Biochemical Pharmacology, Vol. 22, pp. 1781–1791. Pergamon Press, 1973. Printed in Great Britain.

FORMATION OF GLUTATHIONE CONJUGATES AS METABOLITES OF 7,12-DIMETHYLBENZ-[a]ANTHRACENE BY RAT-LIVER HOMOGENATES

JOAN BOOTH, GERALD R. KEYSELL and PETER SIMS

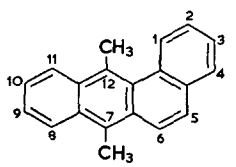
The Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, SW3 6JB, England

(*Received* 4 *November* 1972; *accepted* 19 *January* 1973)

Abstract—The structures of the water-soluble derivatives of ^{3}H-labelled 7,12-dimethylbenz[a]anthracene that are formed by rat-liver homogenates have been investigated. Paper chromatography resolved the mixture of metabolites into two regions of high radioactivity. The products of the alkaline hydrolyses of the material eluted from both of these regions contained cysteine, glycine and glutamic acid, indicating that GSH conjugates were present. The material forming the faster moving region appeared to consist of a single compound that was identified as *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione by comparisons with the synthetic conjugate.

The slower-moving region of radioactivity may contain a mixture of conjugates since the dihydrodiols that are formed during the metabolism of 7,12-dimethylbenz[a]anthracene by rat-liver homogenates are all converted by further metabolism into water-soluble derivatives that are located in this region of the chromatograms. The possible structures of these conjugates is discussed.

THE CARCINOGENIC hydrocarbon, 7,12-dimethylbenz[a]anthracene, is converted into water-soluble derivatives by mammalian liver[1–5] and by cells grown in culture,[6–8] but these metabolites have not been fully characterized. Much of the previous work on the metabolism of 7,12-dimethylbenz[a]anthracene has involved the incubation of the ^{3}H-labelled hydrocarbon with rat-liver preparations and NADPH, followed by extraction of the non-polar metabolites into organic solvents for identification.[1,3,9–14] However, a considerable part of the radioactivity remains in the aqueous phase because of the formation of water-soluble derivatives. Evidence that rat-liver preparations form a small peptide conjugate during the metabolism of the hydrocarbon has been presented.[5] It was, therefore, suggested that GSH is involved in the formation of the water-soluble metabolites produced from the hydrocarbon by preparations of the livers from rats that had been pretreated with 3-methylcholanthrene. This paper describes experiments designed to elucidate the structure of these water-soluble metabolites.



7, 12-Dimethylbenz[a]anthracene

EXPERIMENTAL

Materials. ^{3}H-Labelled benz[a]anthracene (sp.act. 714 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. The purification of 7,12-dimethylbenz[a]anthracene and the preparation of 7-hydroxymethyl-12-methylbenz[a]anthracene and 12-hydroxymethyl-7-methylbenz[a]anthracene, all generally labelled with tritium, have been previously described.[14] The specific activity of these compounds was 81·5 mCi/mmole. Unlabelled *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene,[11] *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione[15] and *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione and *S*-(5,6-dihydro-6-hydroxy-7-hydroxymethyl-12-methylbenz[a]anthracen-5-yl)glutathione[16] were prepared by published methods.

Chromatography. Thin-layer chromatography (TLC) was carried out on glass plates coated with layers (0·25 mm thick) of Silica gel G (E. Merck A.-G. Darmstadt, Germany). The chromatograms were developed for 15 cm with either solvent (a), benzene–ethanol (9:1, v/v); solvent (b), light petroleum (b.p. 60–80°)–benzene (19:1, v/v); solvent (c), butan-1-ol–propan-1-ol–aqueous 2M-NH_4OH, (2:1:1, v/v); solvent (d), chloroform–2-methylbutan-2-ol–acetic acid (6:3:1, v/v) or solvent (e), chloroform–ethanol–acetic acid (38:4:3, v/v). The hydrocarbon metabolites were located on chromatograms in u.v.-light both before and after exposure to NH_3.[12] The Dns-derivatives, prepared as described below, were examined by TLC using solvents (c), (d) or (e) and they were located by inspecting the chromatograms in u.v.-light.

