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FORMATION OF K-REGION EPOXIDES AS MICROSOMAL METABOLITES OF PYRENE AND BENZO[A]PYRENE

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Abstract—Epoxides of pyrene and benzo[a]pyrene have been detected for the first time as microsomal metabolites of these polycyclic hydrocarbons. The epoxides, which have been identified as the K-region derivatives, pyrene 4,5-oxide and benzo[a]pyrene 4,5-oxide respectively, were formed by the NADPH-dependent mixed function oxidase of a rat-liver microsomal incubation system where the epoxide hydrase was inhibited. In the absence of hydrase inhibition, benzo[a]pyrene was converted into a metabolite with the chromatographic properties of a K-region dihydrodiol, *trans*-4,5-dihydro-4,5-dihydroxybenzo[a]pyrene, which has not previously been described as a metabolite of this hydrocarbon. Pyrene 4,5-oxide and benzo[a]pyrene 4,5-oxide rearrange in acid to compounds with the properties of 4-pyrenol and 4-benzo[a]pyrenol respectively, are converted by microsomal epoxide hydrase into compounds indistinguishable from the corresponding 4,5-dihydro-4,5-dihydroxy derivatives and react with glutathione to yield conjugates. Both epoxides are reactive towards polyguanylic acid.

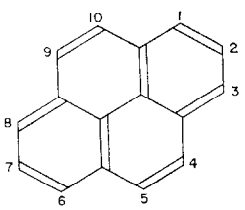
The significance of these results is discussed in relation to our hypothesis that polycyclic hydrocarbon carcinogenesis results from somatic mutations caused by epoxides that are formed from the hydrocarbons by metabolism.

THE METABOLISM of pyrene or benzo[a]pyrene by rat-liver microsomes leads to the formation of intermediates that react with DNA.[1] These and other polycyclic hydrocarbons are mainly metabolized to hydroxylated derivatives[2] which are now known to arise from epoxides formed by the action of the NADPH-dependent microsomal mixed function oxidase.[3–5] The synthetic K-region epoxides of several polycyclic hydrocarbons have been found to possess properties very relevant to the problem of polycyclic hydrocarbon carcinogenesis. They have been shown to be alkylating agents that react covalently with nucleic acids and with protein both chemically[6] and in cells in culture.[7,8] Moreover, K-region epoxides are more active than the parent hydrocarbons[9,10] in inducing malignant transformation of cells in culture and are effective mutagens in bacteriophage,[11] in bacteria,[12] in mammalian cells[13] and in *Drosophila**.

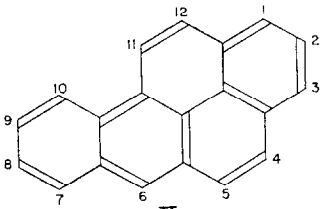
These results have led to the formulation of our working hypothesis concerning the mechanism of carcinogenesis by polycyclic hydrocarbon which proposes that these relatively inert compounds exert their carcinogenic effects through somatic mutations caused by epoxides produced by metabolism. Up to now the range of hydrocarbons studied with this hypothesis in mind has not included pyrene or benzo[a]pyrene, mainly because the K-region epoxides of these two hydrocarbons have not been prepared synthetically and have not been detected as metabolites.

* O. G. Fahmy and M. J. Fahmy, unpublished observations.

This paper presents the results of experiments designed firstly to detect the presence of K-region epoxides as microsomal metabolites of pyrene (I) and benzo[a]pyrene (II) and secondly to show that such epoxides are capable of reacting with macromolecules.



I



II

MATERIALS AND METHODS

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Boehringer, Mannheim, Germany and ^{3}H-labelled hydrocarbons (specific activities; benz[a]anthracene, 450 mc/mmole; pyrene, 94 mc/mmole; benzo[a]pyrene, 315 mc/mmole), from the Radiochemical Centre, Amersham, Bucks. ^{3}H-labelled benz[a]anthracene 5,6-oxide (specific activity 57 mc/mmole) and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene were prepared as previously described.[7,14] Polyguanylic acid was obtained from Miles Seravac, Maidenhead, Berks., and alumina, Type H, 100–200 mesh from P. Spence & Sons, Widnes, Lancs.

Other reagents were of AnalaR grade and all solvents used in experiments involving u.v. spectra were purified by fractional distillation before use. Rat-liver microsomes were prepared from animals pretreated with 3-methylcholanthrene.[2]

Inhibition of microsomal epoxide hydrase. The incubation system previously described[5] for the detection of polycyclic hydrocarbon epoxides as microsomal metabolites contained styrene oxide added in an attempt to improve the yield of epoxides by inhibiting the enzyme-catalysed conversions of epoxides to dihydrodiols. Further investigation of benz[a]anthracene metabolism has shown that 1,2,3,4-tetrahydronaphthalene 1,2-oxide is a more effective inhibitor of the microsomal epoxide hydrase than styrene oxide (Fig. 1); consequently this naphthalene derivative has been used in the experiments described below.

