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METABOLISM OF BENZ[a]ANTHRACENE EPOXIDES BY RAT-LIVER

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Abstract—Benz[a]anthracene is metabolized to 5,6-dihydro-5,6-dihydroxybenz[a]anthracene and 8,9-dihydro-8,9-dihydroxybenz[a]anthracene by the microsomal fraction of rat-liver and NADPH and these metabolites are formed at similar rates. When the soluble liver fraction and GSH are also present, the rate of formation of the 8,9-dihydrodiol is not affected while that of the 5,6-isomer is considerably reduced. Since epoxides are the intermediates formed in the metabolism of aromatic hydrocarbons, the further metabolism of benz[a]anthracene 5,6- and 8,9-oxide into dihydrodiols and into GSH conjugates has been investigated. Each epoxide is converted into a dihydrodiol by microsomal "epoxide hydrase" at a similar rate, whereas conjugation with GSH, a reaction catalysed by "glutathione *S*-epoxide transferase" in the soluble liver fraction, proceeds considerably more rapidly with the 5,6- than with the 8,9-isomer. When a mixture of the two epoxides of benz[a]anthracene is incubated both with "epoxide hydrase" and with "GSH transferase", the 5,6-isomer is converted largely into the GSH conjugate while the main route of metabolism of the 8,9-isomer is by hydration to the dihydrodiol.

THE *in vitro* metabolism of aromatic hydrocarbons into dihydrodiols and GSH conjugates takes place via epoxide intermediates,[1,2] and the identification of dihydrodiols as metabolites of benz[a]anthracene and related compounds[3,4] has demonstrated that the oxidation of various bonds of the hydrocarbon molecule is catalysed by the microsomal mixed-function oxidases in liver. Direct evidence that the dihydrodiols of polycyclic hydrocarbons are formed through epoxide intermediates has, so far, only been obtained for "K-region" isomers,[5–7] the "*K*-region" double bonds being regions of high electron density similar to the 9,10-bond of phenanthrene. Since synthetic benz[a]anthracene 5,6-oxide[8] ("*K*-region") and benz[a]anthracene 8,9-oxide[9] (non-"*K*-region") are both converted by microsomal "epoxide hydrase" into the corresponding dihydrodiols that are identical with those formed from the parent hydrocarbon by the microsomal fraction of rat liver in the presence of NADPH, it is likely that both dihydrodiols are formed by a similar mechanism. Other non-"*K*-region" oxides that exhibit similar properties are dibenz[a,c]anthracene 10,11-oxide,[10] 9,10-dihydrobenzo[a]pyrene 7,8-oxide and 7,8-dihydrobenzo[a]pyrene 9,10-oxide.[11] Further evidence that non-"*K*-region" dihydrodiols are derived from epoxide intermediates is supplied by the finding that the enzymic synthesis of the 8,9- and 10,11-dihydrodiols from 7-hydroxymethyl-12-methylbenz[a]anthracene is inhibited by cyclohexene oxide,[12] an established inhibitor of "epoxide hydrase".[13]

Another route of metabolism of benz[a]anthracene 5,6- and 8,9-oxide is catalysed by the "glutathione transferase" present in the soluble fraction of rat-liver.[14] In this

reaction, the 5,6- and 8,9-oxides are conjugated with GSH to form *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione[8] and *S*-(8,9-dihydro-9-hydroxybenz-[a]anthracen-8-yl)glutathione[9] respectively.

In previous studies on the metabolism of the carcinogenic hydrocarbon 7,12-dimethylbenz[a]anthracene by rat-liver homogenates, the identification of the 8,9-dihydrodiol[4] and the 5,6-GSH conjugate[15] as major metabolites led to the suggestion that the oxides of 7,12-dimethylbenz[a]anthracene were metabolized largely by different routes, the 5,6-isomer ("*K*-region") being conjugated with GSH and the 8,9-oxide (non-"*K*-region") being hydrated to the dihydrodiol.[15]

The synthesis of benz[a]anthracene 5,6-[8] and 8,9-oxides[9] has now made the comparison of the further metabolism of a "*K*-region" and a non-"*K*-region" epoxide possible.