Paper chromatography was carried out on Whatman no. 1 paper and the chromatograms were developed downwards for approximately 25 cm with solvent (f), butan-1-ol–acetic acid–water (12:3:5, v/v). The dried chromatograms were examined in u.v.-light, dipped in a solution of ninhydrin (0·2%) in acetone and heated at 100° for 5 min.

Incubations with rat-liver homogenates. Homogenates were prepared[12] from the livers of pretreated Chester Beatty or Wistar strain male rats (body weight approx. 200 g). Pretreatment consisted of an intraperitoneal injection of 3-methylcholanthrene (5 mg) in arachis oil (0·5 ml) administered 48 hr before the animals were killed. Incubations were carried out in 50 mM-phosphate buffer (NaH_2PO_4–Na_2HPO_4), pH 7·4. Standard incubation mixtures contained rat-liver homogenate (250 mg liver), $NADP^+$ (3 mg), glucose 6-phosphate (15 mg) and substrate (100 μg in 0·1 ml ethanol) in 10 ml of reaction mixture. The total volumes of incubation mixtures are indicated in each individual experiment. After warming the mixtures to 37°, the reactions were started by the addition of the substrate.

Samples of ^{3}H-labelled dihydrodiols were prepared enzymically by isolating the products from standard incubations (200 ml) using either ^{3}H-labelled 7,12-dimethylbenz[a]anthracene or one of its ^{3}H-labelled hydroxymethyl derivatives as substrate. In these experiments, incubations were for 15 min. At the end of the incubation period, each reaction mixture was shaken with 2 vol. of ethyl acetate and the phases separated by centrifugation. The ethyl acetate phase was dried over anhydrous Na_2SO_4, concentrated under reduced pressure and the solution applied as a band to the base of a chromatogram that was developed with solvent (a). The dihydrodiols were located in u.v.-light and the appropriate bands of Silica gel removed from the plate. The compounds were eluted from the Silica gel with ethanol. The u.v.-absorption spectrum,

measured on a Unicam SP-800 recording spectrophotometer, of each dihydrodiol was compared with published data[13,14] to confirm its identity.

Estimation of metabolites. In time-course experiments, portions (3 ml) of standard incubation mixtures were removed at various intervals of time and shaken immediately with ethyl acetate (6 ml). After centrifugation to separate the phases, ethyl acetate (5 ml) was removed and evaporated to dryness under reduced pressure. The residue was dissolved in ethanol (60 μl) and 40 μl of this solution applied to a thin-layer chromatogram with a"Microcap", Shandon Southern Instruments Ltd., Camberley, Surrey. After development of the chromatogram with solvent (a), the bands of Silica gel containing the various metabolites were removed from the plate and transferred to vials for the estimation of radioactivity by liquid scintillation counting with a Packard Tri-Carb spectrometer (model 3320 or 3375).

The amounts of water-soluble metabolites formed were determined by estimation of the radioactivity remaining in the aqueous phase after the extraction with ethyl acetate. Portions (0·1–0·5 ml) of the aqueous phase were transferred to vials and the radioactivity determined as before.

Qualitative examinations of the water-soluble derivatives of 7,12-dimethylbenz[a]-anthracene. These metabolites were prepared using incubation mixtures (200 ml) containing rat-liver homogenate (10 g liver) and 7,12-dimethylbenz[a]anthracene (2 mg) together with cofactors added in the proportions described for standard incubations. Incubations were for 1 hr. The mixture was extracted with 2 vol. of ethyl acetate and the water-soluble derivatives removed from the aqueous phase by absorption onto charcoal.[16] The aqueous phase was adjusted to pH 4 with acetic acid and the precipitated protein removed by centrifugation. Activated charcoal (2 g) was added to the supernatant. The mixture was filtered and the charcoal was washed with water (200 ml). The metabolites were eluted from the charcoal with methanol (500 ml) containing 5% (v/v) aqueous NH_3 (sp. gr. 0·88). The solvent was concentrated under reduced pressure to approximately 0·5 ml and the solution applied as a band to the base of a thin-layer chromatogram that was developed in solvent (c). To locate the metabolites, twenty horizontal bands were marked on the chromatogram and a small portion of Silica gel removed from each for radioactivity determinations. The band of Silica gel containing radioactivity was removed and the metabolites were eluted with methanol (20 ml) containing 5% (v/v) aqueous NH_3 followed by water (20 ml). The Silica gel was removed by filtration after each elution. The combined eluates were concentrated to approximately 0·5 ml under reduced pressure and the solution applied as a band to Whatman no. 1 chromatography paper. The chromatogram was developed with solvent (f). The metabolites were located by cutting a small vertical strip from the dried chromatogram and dividing it into twenty equal fractions for radioactivity determinations. The metabolites were eluted as before from the bands containing radioactivity.