Microsomal metabolism of polycyclic hydrocarbons. Incubation mixtures consisting of rat-liver microsomes ($\equiv$10 g liver), resuspended in pyrophosphate buffer (0·1 M, pH 8·0, 80 ml) containing glucose 6-phosphate (1 mmole), glucose 6-phosphate dehydrogenase (96 units), NADPH (48 μmole), nicotinamide (2 mmole), $MgCl_2$ (1 mmole) and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (60 μmole) were preincubated at 30° for 10 min. ^{3}H-labelled hydrocarbon (400 μg) was added in ethanol (0·5 ml) and, after incubation at 30° for 10 min, the mixture was extracted with ether (1 vol). The ether extract was dried (Na_2SO_4), evaporated to dryness under reduced pressure and the residue redissolved in 1 ml of a mixture of petroleum ether (b.p. 40–60°)–benzene (4:1, v/v).

Column chromatography of metabolites. Concentrated solutions of metabolites were chromatographed on alumina columns as previously described[5] except that, in the experiments with ^{3}H-labelled pyrene and benzo[a]pyrene, unlabelled K-region epo-

xides could not be used as carriers since such compounds have not yet been prepared synthetically.

Enzyme–catalysed conversion of epoxides to dihydrodiols. Fractions containing the radioactive pyrene or benzo[a]pyrene epoxide eluted from alumina columns were pooled and evaporated to dryness under reduced pressure. The residue was redissolved in acetone and incubated with microsomes in the absence of cofactors as previously described.[5] Ether-soluble products were examined by thin-layer chromatography (t.l.c.) on Silica gel G developed in benzene–ethanol (19:1, v/v) with authentic samples of the dihydrodiols of pyrene or of benzo[a]pyrene as reference compounds.[16,17] The radioactivity present on samples of Silica gel removed from these chromatograms was determined by liquid scintillation counting.

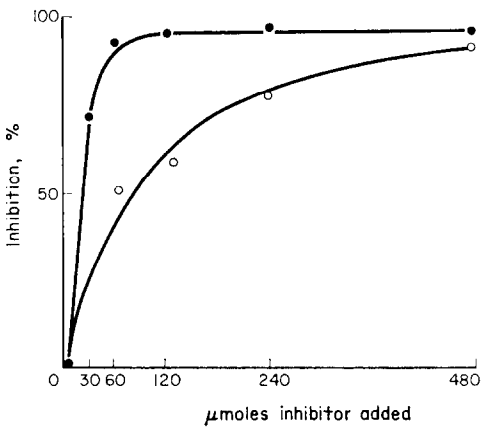


FIG. 1. Inhibition of formation of hydroxylated metabolites of benz[a]anthracene. Microsomal mixtures set up as described in the text and containing ^{3}H-benz[a]anthracene were incubated for 10 min in the presence of various concentrations of either styrene oxide (○——○) or 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (●——●). An ether extract of each incubation mixture was then chromatographed on an alumina column and the radioactivity in the peak containing the hydroxylated products estimated.

Acid-catalysed rearrangement of epoxides to phenols. Fractions from alumina columns that contained radioactive epoxides of either pyrene or of benzo[a]pyrene were pooled and evaporated to dryness under reduced pressure and the residue redissolved in methanol (5 ml). The solution was acidified with HCl (0·1 ml), diluted with water (20 ml) and the ether soluble rearrangement products examined using t.l.c. on Silica gel G developed with benzene or with benzene–ethanol (19:1, v/v) as previously described.[15,16] Authentic pyrenols and benzo[a]pyrenols were used as reference compounds and the radioactivity present in silica gel samples was determined as before.

Formation of acid-labile glutathione derivatives of epoxides. Fractions from alumina columns containing either radioactive pyrene or benzo[a]pyrene epoxide were pooled and evaporated to dryness. Each residue was redissolved in acetone (5 ml) and benz-[a]anthracene 5,6-oxide (2 mg) was added and the solution stirred in the dark at room temperature for 18 hr with an aqueous solution (5 ml) containing glutathione (2·5 mg)

and sodium bicarbonate (5 mg). The products were chromatographed on Whatman 3MM paper developed with butan-1-ol–propan-1-ol–aq. 2 M NH_3 (2:1:1, by vol) followed by examination under u.v. light and treatment with ninhydrin. The radioactivity present on strips cut from these chromatograms was measured by liquid scintillation counting.