EXPERIMENTAL

Materials. Benz[a]anthracene (sp. act. 112 mCi/m-mole), generally labelled with tritium, was prepared by diluting a more highly labelled sample, obtained from the Radiochemical Centre, Amersham, Bucks, with unlabelled carrier hydrocarbon. ^{3}H-Labelled benz[a]anthracene 5,6-oxide[8] (sp. act. 93 mCi/m-mole), unlabelled benz-[a]anthracene 5,6-oxide,[8] 5,6-dihydro-5,6-dihydroxybenz[a]anthracene,[16] *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione,[8] benz[a]anthracene 8,9-oxide[9] and *S*-(8,9-dihydro-9-hydroxybenz[a]anthracen-8-yl)glutathione[9] were prepared by published methods. Samples of 8,9-dihydro-8,9-dihydroxybenz[a]anthracene were prepared enzymically by isolating the products from incubations using benz[a]anthracene and rat-liver preparations as previously described.[3]

Thin-layer chromatography (TLC). This was carried out on glass plates coated with a layer (0·25 mm thick) of Silica gel G (E. Merck A.-G, Darmstadt, Germany). The chromatograms were developed for 15 cm with benzene–ethanol (9:1 v/v) and the metabolites located by examination of the chromatograms in u.v. light.

Ultraviolet absorption spectra. These were measured on a Unicam SP 800 recording spectrophotometer.

Radioactivity. Radioactivity was measured by liquid scintillation counting using a Packard Tri-Carb spectrometer (model 3320 or 3375).

Preparation of subcellular fractions of rat-liver. Homogenates were prepared from the livers of pretreated Wistar strain male rats (body wt 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. Livers of five rats were homogenized in 4 vol. isotonic KCl (1·15% w/v) for the preparation of microsomal, soluble and dialysed soluble fractions as previously described.[12]

Incubation of ^{3}H-labelled benz[a]anthracene with subcellular fractions of rat-liver. Incubation mixtures (10 ml) in 50 mM-phosphate buffer (pH 7·4) contained microsomal fraction (≡ 1 g, liver), $NADP^+$ (3 mg), glucose 6-phosphate (15 mg) glucose 6-phosphate dehydrogenase (2 units) and ^{3}H-labelled benz[a]anthracene (2 μmoles added in 0·4 ml ethanol). In other experiments, similar mixtures also contained soluble liver fraction (0·5 ml) and GSH (1·5 mg) as indicated. The mixtures were heated to 37° and the reaction started by the addition of substrate. Reactions were stopped by shaking the mixtures with ethyl acetate (15 ml) and centrifuging to separate the phases. Zero time values were obtained by shaking incubation mixtures with

TABLE 1. METABOLITES FORMED DURING INCUBATIONS OF MIXTURES OF BENZ[a]ANTHRACENE 5,6- AND 8,9-OXIDE WITH SUBCELLULAR FRACTIONS OF RAT-LIVER

Expt. no.	Additions to reaction mixtures	Amounts (nmoles) of metabolites formed from 5,6-Oxide: Dihydrodiol	5,6-Oxide: GSH-Conjugate	8,9-Oxide: Dihydrodiol	8,9-Oxide: GSH-Conjugate
1.	Microsomal fraction (heated*)	0	—	0	—
2.	Microsomal fraction	207	—	228	—
3.	Soluble fraction (heated*) + GSH (1·5 mg)	—	4	—	0
4.	Soluble fraction + GSH (1·5 mg)	—	368	—	76
5.	Soluble fraction	174	174	—	38
6.	Soluble fraction + GSH (2 mg)	—	366	—	75
7.	Soluble fraction (dialysed)	—	32	—	10
8.	Soluble fraction (dialysed) + GSH (1·5 mg)	—	360	—	74

* Heated at 100° for 5 min.