Alkaline hydrolyses of water-soluble products. The water-soluble metabolites and synthetic *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione were each hydrolysed in 2 M-NaOH for 15 min at 100°. The solutions were cooled, acidified with HCl and extracted with 2 vol. of diethyl ether. The organic phases were dried over Na_2SO_4 and concentrated for examination by TLC. The aqueous phases were neutralized and used in the preparation[17] of Dns–amino acids, which were examined by TLC.[17,18]

RESULTS

Time-course studies. 7,12-Dimethylbenz[a]anthracene is converted by rat-liver homogenate and NADPH into 7-hydroxymethyl-12-methylbenz[a]anthracene and 12-hydroxymethyl-7-methylbenz[a]anthracene[9] and both the hydrocarbon and its hydroxymethyl derivatives are metabolized into 8,9-dihydrodiols.[13,14] The 10,11-dihydrodiol has also been identified as a metabolite of 7-hydroxymethyl-12-methylbenz[a]anthracene[14] that is formed by rat-liver homogenates.

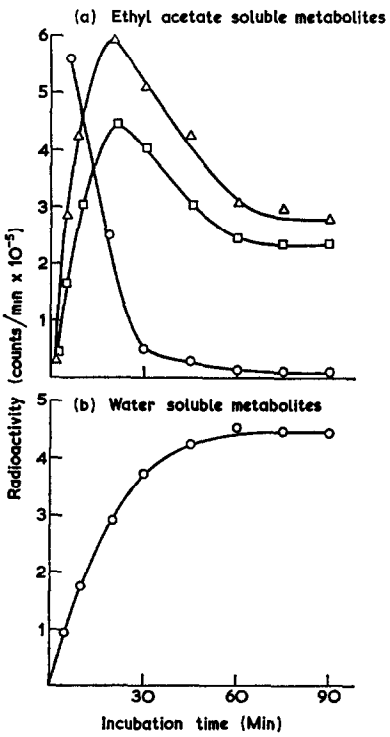


FIG. 1. Time course for the metabolism of 7-hydroxymethyl-12-methylbenz[a]anthracene by rat-liver homogenate. Reaction mixtures (40 ml) contained liver homogenate (1 g liver), $NADP^+$ (12 mg) and glucose 6-phosphate (60 mg) in 50 mM phosphate buffer pH 7·4. After heating to 37° the reaction was started by the addition of 7-hydroxymethyl-12-methylbenz[a]anthracene (400 μg) in ethanol (0·4 ml). Portions of the mixtures were withdrawn at various intervals of time, extracted with ethyl acetate and the radioactivity in the ethyl acetate and aqueous phases measured. (a) Ethyl acetate soluble compounds. ○——○, Unchanged 7-hydroxymethyl-12-methylbenz[a]anthracene; △——△ 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene; □——□, 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene. (b) Water-soluble metabolites.