U.V. spectroscopy of epoxide solutions. Large-scale incubations of ^{3}H-pyrene or ^{3}H-benzo[a]pyrene were carried out that contained four times the amounts of materials noted earlier. These incubation mixtures were extracted with ether and the dried, concentrated ether extract chromatographed on an alumina column as described before[5] and fractions containing the radioactive epoxide collected. Ethanol (10 ml) was added to the pooled benzene–pet. ether fractions and the solution evaporated almost to dryness under reduced pressure. Ethanol (10 ml) was again added and the solution re-evaporated to low volume. The u.v. spectra of these ethanol solutions, both before and after acidification, were examined using a Unicam S.P. 800 spectrophotometer fitted with silica micro cells of 2 cm path length.

Reactions of epoxides with polyguanylic acid. Fractions that contained radioactive epoxides of either pyrene or of benzo[a]pyrene eluted from alumina columns were combined and evaporated to small volume under reduced pressure and acetone (10 ml) was added. The solution was re-evaporated to low volume, the volume adjusted to 1 ml with acetone and the solution added to a solution of polyguanylic acid (2 mg) in water (2 ml, pH 7·4). The mixture was incubated for 2 hr at 37° and then extracted with ether (3 × 1 vol). The aqueous portion was added to the top of a G25 Sephadex column (0·9 × 60 cm) which was eluted with distilled water of pH 7·4. Fractions (0·5 ml) were collected and analysed both for u.v. absorbance at 260 nm and for radioactivity.

RESULTS

Alumina column chromatography of metabolites. When ^{3}H-pyrene or ^{3}H-benzo[a]pyrene were incubated with the NADPH-dependent microsomal mixed function oxidase of rat-liver in the presence of an epoxide hydrase inhibitor and the ether-soluble products chromatographed on an alumina column, radioactive metabolites were found which behaved in a similar manner to the K-region epoxides previously detected as metabolites of other hydrocarbons[5] using the same system. These metabolites of pyrene or of benzo[a]pyrene, which were eluted from a column after the peak of unchanged hydrocarbon but before the peak containing the hydroxylated derivatives (Fig. 2), were not present if the epoxide hydrase inhibitor, 1,2-epoxy-1,2,3,4-tetrahydronaphthalene was omitted from the incubation mixture. This indicated that they were most probably epoxides of pyrene and of benzo[a]pyrene and further investigations confirmed this view.

Conversion of epoxides to dihydrodiols, phenols and glutathione derivatives. Radioactive peaks eluted from alumina columns and which were thought to contain epoxides of either pyrene or of benzo[a]pyrene were subjected to procedures known to convert K-region epoxides into dihydrodiols, into phenols or into glutathione conjugates[17,18] and the products examined by chromatography together with the relevant authentic reference compounds. Figure 3 shows that the radioactive epoxides formed as metabolites of pyrene and benzo[a]pyrene can be converted by incubation with rat-liver

microsomal epoxide hydrase[18,19] in the absence of cofactors, into products indistinguishable on t.l.c. plates from the K-region dihydrodiols or pyrene and benzo-[a]pyrene, *trans*-4,5-dihydro-4,5-dihydroxypyrene and *trans*-4,5-dihydro-4,5 dihydro-xybenzo[a]pyrene. Figure 4 shows that metabolitically-formed epoxides derived from pyrene and benzo[a]pyrene are rearranged by treatment with acid into products with the thin-layer chromatographic characteristics of 4-pyrenol and 4-benzo[a]pyrenol respectively.

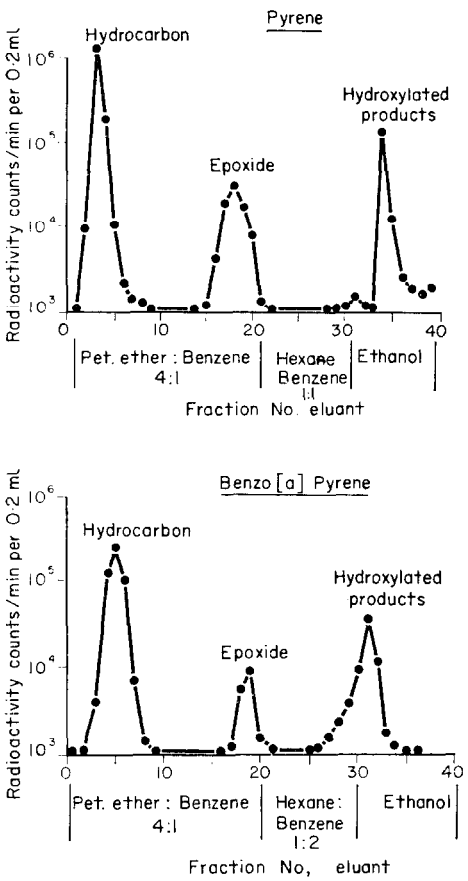


FIG. 2. Alumina column chromatography of ^{3}H-pyrene and ^{3}H-benzo[a]pyrene metabolites. The concentrated ether extract from a rat liver microsomal incubation (see text) was applied to a column (1·3 × 4 cm) of activated alumina (100–200 mesh, Type H) and eluted with solvent. 100-drop fractions were collected and radioactivity measured.