All reaction mixtures (10 ml) were in 50 mM-phosphate buffer pH 7·4 and contained benz[a]anthracene 5,6- (1 μmole) and 8,9-oxide (1 μmole). Microsomal fraction (≡ 1 g liver), soluble fraction (0·5 ml) and GSH were added as indicated. Incubations were at 37° for 3 min. Reactions were stopped by shaking the mixtures with ethyl acetate (15 ml) and the dihydrodiols in the ethyl acetate phases or the GSH conjugates in the aqueous phases estimated as described in the text. Dashes indicate that the amount of products were not estimated.

ethyl acetate (15 ml) before the addition of substrate. After the addition of substrate, shaking was repeated and the phases separated by centrifugation. The amounts of 5,6- and 8,9-dihydrodiols in the ethyl acetate were estimated as described.

Incubation of benz[a]*anthracene 5,6- and 8,9-oxides with subcellular fractions of rat-liver.* Incubations were in 50 mM-phosphate buffer (pH 7·4) and complete mixtures (10 ml) contained microsomal fraction (≡ 1 g liver), soluble fraction (0·5 ml) and GSH (1·5 mg). After warming the mixtures to 37°, the reactions were started by the addition of ethanolic solutions (0·4 ml) containing benz[a]anthracene 5,6-oxide (1 μmole) and 8,9-oxide (1 μmole). In some experiments, one or more of these constituents were omitted as indicated in the results of the individual experiments presented in Table 1. Reactions were stopped by shaking the mixtures with ethyl acetate (15 ml) and the phases separated by centrifugation. The amounts of dihydrodiols in the ethyl acetate phases and of GSH conjugates in the aqueous phases were estimated as described.

Estimation of metabolites. (a) *Estimation of* ^{3}*H-labelled benz*[a]*anthracene 5,6- and 8,9-dihydrodiols formed in incubations of* ^{3}*H-labelled benz*[a]*anthracene with subcellular liver fractions.* The incubation mixtures were extracted with ethyl acetate, and a portion of the extract (6 ml) was filtered through Whatman no. 1 filter paper and evaporated to dryness under reduced pressure. The residue was dissolved in ethanol (60 μl) and 10 μl of the solution applied to the base of a thin-layer chromatogram with a "Microcap", Shandon Southern Instruments Ltd., Camberley, Surrey. Samples of unlabelled 5,6- and 8,9-dihydrodiols were also applied to each chromatogram as reference compounds and, after development of the chromatogram, the dihydrodiols were located in u.v. light. The areas of silica gel containing the dihydrodiols were removed from the plate and the silica gel transferred to vials for the estimation of radioactivity. In each experiment, zero time values were subtracted from those obtained after incubation.

(b) *Estimation of unlabelled dihydrodiols formed from benz*[a]*anthracene 5,6- and 8,9-oxides by incubation with subcellular liver fractions.* The incubation mixtures

were extracted with ethyl acetate, and the extracts prepared and examined by TLC as described for the tritiated samples, except that 12 ml ethyl acetate phase was used and 40 μl portions of ethanolic solutions of metabolites were applied to the thin-layer chromatograms. The chromatograms were developed and the areas of silica gel containing the dihydrodiols were each transferred to tubes containing ethanol (3 ml). The tubes were shaken and allowed to stand for at least 30 min. The shaking was repeated, the silica gel removed by centrifugation and the absorption spectra of the dihydrodiols in the supernatant phases recorded. The concentration of the 5,6-dihydrodiol was calculated from the absorbance at 309 nm[16] and that of the 8,9-dihydrodiol from the absorbance at 318 nm.[9] In each experiment zero time values were subtracted from those obtained after incubation.

(c) *Estimation of GSH-conjugates formed from benz*[a]*anthracene 5,6- and 8,9-oxides on incubation with subcellular liver fractions.* After extraction with ethyl acetate, a portion of each of the aqueous phases (8 ml) was treated with 40% (w/v) trichloroacetic acid (0·5 ml), the precipitated protein removed by centrifugation and the u.v. absorption spectra of mixtures of the isomeric GSH conjugates in the supernatant phases measured between 308 and 335 nm. The amounts of GSH-conjugates present could be measured by the absorbance-ratio method for two-component spectrophotometric analysis,[17] since the two isomers exhibit characteristic but significantly different absorption spectra.[8,9] Figure 1 shows the u.v. absorption spectra between 308 and 335 nm of solutions of synthetic benz[a]anthracene 5,6- and 8,9-GSH conjugates mixed in various proportions. The ratio of the absorptivities at 315 nm (isoabsorptive point) and 321 nm (where the 8,9-isomer exhibits maximum absorption and the 5,6-isomer little absorption) was used to calculate the ratio of each isomer present, by the method described for the determination of the distribution of isomers in a mixture.[18] Using the absorbance-ratio method, the total amount of GSH conjugates present in a mixture was obtained from the absorbance of solutions of known concentrations at the isoabsorptive point.[17,18] Since the GSH conjugates of benz[a]anthracene oxides have not, so far, been obtained in crystalline form, the