Time-course studies showed that when either 7,12-dimethylbenz[a]anthracene or one of its hydroxymethyl derivatives was used as substrate, the amount of each dihydrodiol in the incubation mixtures increased to a maximum value and then decreased on further incubation. On the other hand, the amounts of water-soluble metabolites as represented by the radioactivity remaining in the aqueous phase after extraction with ethyl acetate increased to a maximum and remained at this level. Neither the formation of dihydrodiols nor the increases in the amounts of radio-

activity in the aqueous phase occurred if the rat-liver homogenates were boiled before incubation. The example in Fig. 1 shows the rates of conversion of 7-hydroxymethyl-12-methylbenz[a]anthracene into its 8,9- and 10,11-dihydrodiols and into water-soluble derivatives. These results suggest that the dihydrodiols may themselves be further metabolized into water-soluble derivatives by this system. To investigate this possibility, samples of the following dihydrodiols were prepared enzymically as described above; (i), 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene, (ii), 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]anthracene, (iii), 8, 9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene, (iv), 10,11-dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene. Each dihydrodiol was then incubated in a standard incubation mixture as described above. Portions

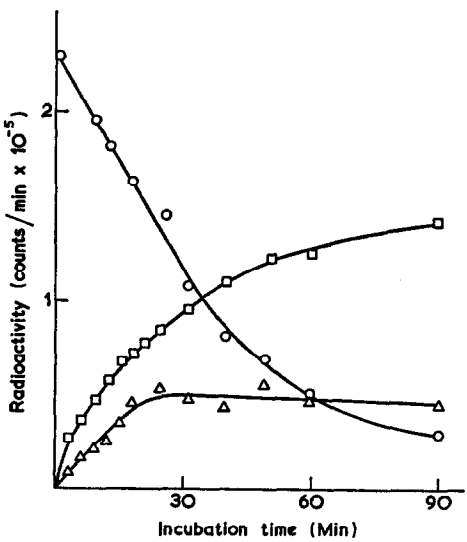


FIG. 2. Time-course of the conversion of 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene into water-soluble metabolites. Experimental conditions were as described for Fig. 1. ○——○, Unchanged 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene; △——△, unidentified ethyl acetate-soluble compound; □——□, water-soluble metabolites.

of the incubation mixtures were removed at various times and the metabolites were estimated as before. All the dihydrodiols behaved in a similar manner and a typical example, that of the incubation of 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene, is shown in Fig. 2. As the incubations proceeded, the amounts of radioactivity present in the aqueous phases due to the presence of water-soluble derivatives increased while the amounts of dihydrodiols remaining in the ethyl acetate phases decreased. With each substrate, the formation of small amounts of unidentified ethyl acetate-soluble metabolites was also observed. In the example shown in Fig. 2, the metabolite had R_f 0·12 on a thin-layer chromatogram developed with solvent (a).

Structures of the water-soluble derivatives. When examined on thin-layer or paper chromatograms, the water-soluble metabolites of 7,12-dimethylbenz[a]anthracene

formed purple complexes when the chromatograms were treated with ninhydrin and the metabolites could also be converted into Dns-derivatives. These results indicate that the metabolites contain amino acid or peptide side chains. On thin-layer chromatograms developed with solvent (c), the metabolites formed one rather diffuse area of radioactivity with R_f 0·25: the synthetic conjugate *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione, also had R_f 0·25 on thin-layer chromatograms developed with solvent (c). However, as shown in Fig. 3, paper chromatography in solvent (f) resolved the metabolites into two distant regions of radioactivity having R_f 0·58 and 0·28. In this system, the synthetic conjugate had R_f 0·58 and was thus chromatographically identical with the less polar water-soluble

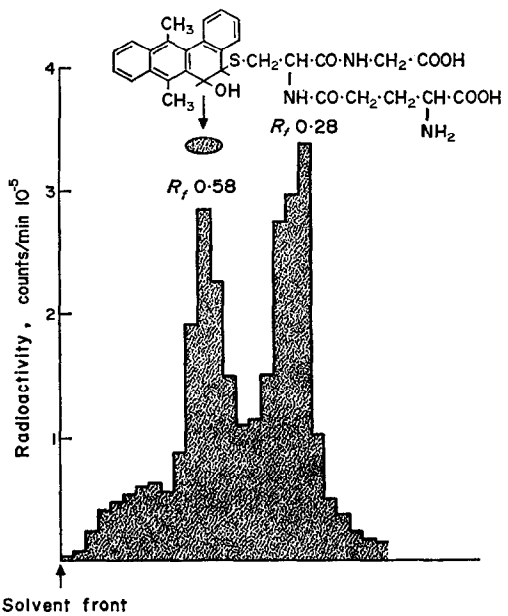


FIG. 3. Paper chromatography of water-soluble metabolites of 7,12-dimethylbenz[a]anthracene with butan-1-ol–acetic acid–water (12:3:5, v/v). Experimental conditions as for Table 2. The GSH conjugate applied as reference compound is *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenza]anthracen-5-yl)glutathione.