The epoxides formed as metabolites of pyrene and of benzo[a]pyrene also react with glutathione to give radioactive products with paper-chromatographic characteristics very similar to those of the acid-labile glutathione derivative formed from benz[a]-anthracene 5,6-oxide (Fig. 5).[17]

Metabolic formation of 4,5-*dihydro*-4,5-*dihydroxybenzo*[*a*]*pyrene*. Previous investigations of the metabolism of benzo[a]pyrene have failed to detect either of the two

K-region dihydrodiols derived from benzo[a]pyrene, 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene and 11,12-dihydro-11,12-dihydroxybenzo[a]pyrene as metabolites.[2,16] Since evidence obtained in the present work indicated that the epoxide detected as a metabolite of benzo[a]pyrene was the K-region derivative, benzo[a]pyrene 4,5-oxide, the metabolic formation of the corresponding K-region dihydrodiol was re-examined.

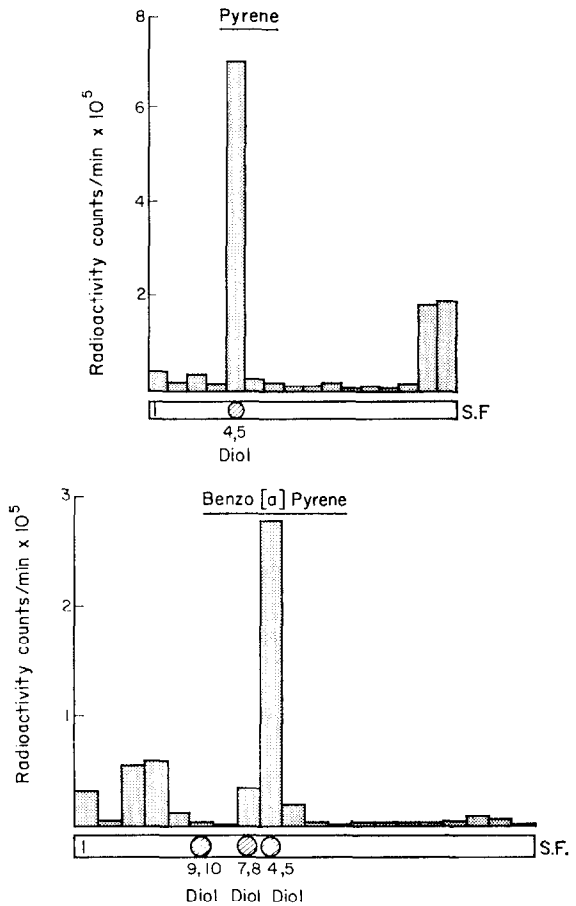


FIG. 3. Enzyme-catalysed conversions of radioactive pyrene and benzo[a]pyrene epoxides to the corresponding dihydrodiols. Fractions eluted from alumina columns that contained an epoxide were pooled and evaporated to dryness. The residue was redissolved in acetone (0·2 ml) and incubated for 1 hr at 37° with the epoxide hydrase of rat-liver microsomes (≡ 10 g liver) resuspended in Tris-buffer (pH 7·4 0·1 M, 50 ml). Ether soluble products were examined as previously described[15,16] using t.l.c. on Silica gel G developed with benzene–ethanol (19:1, v/v).

^{3}H-benzo[a]pyrene was incubated with the microsomal mixed function oxidase system in the absence of the epoxide hydrase inhibitor. The ether soluble products were chromatographed on an alumina column in the usual way and the eluted peak containing the hydroxylated derivatives further examined by thin layer chromatography using authentic unlabelled dihydrodiols derived from benzo[a]pyrene as markers. Figure 6 shows that, in addition to the expected 9,10-dihydrodiol, there was a

smaller peak of radioactivity on these chromatograms coincident with authentic *trans*-4,5-dihydro-4,5-dihydroxybenzo[a]pyrene but separable from the 7,8-dihydrodiol.

U.V. spectroscopy of metabolites

Pyrene. The u.v. spectrum of an ethanol solution of the pyrene epoxide obtained from a large-scale incubation was examined but the presence of contaminating u.v.-light absorbing material, probably originating from the microsomal incubation

