In addition to poly [G], these other epoxides also react with DNA and RNA, react to a lesser extent with apurinic acid, poly [A], poly [X] and poly [I]; but do not react appreciably with poly [U] and poly [C].[31] It is clear, therefore, that like non-methylated hydrocarbons, the hydrocarbons (I; R = H and Me) and their hydroxymethyl derivatives (II; R = H and Me) are all metabolized at their "K-regions" to yield similar reactive epoxides. There is now evidence to suggest that carcinogenic polycyclic hydrocarbons exert their biological effects only after conversion into epoxides; some of this evidence has been provided by studies on the malignant transformation of rodent cells in culture by a number of hydrocarbons and their "K-region" epoxides. Although in most cases the epoxides proved more active than the parent hydrocarbons, the epoxide (III; R = H)[21,22] and (III; R = Me)* were usually no more active than the parent compounds. Similar results were obtained when the transforming abilities of the hydroxymethyl derivatives (II; R = H) and its "K-region" epoxide (IV; R = H) were compared*.

It has been suggested[39] that the formation of 7-hydroxymethyl-12-methylbenz[a]anthracene (II; R = Me) may be the first step in the initiation of carcinogenesis by 7,12-dimethylbenz[a]anthracene (I; R = Me) but the results now described show that both compounds are metabolized to reactive epoxides. In the induction of adrenal necrosis in rats,[23] however, the hydroxymethyl derivative (II; R = Me) is more active than 7,12-dimethylbenz[a]anthracene (I; R = Me):[5,24] since epoxides are formed by metabolism in tissues other than liver† the possibility that the epoxide (IV; R = Me) is involved in this biological effect was investigated. It has now been shown that the epoxide (IV; R = Me) is inactive under conditions where the hydrocarbon (I; R = Me) induces adrenal necrosis in rats.

It is clear, therefore, that both methyl-substituted hydrocarbons (I; R = H and Me) and their hydroxymethyl derivatives (II; R = H and Me) are converted into reactive "K-region" epoxides by microsomal enzyme systems and that these intermediates may be further metabolized to phenols, dihydrodiols of GSH conjugates. The role played by these intermediates in the induction of cancer by their parent hydrocarbons has yet to be determined.

* H. Marquardt, P. L. Grover and P. Sims, unpublished observations.
† P. L. Grover, unpublished observations.

*Acknowledgements*—This investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the Cancer Research Campaign.

## REFERENCES

1. E. Boyland and P. Sims, *Biochem. J.* **95,** 780 (1965).
2. P. H. Jellinck and B. Goudy, *Science, N.Y.* **152,** 1375 (1966).
3. W. Levin and A. H. Conney, *Cancer Res.* **27,** 1931 (1967).
4. J. W. Flesher, S. Soedigdo and D. R. Kelley, *J. med. Chem.* **10,** 932 (1967).
5. E. Boyland, P. Sims and C. Huggins, *Nature, Lond.* **207,** 816 (1965).
6. J. Booth, G. R. Keysell and P. Sims, *Biochem. Pharmac.* **22,** 1781 (1973).
7. E. Boyland and P. Sims, *Biochem. J.* **104,** 394 (1967).
8. E. Boyland, *Symp. Biochem. Soc.* **5,** 40 (1950).
9. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenburg and S. Udenfriend, *Biochemistry, Easton* **9,** 147 (1970).
10. P. L. Grover, A. Hewer and P. Sims, *FEBS Lett.* **18,** 76 (1971).
11. J. K. Selkirk, E. Huberman and C. Heidelberger, *Biochem. biophys. Res. Commun.* **43,** 1010 (1971).
12. P. L. Grover, A. Hewer and P. Sims, *Biochem. Pharmac.* **21,** 2713 (1972).
13. G. R. Keysell, J. Booth, P. Sims, P. L. Grover and A. Hewer, *Biochem. J.* **129,** 41P (1972).
14. P. L. Grover and P. Sims, *Biochem. Pharmac.* **19,** 2251 (1970).
15. P. L. Grover, J. A. Forrester and P. Sims, *Biochem. Pharmac.* **20,** 1297 (1971).
16. T. Kuroki, E. Huberman, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, *Chem.-Biol. Inter.* **4,** 389 (1972).
17. B. N. Ames, P. Sims and P. L. Grover, *Science, N.Y.* **176,** 47 (1972).
18. M. J. Cookson, P. Sims and P. L. Grover, *Nature, Lond.* **234,** 186 (1971).
19. E. Huberman, L. Aspiras, C. Heidelberger, P. L. Grover and P. Sims, *Proc. natn. Acad. Sci. U.S.A.* **68,** 3195 (1971).
20. P. L. Grover, P. Sims, E. Huberman, H. Marquardt, T. Kuroki, and C. Heidelberger, *Proc. natn. Acad. Sci. U.S.A.* **68,** 1098 (1971).
21. H. Marquardt, T. Kuroki, E. Huberman, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims. *Cancer Res.* **32,** 716 (1972).
22. E. Huberman, T. Kuroki, H. Marquardt, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, *Cancer Res.* **32,** 1391 (1972).
23. C. Huggins, T. F. Deuel and R. Fukunishi, *Biochem. Z.* **338,** 106 (1963).
24. D. N. Wheatley, A. G. Hamilton, A. R. Currie, E. Boyland and P. Sims, *Nature, Lond.* **211,** 1311 (1966).
25. M. S. Newman and S. Blum, *J. Am. chem. Soc.* **86,** 5598 (1964).
26. P. Sims, *Biochem. J.* **105,** 591 (1967).
27. P. Sims, *Biochem. J.* **131,** 405 (1973).
28. P. Sims, *Xenobiotica* **2,** 469 (1972).
29. E. Boyland and P. Sims, *Biochem. J.* **104,** 394 (1967).
30. P. L. Grover and P. Sims, *Biochem. J.* **110,** 159 (1968).
31. P. L. Grover and P. Sims, *Biochem. Pharmac.* **22,** 661 (1973).
32. C. Huggins and S. Morii, *J. exp. Med.* **114,** 741 (1961).
33. P. Sims, *Biochem. Pharmac.* **19,** 795 (1970).
34. P. Sims, *Biochem. Pharmac.* **19,** 2261 (1970).
35. J. F. Waterfall and P. Sims, *Biochem. J.* **128,** 265 (1972).
36. P. Sims, *Biochem. J.* **125,** 159 (1971).
37. P. Sims and P. L. Grover, *Biochem. Pharmac.* **17,** 1751 (1968).
38. E. Boyland and K. Williams, *Biochem. J.* **94,** 190 (1965).
39. J. W. Flesher and K. L. Sydnor, *Cancer Res.* **31,** 1951 (1971).