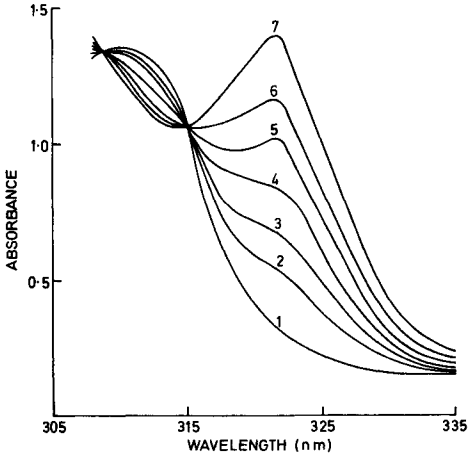


FIG. 1. Ultraviolet absorption spectra of mixtures of isomeric benz[a]anthracene 5,6- and 8,9-GSH conjugates. Spectra 1, 2, 3, 4, 5, 6 and 7 contain 100, 80, 66, 50, 33, 20 and 0% of the 5,6-isomer respectively and show an isoabsorptive point at 315 nm.

relationship between concentration and absorbance at 315 nm was determined by using the conjugate synthesized from ^{3}H-labelled benz[a]anthracene 5,6-oxide.[8] This oxide was incubated in standard incubation mixtures and the aqueous phase was extracted with ethyl acetate and prepared for spectroscopy as described. Portions of the aqueous phase (0·5 ml) were also used to determine the radioactivity of the water soluble conjugate present in this phase. A standard curve relating nmoles of GSH-conjugate to absorbance at 315 nm was constructed and used to determine the total amounts of the conjugates in the mixtures.

RESULTS

Metabolism of ^{3}H-labelled benz[a]anthracene. Previous experiments have shown that when benz[a]anthracene is incubated with the microsomal fraction of rat-liver in the presence of an NADPH generating system, both the 5,6- and the 8,9-dihydrodiols are synthesized.[3] Time course studies on the rates of formation of 5,6-dihydrodiol from ^{3}H-labelled benz[a]anthracene in two systems is shown in Fig. 2a. In one system, in which incubation mixtures contained ^{3}H-labelled benz[a]anthracene, microsomal fraction and NADPH, the hydrocarbon is presumably converted by the microsomal mixed-function oxidase into benz[a]anthracene 5,6-oxide, which is then hydrated to the 5,6-dihydrodiol by the microsomal "epoxide hydrase". The other system contained similar incubation mixtures together with soluble liver fraction and GSH. Since the soluble liver fraction contains "glutathione *S*-epoxide transferase", both this enzyme and the microsomal "epoxide hydrase" are competing for the epoxide intermediates. Hence the finding that the rate of formation of the 5,6-dihydrodiol is considerably reduced by the addition of "glutathione *S*-epoxide transferase" and GSH (Fig. 2a) indicates that some of the epoxide intermediate is further metabolized by conjugation with GSH. On the other hand, the rate of formation of 8,9-dihydro-