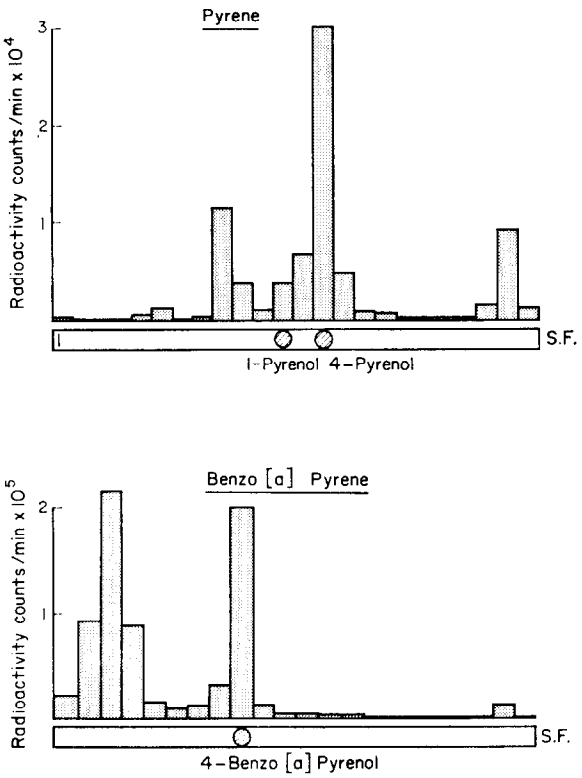


FIG. 4. Acid-catalysed rearrangement of radioactive epoxides of pyrene and of benzo[a]pyrene to the corresponding phenols. Fractions eluted from an alumina column that contained a radioactive epoxide were pooled, evaporated to dryness and the residue redissolved in methanol. The solution was acidified and the rearrangement products examined as previously described[15,16] using t.l.c. on Silica gel G developed for pyrenols, with benzene–ethanol (19:1, v/v) or for benzo[a]pyrenols, with benzene.

mixture, precluded the recognition of u.v. spectral structure below 300 nm. Consequently the epoxide solution was treated with acid and the u.v. spectrum of the resulting pyrenol measured.

This spectrum showed the peak at 378 nm and the inflexion at 358 nm that are characteristic of the spectrum of authentic 4-hydroxypyrene but which are not present in the spectra of 1- or 2-hydroxypyrene, the only alternative mono-hydroxy derivatives of pyrene.[15] When the solution was subjected to t.l.c. on Silica gel developed with

benzene–ethanol (19:1) and the plate examined under u.v. light, a violet fluorescent band was visible that corresponded in R_f with 4-hydroxypyrene. Elution of this band with ethanol, however, failed to yield sufficient material for a satisfactory u.v. spectrum.

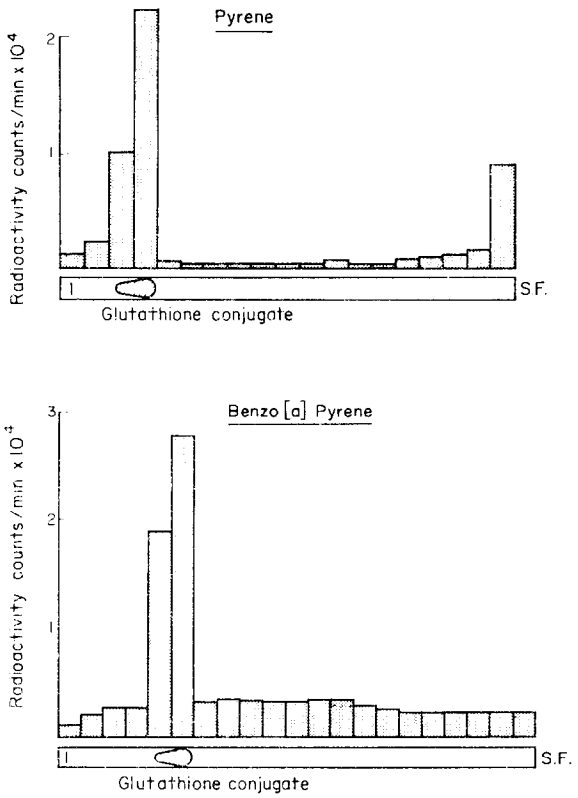


FIG. 5. Formation of acid-labile glutathione conjugates from radioactive epoxides of pyrene and of benzo[a]pyrene. Fractions eluted from an alumina column that contained a radioactive epoxide were pooled and evaporated to dryness. The residue was redissolved in acetone (5 ml), benz[a]anthracene 5,6-oxide (2 mg) was added and the solution mixed with an aqueous solution (5 ml) containing glutathione (2·5 mg) and sodium bicarbonate (5 mg) and stirred for 18 hr at room temperature. The products were examined as previously described[17] using paper chromatography on Whatman 3MM developed with butan-1-ol–propan-1-ol–2 M NH_3 (2:1:1, by vol).