metabolite. The structure of this metabolite was confirmed by the identification of the products of alkaline hydrolysis. The ^{3}H-labelled water-soluble metabolite obtained by elution as described above of the appropriate regions cut from paper chromatograms, and the synthetic unlabelled conjugate were both hydrolysed with NaOH and the hydrolysates extracted with diethyl ether as described. Estimations of the radioactivity present in the organic and aqueous phases from the hydrolysate of the metabolite showed that 70–80 per cent of the radioactivity originally associated with the metabolite was now present in the organic phase. The products remaining in the two aqueous phases were identified as the three amino acid components of GSH. Table 1 shows that the Dns-derivatives of the hydrolysis products both from the water-soluble metabolites and from the synthetic conjugate contained products that are

chromatographically indistinguishable from the Dns-derivatives of glycine, glutamic acid and cysteine in three solvent systems. The possibility of contamination of the water-soluble derivatives with endogenous liver GSH or amino acids was excluded by liver blank experiments. Standard incubation mixtures from which 7,12-dimethylbenz[a]anthracene was omitted were treated by the complete process used to prepare the alkaline hydrolysis products of the water-soluble metabolites and Table 1 shows

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF DNS-AMINO ACIDS

Compounds converted into Dns-derivatives	R_f (× 100) Dns-derivatives in		
	Solvent (c)	Solvent (d)	Solvent (e)
Glycine	43	75	74
Glutamic acid	26	61	60
Cysteine	30	6	32
GSH	22	0	0
Synthetic conjugate	28		5
Water-soluble metabolite (R_f 0·58)*	28		5
Water-soluble metabolite (R_f 0·28)*	28		5
Alkaline hydrolysis products of synthetic conjugate	27, 30 42	6 60, 74	6, 32 60, 77
Alkaline hydrolysis products of water-soluble metabolite (R_f 0·58)*	28, 30 42	6 60, 75	5, 32 60, 76
Alkaline hydrolysis products of water-soluble metabolite (R_f 0·28)*	27, 30, 37, 43, 48	6, 20, 60, 75	5, 32, 50, 60, 76
Alkaline hydrolysis products of liver blank	37 48	20	50

Compounds were converted into Dns-derivatives either immediately or after alkaline hydrolysis as indicated. The synthetic conjugate, *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione, and the water-soluble metabolites from incubations of 7,12-dimethylbenz[a]anthracene with rat-liver homogenate and NADPH were hydrolysed by heating at 100° for 15 min in 2 M-NaOH. The experiment representing Dns-derivatives from a liver blank is explained in the text.
* R_f on paper chromatograms with butan-ol–acetic acid–water (12:3:5, v/v).

that Dns-derivatives with the chromatographic properties of GSH amino acids are not present in these preparations. Furthermore, the initial purification of the water-soluble metabolites involves TLC with solvent (c). Since GSH, cysteine, glutamic acid and glycine all have R_f-values below 0·12 in this system they are separated from the metabolite (R_f 0·25) at this stage.