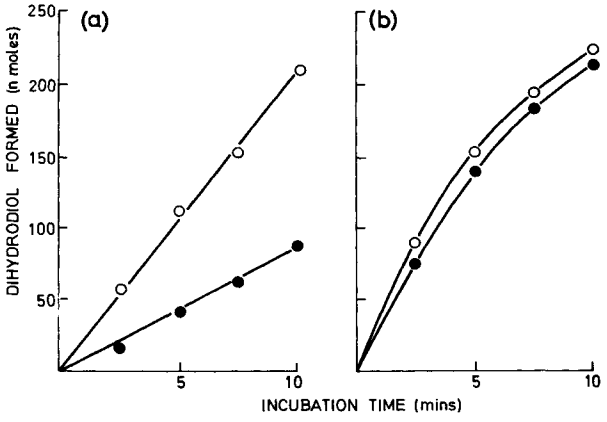


FIG. 2. (a) Conversion of ^{3}H-labelled benz[a]anthracene into 5,6-dihydrodiol by microsomal fraction (○), or microsomal and soluble fractions and GSH (●). (b) Conversion of ^{3}H-labelled benz[a]anthracene into 8,9-dihydrodiol by microsomal fraction (○), or microsomal and soluble fractions and GSH (●). Incubations were carried out in 50 mM-phosphate buffer pH 7·4 and contained $NADP^+$ (3 mg), glucose 6-phosphate (15 mg), glucose 6-phosphate dehydrogenase (2 units), microsomal fraction (≡ 1 g liver) and ^{3}H-labelled benz[a]anthracene (2 μmoles) in a total volume of 10 ml. Values represented by solid circles (●) also contained soluble liver fraction (0·5 ml) and GSH (1·5 mg). At the end of the incubation period reactions were stopped by shaking with ethyl acetate (15 ml) and the amounts of dihydrodiol in the ethyl acetate phases estimated as described in the text.

diol is not affected by the addition of "glutathione *S*-epoxide transferase" and GSH to incubation mixtures (Fig. 2b), indicating that the 8,9-oxide is a poor substrate for the "transferase".

This is in agreement with the finding that benz[a]anthracene is converted by rat-liver homogenates into a water soluble metabolite that is identical in its u.v. spectrum and properties on paper chromatograms with *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)glutathione.[8] Thus it appears that whereas the major route of metabolism of benz[a]anthracene 8,9-oxide is one of conversion into the dihydrodiol, that of the 5,6-oxide is by conjugation with GSH. This possibility was investigated in more detail by comparing the metabolism of synthetic benz[a]anthracene 5,6- and 8,9-oxide.

Metabolism of benz[a]*anthracene 5,6- and 8,9-oxide by subcellular fractions of rat-liver.* (a) *Incubations of equimolar mixtures of the oxides with either the microsomal fraction or the soluble fraction and GSH.* The isomeric oxides react chemically and enzymically with water or GSH to yield dihydrodiols or GSH conjugates respectively.[8,9] Table 1 shows that, with the concentrations of reactants and incubation times used in the present work, the amounts of chemical reaction taking place were negligible. Table 1 also summarizes the results of experiments in which equimolar mixtures of benz[a]anthracene 5,6- and 8,9-oxides were incubated either with the microsomal fraction of rat-liver, containing "epoxide hydrase", or with the soluble fraction, containing "glutathione *S*-epoxide transferase" and GSH. Thus experiments 1 and 2 demonstrate that the microsomal "epoxide hydrase" is able to convert about equal amounts of each oxide into the corresponding dihydrodiol and that this activity is destroyed by heating the microsomal fraction. The enzymic nature of the GSH conjugation measured in these studies is shown by experiments 3 and 4, which demonstrate that "GSH transferase" activity is destroyed by heating the soluble liver