Benzo[a]pyrene. When the u.v. spectra of ethanol solutions of the benzo[a]pyrene epoxide obtained from large scale incubations were measured, difficulties associated with contamination by extraneous material that absorbed u.v.-light below 300 nm were again experienced. At longer wavelengths, however, the u.v. spectra of these epoxide solutions showed two peaks, at 327 and 315 nm, which were close to the peaks at 323 and 310 nm that are present in the u.v. spectrum of authentic 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene.[16] Similar shifts to longer wavelengths have previously been found in comparisons between the spectra of epoxides and the related dihydrodiols derived from other hydrocarbons.[20]

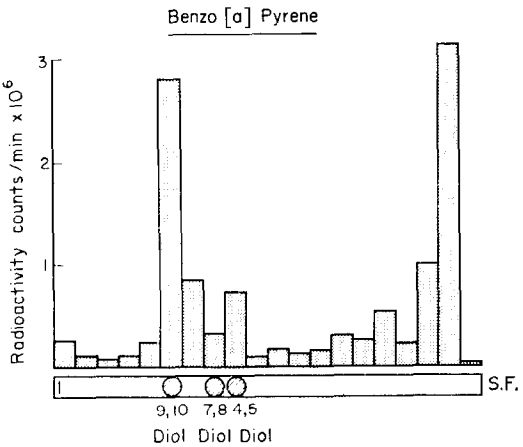


FIG. 6. Thin-layer chromatography of radioactive dihydrodiols derived from benzo[a]pyrene. Fractions containing the radioactive hydroxylated products obtained by alumina column chromatography of the ether soluble products of a microsomal incubation of ^{3}H-benzo[a]pyrene, in the absence of the epoxide hydrase inhibitor, were collected. After evaporation, these products were examined as previously described[16] on thin layer chromatograms of Silica gel G developed in benzene–ethanol (19:1, v/v). Authentic benzo[a]pyrene dihydrodiol markers were added to the solution of metabolites prior to spotting on the plate.

Solutions of the metabolically-formed benzo[a]pyrene epoxide were then treated with acid in order to rearrange epoxide to phenol and the u.v. spectra were remeasured. At longer wavelengths these spectra (Fig. 7A) were identical with that of the phenol obtained chemically from authentic 4,5-dihydro 4,5-dihydroxybenzo[a]pyrene (Fig. 7B) but were distinct from the u.v. spectra of the 1-, 3-, 6-, 7-, 8- and 9-hydroxy derivatives of benzo[a]pyrene and from that of the phenol obtained chemically from the 11,12-dihydrodihydroxy derivative of benzo[a]pyrene.[16,21]

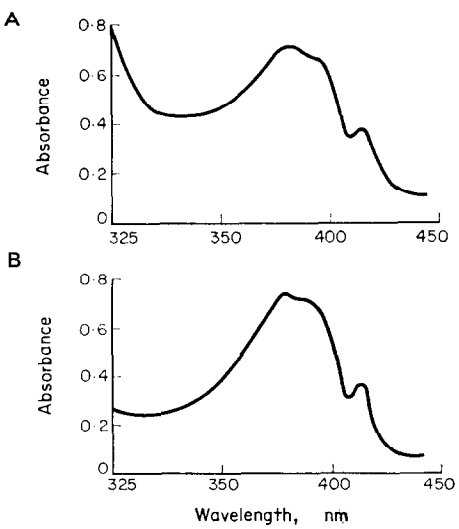


FIG. 7. Ultra-violet spectra. (A) phenol obtained by treating metabolically-formed benzo[a]pyrene epoxide with acid. (B) phenol obtained from 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene.