The diethyl ether-soluble decomposition products of the less polar water-soluble metabolite were examined by TLC with solvent (a) and areas of radioactivity were located at R_f-values 0·32, 0·61, 0·71 and 0·95. Chromatograms, examined in u.v.-light both before and after exposure to NH_3, showed that the metabolite and the synthetic conjugate each yielded diethyl ether-soluble products that were identical in chromatographic properties. The slowest moving product in each extract had the following properties in common with *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene: (i) ,R_f 0·32 when examined by TLC using solvent (a), (ii), violet fluorescence in u.v.-light which was unchanged by exposure to NH_3 and (iii), u.v.-absorption maxima at 260, 268 and 303 nm.[9,11] The fastest-moving hydrolysis product

of each conjugate was examined by TLC in solvent (b) using 7,12-dimethylbenz[a]-anthracene as a reference compound. Both hydrolysis products had R_f 0·54 and showed a violet fluorescence in u.v.-light and were thus indistinguishable from 7,12-dimethylbenz[a]anthracene. Furthermore, when the hydrolysis products of the water-soluble metabolites were examined by TLC, radioactivity was present in the band at R_f 0·54. Both diethyl ether extracts also contained compounds, with R_f values of 0·61 and 0·71, each of which showed an orange fluorescence under u.v.-light after exposure to NH_3, but these products were not identified. There was no evidence for the presence in these incubation mixtures of a GSH conjugate formed on the 8,9-bond of 7,12-dimethylbenz[a]anthracene.

The material in the slower-moving region of the paper chromatograms was similarly hydrolysed with NaOH and the hydrolysate extracted with diethyl ether. The aqueous phase was examined as before and, as shown in Table 1, products with the properties

TABLE 2. PAPER CHROMATOGRAPHY OF WATER-SOLUBLE METABOLITES OF BENZ[a]ANTHRACENE DERIVATIVES

Compound	R_f
Water-soluble metabolites from:	
7,12-Dimethylbenz[a]anthracene	0·58 0·28
8,9-Dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene	0·27
8,9-Dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]anthracene	0·27
8,9-Dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene	0·21
10,11-Dihydro-10,11-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene	0·22
Benz[a]anthracene	0·58
S-(5,6-Dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione	0·58
S-(5,6-Dihydro-6-hydroxy-7-hydroxymethyl-12-methylbenz[a]anthracen-5-yl)glutathione	0·47
S-(5,6-Dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione	0·58

Incubation mixtures (20 ml) contained liver homogenate (1 g liver), $NADP^+$ (6 mg) and glucose 6-phosphate (30 mg) in 50 mM-sodium phosphate buffer pH 7·4. Reactions were started by the addition of the substrate (300 μg) in ethanol (0·3 ml). The water-soluble metabolites remaining in the aqueous phase after extraction of the mixtures with two volumes of ethyl acetate were prepared for chromatography and located on the chromatogram as described in the text. Solvent system, butan-1-ol–acetic acid–water (12:3:5, v/v).

of glycine, glutamic acid and cysteine were present. The diethyl ether-soluble hydrolysis products have not yet been identified and hence the metabolites have not been fully characterized. However, Table 2 shows that the conjugates derived from each of the dihydrodiols that are formed during the metabolism of 7,12-dimethylbenz[a]anthracene were located in the same region of the chromatogram as that of the slower-moving metabolite of the hydrocarbon. This suggests that the radioactivity associated with this region probably represents a mixture of several conjugates.

The chromatographic properties of the GSH conjugates of 7-hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide and benz[a]anthracene 5,6-oxide and of the conjugate obtained from the incubations of benz[a]anthracene with rat-liver homogenate are included in Table 2 for comparison with the analogous derivatives of 7,12-dimethylbenz[a]anthracene.

DISCUSSION

Evidence for the participation of GSH in the formation of water-soluble derivatives of [$^{14}C_{12}$]7,12-dimethylbenz[a]anthracene has been described in experiments using the microsomal fractions of livers from rats pretreated with 3-methylcholanthrene.[5] The yield of these conjugates was increased by the addition of GSH and the soluble cell fraction and gel filtration of the products on columns of Sephadex G-25 showed that the water-soluble derivatives of the hydrocarbon were eluted in the same fraction as the GSH conjugate of 2-hydroxyoestradiol.