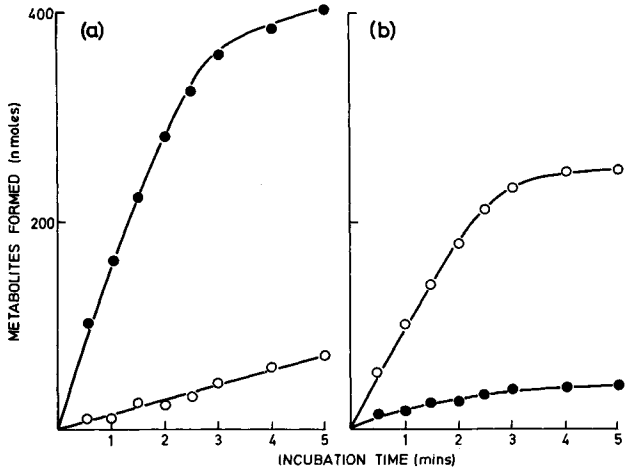
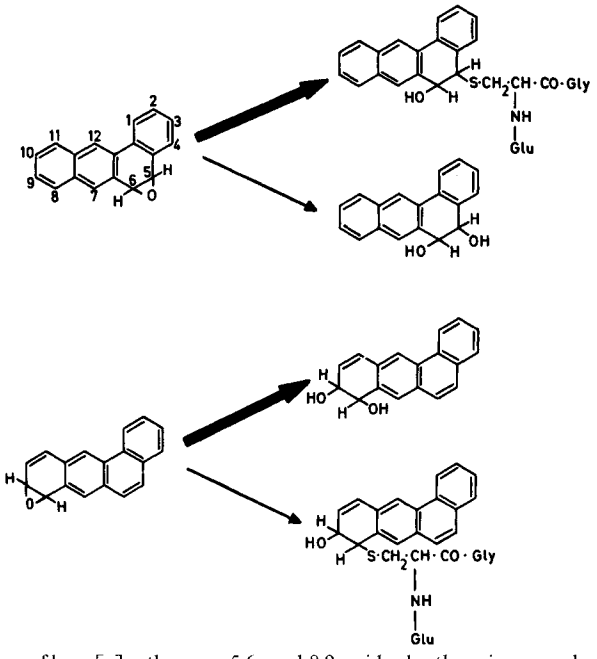


FIG. 3. (a) Conversion of benz[a]anthracene 5,6-oxide into dihydrodiol (○), or into GSH-conjugate (●). (b) Conversion of benz[a]anthracene 8,9-oxide into dihydrodiol (○) or into GSH conjugate (●). Incubations were in 50 mM-phosphate buffer pH 7·4 and contained microsomal fraction (≡ 1 g liver), soluble fraction (0·5 ml) GSH (1·5 mg), benz[a]anthracene 5,6- (1 μmole) and 8,9-oxide (1 μmole) in a total volume of 10 ml. Reactions were stopped by shaking with ethyl acetate (15 ml) and dihydrodiols in the ethyl acetate phases and GSH conjugates in the aqueous phases estimated as described in the text.

fraction. Although unfortified soluble fraction supports some conjugation (expt. 5), addition of GSH (1·5 mg) is required for maximum activity (expt. 4, 5 and 6). Dialysis of the soluble fraction removes most of the conjugating activity but this can be restored by the addition of GSH (expts. 7 and 8).

It is also of interest that whereas similar amounts of the two oxides are hydrated to dihydrodiols by the microsomal fraction (expt. 2), considerably more of the 5,6- than the 8,9-isomer is conjugated with GSH in the presence of the soluble fraction (expt. 4).



SCHEME 1. Metabolism of benz[a]anthracene 5,6- and 8,9-oxides by the microsomal and soluble fractions of rat-liver. Thick arrows represent major and thin arrows minor routes.

(b) *Incubations of equimolar mixtures of the oxides with both microsomal and soluble fractions in the presence of GSH.* When liver preparations containing both "epoxide hydrase" and "glutathione *S*-epoxide transferase" are incubated with the isomeric benz[a]anthracene oxides, each oxide is converted into a dihydrodiol and a GSH conjugate. Time course studies on the rates of formation of these four metabolites show that, under the conditions used, reaction rates are maintained only for 3 min (Fig. 3). This may be due to loss of substrates by other routes, one possibility being non-enzymic rearrangement to phenolic derivatives.[1,8,9,19] Figure 3 also shows that benz[a]anthracene 5,6-oxide is metabolized mainly by conjugation with GSH, while the major route of metabolism of the 8,9-oxide is by hydration to the dihydrodiol as shown in Scheme 1.