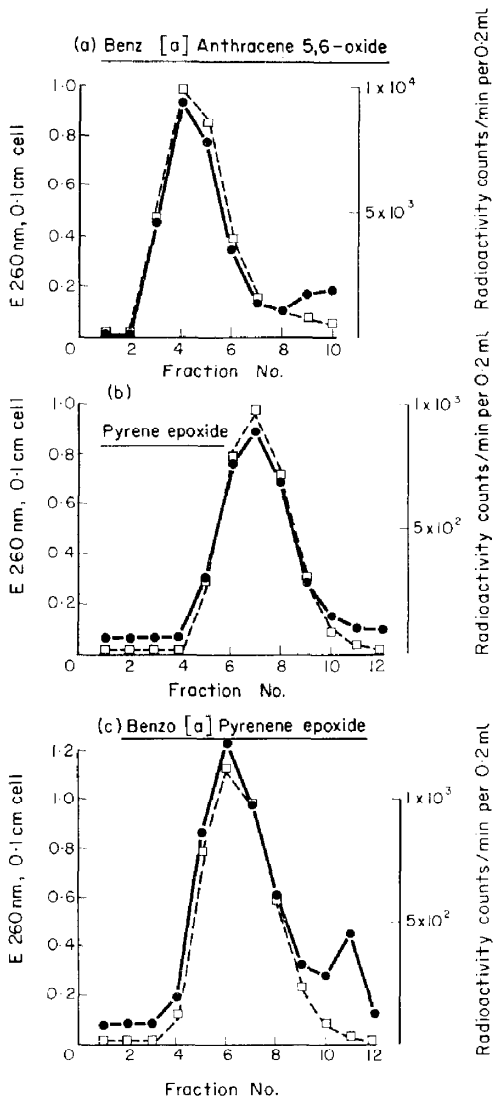


FIG. 8. Sephadex G. 25 column elution profiles of the products of reaction between polyguanylic acid and, (a) ^{3}H-benz[a]anthracene 5,6-oxide. (b) radioactive epoxide formed metabolically from ^{3}H-pyrene, and (c) radioactive epoxide formed metabolically from ^{3}H-benzo[a]pyrene. Acetone solutions of epoxide (1 ml) were added to an aqueous solution (2 ml, pH 7·4) of polyguanylic acid (2 mg) and the mixture incubated at 37° for 2 hr. After extraction with ether (3 × 1 vol) the aqueous portion was added to the top of a G. 25 Sephadex column (0·9 × 60 cm) and eluted with distilled water (pH 7·4). Fractions (0·5 ml) were collected and analysed for u.v. absorbance at 260 nm (□- - -□) and for radioactivity (●——●).

Reactions with polyguanylic acid. Figure 8 shows the G 25 Sephadex column elution profiles obtained when acetone solutions of metabolically-formed epoxides of pyrene or of benzo[a]pyrene were reacted with polyguanylic acid as described. For comparison a third elution profile is shown (Fig. 8) of the products of the reaction between

the ^{3}H-labelled K-region epoxide of benz[a]anthracene (benz[a]anthracene 5,6-oxide) and polyguanylic acid. The profiles show that, in all three cases, the peaks of radioactivity and of u.v. absorbance at 260 nm are superimposable. This indicates that the epoxides formed from pyrene and from benzo[a]pyrene and the K-region epoxide of benz[a]anthracene are reactive towards polyguanylic acid: similar results have been obtained with other ^{3}H-labelled K-region epoxides.*

DISCUSSION

The metabolism of ^{3}H-pyrene and of ^{3}H-benzo[a]pyrene by the NADPH-dependent microsomal mixed function oxidase of rat-liver has been examined in the presence of an inhibitor of the microsomal epoxide hydrase. Under these conditions, radioactive metabolites have been detected which, when chromatographed on alumina columns (Fig. 2), had characteristics that are very similar to those of K-region epoxides derived from other polycyclic hydrocarbons.[5] These radioactive metabolites of pyrene and of benzo[a]pyrene have been identified as epoxides since they have been shown to, (a) be converted by rat-liver microsomal epoxide hydrase, in the absence of co-factors, into compounds with the thin-layer chromatographic characteristics of dihydrodiols (Fig. 3); (b) rearrange on treatment with acid into products with the chromatographic characteristics of phenols (Fig. 4) and, (c) react with glutathione in aqueous alkaline solution to give products with the paper chromatographic characteristics of acid-labile glutathione conjugates (Fig. 5).

Further examination of the epoxide metabolite formed from pyrene has shown that the pyrenol, resulting from treatment of the epoxide with acid, is indistinguishable on t.l.c. plates from and has some of the u.v. spectral characteristics of 4-pyrenol. The dihydrodiol formed enzymically from the pyrene epoxide metabolite co-chromatographs on t.l.c. plates with authentic *trans*-4,5-dihydro-4,5-dihydroxypyrene. Consequently we feel justified in identifying this epoxide metabolite as pyrene 4,5-oxide.

The epoxide metabolite formed from benzo[a]pyrene has been found to rearrange in acid to a benzo[a]pyrenol that co-chromatographs on t.l.c. plates with the phenol formed chemically from authentic 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene. In addition, the phenol formed from the epoxide metabolite of benzo[a]pyrene has u.v. spectral characteristics identical with those of this authentic K-region phenol (Fig. 7) but distinct from those of other benzo[a]pyrenols. The dihydrodiol of benzo[a]pyrene, formed enzymically from the epoxide metabolite, co-chromatographed on t.l.c. plates with *trans*-4,5-dihydro-4,5-dihydroxybenzo[a]pyrene but was separable from the 7,8- and 9,10-dihydrodiols. We feel therefore that sufficient evidence has been obtained to permit identification of the benzo[a]pyrene epoxide eluted from alumina columns in these experiments as benzo[a]pyrene 4,5-oxide.

Since the microsomal metabolism of polycyclic hydrocarbons usually results in the formation of a mixture of isomeric dihydrodiols and since dihydrodiols arise from epoxide precursors, it is reasonable to expect that, in the presence of an epoxide hydrase inhibitor, a mixture of isomeric epoxides would be formed. K-region epoxides of polycyclic hydrocarbons are stable when chromatographed on alumina columns but attempts to chromatograph the non-K-region epoxides, benz[a]anthracene 8,9-oxide, benzo[a]pyrene 7,8-oxide and benzo[a]pyrene 9,10-oxide in this way, have not been successful and have resulted in decomposition of the epoxide.[20,22] This apparent

* P. L. Grover and P. Sims, unpublished observations.

stability of K-region epoxides relative to epoxides formed on other bonds probably explains why, when the metabolites have been separated on alumina columns, K-region epoxides have been the only epoxides detected as microsomal metabolites of phenanthrene and benz[a]anthracene[5] and of pyrene and benzo[a]pyrene.