It has now been shown that rat-liver homogenates convert ^{3}H-labelled 7,12-dimethylbenz[a]anthracene into a mixture of GSH conjugates, which can be resolved into two distinct radioactive regions by paper chromatography. The less polar region contains only one compound and this has been identified as *S*-(5,6-dihydro-6-hydroxy-7,12-dimethylbenz[a]anthracen-5-yl)glutathione. In the metabolism of polycyclic hydrocarbons to this type of conjugate, the initial step is believed to involve the formation of an epoxide by the NADPH dependent mixed function oxidase of liver microsomes, and the formation of epoxides from several polycyclic hydrocarbons,[19-22] including 7,12-dimethylbenz[a]anthracene,[23] by this system has now been established. The conjugation of the epoxides with GSH is probably catalysed by glutathione *S*-epoxide-transferase[24] that is present in the soluble fraction of rat liver. Synthetic 7,12-dimethylbenz[a]anthracene 5,6-oxide reacts both chemically and enzymically with GSH[16] to form conjugates that are identical with the less polar water-soluble metabolite of 7,12-dimethylbenz[a]anthracene. Epoxides are also further metabolized to dihydrodiols by rat-liver homogenates by a reaction that is catalysed by the microsomal enzyme "epoxide hydrase".[25-28] Presumably all dihydrodiols are formed through the corresponding epoxy derivatives, although so far only "K-region" epoxides have been detected as intermediate in the metabolism of polycyclic hydrocarbons, probably because of the greater stability of "K-region" epoxides under the experimental conditions used in their detection.[22] Enzymic oxidation of the 7,12-dimethylbenz[a]anthracene nucleus takes place mainly at the 5,6 and 8,9 bonds. Hence it is of interest that rat-liver homogenates metabolize the isomeric epoxide intermediate by different routes, the 5,6-oxide being conjugated with GSH and the 8,9-oxide hydrated to the dihydrodiol. This may be because there are differences between the affinities of the enzymes glutathione *S*-epoxide transferase and epoxide hydrase for "K-region" and non-"K-region" expoxides.

The more polar metabolites have not been fully characterized but the formation of glycine, glutamic acid and cysteine by alkaline hydrolysis indicates that they also contain GSH. Ring hydroxylation and side-chain oxidation of 7,12-dimethylbenz[a]-anthracene by rat-liver homogenate results in the synthesis of four dihydrodiols as major metabolites[9,13,14] of the hydrocarbon. When each of these derivatives, which are listed in Table 2, were studied separately, they were all further metabolized to derivatives with the chromatographic properties of the more polar type of conjugate. Hence the radioactivity in this area of chromatograms may represent at least four compounds and several possible structures for the more polar conjugates must be considered. Although the 7,12-dimethylbenz[a]anthracene metabolites so far identified have involved reactions on one bond only,[11,13] the enzymic oxidation of both the 5,6- and the 8,9-bonds in the same molecule is possible. If these reactions occurred then the hydrocarbon nucleus may have undergone dihydrodiol formation on one

bond and conjugation with GSH on another or GSH conjugation on both the reactive positions. In either case these derivatives would be more polar than the GSH conjugate of 7,12-dimethylbenz[a]anthracene 5,6-oxide. This possibility is supported by the fact that if the washed microsomal fraction of rat-liver is used as the enzyme source, NADPH is essential for the conversion of the dihydrodiols to water-soluble derivatives,* suggesting that the activation of another bond in the same molecule is necessary in the formation of the more polar water-soluble metabolites.

Another factor to be taken into account in formulating the structure of the more polar metabolites is the possible role of methyl and hydroxymethyl groups in the formation of these derivatives. 7-Hydroxymethyl-12-methylbenz[a]anthracene 5,6-oxide is converted into a GSH conjugate by rat-liver homogenate,[16] but there is no evidence that this conjugate is formed in the experiments with 7,12-dimethylbenz[a]-anthracene described here. Preliminary experiments* have shown, however, that the hydroxymethyl derivative is converted mainly into the more polar type of conjugate by rat-liver homogenate. Some evidence that the methyl groups are involved, however, is provided by a comparison on paper chromatograms of the water-soluble derivatives of 7,12-dimethylbenz[a]anthracene with those of benz[a]anthracene. Although both hydrocarbons form derivatives with the chromatographic properties of the GSH conjugates of the 5,6-oxides, preliminary experiments indicate that the more polar type of conjugate is not synthesised from benz[a]anthracene by rat-liver homogenates, in spite of the fact that this hydrocarbon is also metabolized at both the 5,6- and 8,9-bonds.[13,15] Possibly the methyl and hydroxymethyl groups are oxidized to carboxylic acids[11,13] which are then conjugated with GSH at one of the reactive bonds. Reactions of this type cannot, however, explain the conversion of dihydrodiols into GSH conjugates. The structure of these derivatives is being further investigated.