DISCUSSION

Previous work on the metabolism of benz[a]anthracene[3] has shown that oxidation occurs at both the 5,6- ("*K*-region") and 8,9-bond (non-"*K*-region") and it has

now been demonstrated that the 5,6- and 8,9-oxide that are probably the primary products formed, follow different major routes of metabolism. When the two isomeric oxides are presented to liver preparations that contain both the enzymes concerned in their further metabolism, "epoxide hydrase" and "glutathione *S*-epoxide transferase", the 5,6-oxide is the preferred substrate for GSH conjugation while the 8,9-isomer is more susceptible to hydration to the dihydrodiol. This is presumably because the isomeric epoxides have different affinities for "epoxide hydrase" and "glutathione *S*-epoxide transferase", but the possibility that different enzymes are involved in the metabolism of "*K*-region" and non-"*K*-region" epoxides might also be considered. The existence of several "epoxide hydrases" has already been reported[20,21] and experiments using "epoxide hydrase" inhibitors may help to solve this problem. Using liver homogenates, indirect evidence suggested that the 5,6-oxide (the "*K*-region" epoxide) of another aromatic hydrocarbon, 7,12-dimethylbenz-[a]anthracene, was mainly conjugated with GSH and the 8,9-isomer (a non-"*K*-region" epoxide) mainly hydrated to the dihydrodiol.[15] This is also in agreement with the finding that the GSH conjugates identified as metabolites of other aromatic hydrocarbons have been derived from "*K*-region" double bonds.[8,15,16] Furthermore, in similar studies with dibenz[a,c]anthracene, a molecule that does not contain a "*K*-region" double bond, GSH conjugates were not detected.[10]

After aromatic hydrocarbons are activated by microsomal mixed function oxidase, the active species become bound to proteins.[22,23] Aryl cysteines, including *S*-(5,6-dihydro-6-hydroxybenz[a]anthracen-5-yl)cysteine, and *S*-(5,6-dihydro-6-hydroxydibenz[a,h]anthracen-5-yl)cysteine, can be activated and transferred to tRNA by synthetase enzymes, which suggests a possible pathway by which aromatic hydrocarbons could become bound to ribosomal proteins.[24,25] The aryl cysteine derivatives could arise in tissues from the conjugates of GSH with hydrocarbon epoxides, since these conjugates are degraded to dihydrohydroxyaryl cysteines before acetylation and excretion as premercapturic acids.[26,27] Hence, if this metabolic pathway was established, hydrocarbons could be bound through the "*K*-region" bonds to ribosomal proteins. These bonds are also implicated in protein binding, since a compound that was identical on paper chromatograms with *S*-(5,6-dihydro-6-hydroxydibenz[a,h]-anthracen-5-yl)glutathione was liberated from the skins of mice that had been painted 2 days previously with ^{3}H-labelled dibenz[a,h]anthracene,[28] a hydrocarbon that is metabolized to an epoxide intermediate.[5,29]

There is also the possibility that the epoxide-GSH conjugates become bound to a protein of the soluble liver fraction such as ligandin,[30] which is known to bind with GSH conjugates of aminoazodye metabolites and of dihydroxy oestradiol.[31,32]

Metabolic activation is a prerequisite for binding of aromatic hydrocarbons to DNA[22,23,33,34] and investigations using ^{3}H-labelled "*K*-region" epoxides have shown that these intermediates react chemically with DNA, RNA and polyribonucleotides.[35] Similar studies with non-"*K*-region" isomers must await the synthesis of the ^{3}H-labelled epoxides but in the alkylation of 4-(*p*-nitrobenzyl) pyridine, benz-[a]anthracene 5,6-oxide is a better alkylating agent than the 8,9-isomer[9] and furthermore the "*K*-region" epoxide, dibenz[a,h]anthracene 5,6-oxide is a better alkylating agent than the non-"*K*-region" epoxide, dibenz[a,c]anthracene 10,11-oxide.[10]

It is also of interest that whereas benz[a]anthracene 5,6-oxide is active in producing malignant transformations in cells derived from mouse prostate, the 8,9-isomer

shows only low activity in this respect.[36] However, the hydrocarbon-deoxyribonucleoside adducts, obtained from the DNA of mouse embryo cell cultures that had been treated with ^{3}H-labelled 7-methylbenz[a]anthracene, were not the same as those obtained from similar cultures that had been treated with ^{3}H-labelled 7-methylbenz-[a]anthracene 5,6-oxide.[37]

Thus "epoxide hydrase" and "glutathione *S*-epoxide transferase" activities in various tissues and species may play an important role in the biological activity of hydrocarbons by reducing the amounts of reactive epoxide intermediates available for binding with tissue constituents.

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