As far as the formation of K-region products is concerned, the detection of pyrene 4,5-oxide as a microsomal metabolite of pyrene is not unexpected since earlier work has shown that *trans*-4,5-dihydro-4,5-dihydroxypyrene is formed as a major metabolite in rat liver preparations[2] and is excreted as a urinary metabolite by both rats and rabbits.[15] K-region derivatives have not previously been described as metabolites of benzo[a]pyrene, although, since the K-region double bonds are those most readily oxidized chemically, one might expect these bonds to be oxidized enzymically. In the present experiments, when ^{3}H-benzo[a]pyrene was metabolized, in the absence of epoxide hydrase inhibitor, a radioactive metabolite was formed that co-chromatographed on t.l.c. plates with *trans*-4,5-dihydro-4,5-dihydroxybenzo[a]pyrene (Fig. 6). The amount of radioactive 4,5-dihydrodiol formed was considerably less, however, than that formed at the 9,10 position.

The initial step in the microsomal oxidation of the aromatic double bonds of polycyclic hydrocarbons quite clearly involves the formation of an epoxide,[3–5] a reaction catalysed by the NADPH-dependent mixed function oxidase. As a second step these epoxides can then give rise, by rearrangement, to phenols and, by enzyme catalysed hydration, to dihydrodiols. In some cases phenols have been reported to be the only products resulting from the oxidation of specific double bonds but it is doubtful if these phenols arise by a separate mechanism that does not involve the initial formation of an epoxide. It is much more likely that, in the metabolism of double bonds like the 1,2-bond of pyrene and the 2,3-bond of benzo[a]pyrene to phenols, but not apparently to dihydrodiols, the rate of epoxide rearrangement to phenols proceeds more rapidly than the rate of enzyme-catalysed hydration to give dihydrodiols. Considerable variations in the rates of rearrangement of epoxides to the corresponding phenols* and in the rates of hydration of epoxides to dihydrodiols[18] have been noted in our laboratory. In metabolizing systems, in addition to varying proportions of dihydrodiols and phenols, epoxides give rise to glutathione conjugates, catalysed by an enzyme present in the soluble fraction,[23] and also react with nucleic acids and proteins.[1,7,8] Consequently it now seems inappropriate to continue to use terms such as "benzpyrene hydroxylase" or "aryl hydrocarbon hydroxylase" in connection with enzyme assays that involve the NADPH-dependent mixed function oxidase but where only that proportion of epoxide formed that rearranges to phenol is measured.

The properties so far ascribed to hydrocarbon epoxides include the alkylation of 4-(p-nitrobenzyl) pyridine[6] and reactions with proteins and nucleic acids both chemically[6] and in cells in culture.[7,8] The epoxides formed by the microsomal metabolism of pyrene and of benzo[a]pyrene and identified as the K-region derivatives, pyrene 4,5-oxide and benzo[a]pyrene 4,5-oxide, react with polyguanylic acid (Fig. 8). In this respect they resemble the K-region epoxides of phenanthrene, benz[a]anthracene, 7-methylbenz[a]anthracene and dibenz[a,h]anthracene which have recently been found to react with polyguanylic acid and to react more extensively with this polynucleotide than with some others. Attempts to demonstrate that the epoxides formed

* A. J. Swaisland and P. Sims, unpublished observations.

metabolically from pyrene and from benzo[a]pyrene are also reactive towards 4-(p-nitrobenzyl)pyridine have not been successful so far, however, probably because of the very small amounts of these epoxides that have been available for study.

Covalent reactions have been reported to occur between unidentified microsomal metabolites of pyrene and benzo[a]pyrene and nucleic acids in some *in vitro* experiments.[1,24] Similarly it is generally thought that metabolically activated intermediates are responsible for the covalent reactions that occur with the constituents of mouse skin[25] and of cells in culture[26,27] following the administration of ^{3}H-benzo[a]pyrene. In some *in vitro* systems, reactions of benzo[a]pyrene with nucleic acid have been shown to be catalysed by u.v. light,[28] by a hydrogen peroxide-ferrous ion system[29] or by iodine,[30] but there is little evidence to suggest that these activation mechanisms, which could involve free radicals, occur intracellularly. It seems more likely that the reactive species are formed by those pathways normally involved in the metabolism of foreign compounds. The detection of an epoxide of benzo[a]pyrene as a microsomal metabolite therefore strengthens our belief that both the *in vitro* and *in vivo* effects of benzo[a]pyrene can now be ascribed to the formation of intermediate epoxides, although not necessarily just to the particular benzo[a]pyrene epoxide detected in this work.

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