Although all the GSH conjugates of 7,12-dimethylbenz[a]anthracene have not been fully characterized, these results show that liver from rats pretreated with 3-methylcholanthrene possess a remarkable ability to convert the hydrocarbon and its metabolites into water-soluble derivatives and emphasize the importance of GSH in detoxification mechanisms.

Acknowledgement—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 Campaign for Research.

REFERENCES

1. P. H. JELLINCK and B. GOUDY, *Biochem. Pharmac.* **16,** 131 (1967).
2. P. H. JELLINCK, S. COLES and M. GARLAND, *Biochem. Pharmac.* **16,** 2449 (1967).
3. W. LEVIN and A. H. CONNEY, *Cancer Res.* **27,** 1931 (1967).
4. P. H. JELLINCK and G. SMITH, *Biochem. Pharmac.* **18,** 680 (1969).
5. P. H. JELLINCK, G. SMITH and R. FLETCHER, *Cancer Res.* **30,** 1715 (1970).
6. P. SIMS, *Biochem. Pharmac.* **19,** 285 (1970).
7. L. DIAMOND, *Int. J. Cancer* **8,** 451 (1971).
8. E. HUBERMAN, J. K. SELKIRK and C. HEIDELBERGER, *Cancer Res.* **31,** 2161 (1971).
9. E. BOYLAND and P. SIMS, *Biochem. J.* **95,** 780 (1965).
10. P. H. JELLINCK and B. GOUDY, *Science* **152,** 1375 (1966).
11. E. BOYLAND and P. SIMS, *Biochem. J.* **104,** 394 (1967).
12. P. SIMS and P. L. GROVER, *Biochem. Pharmac.* **17,** 1751 (1968).
13. P. SIMS, *Biochem. Pharmac.* **19,** 795 (1970).
14. P. SIMS, *Biochem. Pharmac.* **19,** 2261 (1970).

* J. Booth, unpublished observation.

15. E. Boyland and P. Sims, *Biochem. J.* **97,** 7 (1965).
16. P. Sims, *Biochem. J.*, **131,** 405 (1973).
17. D. Morse and B. L. Horecker, *Analyt. Biochem.* **14,** 429 (1966).
18. Z. Deyl and J. Rosmus, *J. Chromat.* **20,** 514 (1965).
19. D. M. Jerina, J. W. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend, *Biochemistry* **9,** 147 (1970).
20. J. K. Selkirk, E. Huberman and C. Heidelberger, *Biochem. biophys. Res. Commun.* **43,** 1010 (1971).
21. P. L. Grover, A. Hewer and P. Sims, *FEBS Letts* **18,** 76 (1971).
22. P. L. Grover, A. Hewer and P. Sims, *Biochem. Pharmac.* **21,** 2713 (1972).
23. G. R. Keysell, J. Booth, P. Sims, P. L. Grover and A. Hewer, *Biochem. J.* **129,** 41P (1972).
24. E. Boyland and K. Williams, *Biochem. J.* **94,** 190 (1965).
25. D. Jerina, J. Daly, P. Zaltzman-Nirenberg, B. Witkop and S. Udenfriend, *Archs Biochem. Biophys.* **128,** 176 (1968).
26. H. Pandov and P. Sims, *Biochem. Pharmac.* **19,** 299 (1970).
27. G. T. Brooks, A. Harrison and S. E. Lewis, *Biochem. Pharmac.* **19,** 255 (1970).
28. F. Oesch and J. Daly, *Biochim. biophys. Acta* **227,** 692 (1971).