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THE METABOLISM OF BENZ[a]ANTHRACENE AND DIBENZ[a,h]ANTHRACENE AND THEIR RELATED "K-REGION" EPOXIDES, *CIS*-DIHYDRODIOLS AND PHENOLS BY HAMSTER EMBRYO CELLS

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Abstract—The nature of some of the metabolites present in the cell media from hamster embryo cells that had been grown in the presence of benz[a]anthracene, dibenz[a,h]-anthracene and their related K-region epoxides, phenols and *cis*-dihydrodiols has been investigated. Small amounts of phenols, dihydrodiols and unidentified water-soluble metabolites were detected in the media from the incubations with the hydrocarbons. Benz[a]anthracene 5,6-oxide yielded mainly the related dihydrodiol, whereas dibenz-[a,h]anthracene 5,6-oxide yielded mainly the related phenol and only small amounts of the dihydrodiol. The "K-region" phenols were metabolized to a small extent and both they and the epoxides yielded unidentified water-soluble metabolites. Much of the *cis*-dihydrodiols were recovered unchanged from the cell media, whereas with the other substrates large proportions of the material originally added to the media were not present in the media at the end of the incubation periods. These results are discussed in relation to those obtained in an examination of the levels of binding of the substrates to the protein, DNA and RNA of the cells that had been grown in the media.

THIS PAPER is the final one of a collaborative series in which the biological and biochemical properties of "K-region" epoxides of aromatic polycyclic hydrocarbons have been compared with those of the parent hydrocarbons, from which the epoxides are formed by metabolism,[1,2] and the related "K-region" *cis*- and *trans*-dihydrodiols and phenols. In the earlier work, it was shown that, with a few exceptions, the "K-region" epoxides are more effective than either their related hydrocarbons, their related *cis*- or *trans*-dihydrodiols or their related phenols in inducing malignant

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transformations in hamster embryo cells[3,4] and in cells derived from C3H mouse ventral prostate[3,5] and in causing mutations in Chinese hamster cells.[6]

In experiments in which the levels of binding of the "K-region" epoxides of benz[a]anthracene and dibenz[a,h]anthracene to proteins, DNA and RNA of hamster embryo cells were compared with the levels of binding of benz[a]anthracene, dibenz[a,h]anthracene and their related *cis*-dihydrodiols and phenols, it was found that in the benz[a]anthracene series, the epoxide was bound to all the macromolecules to a much greater extent than was either the hydrocarbon or the other derivatives.[7] In the dibenz[a,h]anthracene series, the levels of epoxide binding were high with protein and RNA, but low with DNA. Moreover, the levels of binding of the phenol, 5-hydroxydibenz[a,h]anthracene, to DNA and RNA were at least as high as those of the epoxide. In the present work, the nature and amounts of the metabolites, formed from the above substrates and present in the media in which the cells were grown, was investigated in order to seek correlations between the observed levels of binding to macromolecules and the patterns of metabolites formed.

EXPERIMENTAL

Materials. ^{3}H-Labelled benz[a]anthracene (sp. act. 560 mCi/m-mole) and dibenz[a,h]anthracene (sp. act. 280 mCi/m-mole) were supplied by the Radiochemical Centre Amersham, England and radioactive K-region epoxides, dihydrodiols and phenols were prepared from these hydrocarbons as described.[7] Unlabelled *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene and *trans*-5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene were prepared as described by Boyland and Sims[8] and *trans*-8,9-dihydro-8,9-dihydroxybenz[a]anthracene as described by Sims.[9]

Cell culture media. Cell culture media in which secondary cultures of hamster embryo cells had been grown in the presence of the substrates that are listed in Table 1, were stored deep-frozen until required. The details of the methods used in growing the cells have been described by Kuroki *et al.*[7] Media in which the cells had been grown in the presence of the substrates for 3, 6 and 24 hr were examined.

Separation and estimation of the metabolites. The media were allowed to thaw out at room temperature and portions (5 ml) were extracted three times with equal volumes of ethyl acetate. The extracts were combined and dried over Na_2SO_4 and portions (1 ml), containing suitable amounts of the compounds indicated below, added to act as chromatographic markers, were applied as 8 cm bands to the base lines of thin-layer chromatograms prepared by coating glass plates (20 × 20 cm) with layers of Silica gel G (E. Merck A.-G, Darmstadt, Germany) of 0·25 mm thickness. The plates were developed for 15 cm with benzene–ethanol (19:1, v/v) and then examined in u.v. light. The positions on the chromatograms of the compounds added as markers were determined by their fluorescence and the chromatograms were then divided into 25 or 30 fractions marked parallel with the base line. The Silica gel forming each fraction was removed from the chromatogram and the radioactivity present determined by liquid scintillation counting.

The aqueous layers remaining after the extraction with ethyl acetate were examined by paper chromatography. Portions (0·6 ml) of each were applied to the base lines of sheets (46 × 57 cm) of Whatman no. 1 chromatography paper. The chromatograms were developed downwards for 18 hr in butan-1-ol–propan-1-ol–aq. 2 M-NH_3 (2:1:1,

TABLE 1. THE METABOLIC PRODUCTS FORMED FROM BENZ[a]ANTHRACENE AND DIBENZ[a,h]ANTHRACENE AND THEIR RELATED EPOXIDES, PHENOLS AND *cis*-DIHYDRODIOLS BY HAMSTER EMBRYO CELL

Substrate	Amounts of substrates added ($\mu\mu$mole $\times 10^{-2}$/ml)	Product	Amounts of products ($\mu\mu$mole $\times 10^{-2}$/ml) present in media after incubating for		
			3 hr	6 hr	24 hr
Benz[a]anthracene	220	Benz[a]anthracene	42	48	43
		Phenolic metabolites	0·04	0·26	0·56
		trans-5,6-Dihydro-5,6-dihydroxybenz[a]anthracene	0·02	0·04	1·10
		trans-8,9-Dihydro-8,9-dihydroxybenz[a]anthracene	0·25	1·70	3·10
		Water-soluble metabolites	0·73	0·95	2·10
Benz[a]anthracene 5,6-oxide	205	5-Hydroxybenz[a]anthracene	< 0·01	< 0·01	< 0·01
		trans-5,6-Dihydro-5,-6-dihydroxybenz[a]anthracene	69	59	63
		Water-soluble metabolites	18	22	16
5-Hydroxybenz[a]anthracene	205	5-Hydroxybenz[a]anthracene	135	147	121
		Unidentified ethyl acetate-soluble product	0·52	1·01	10·40
		Water-soluble metabolites	5·05	6·10	28·30
cis-5,6-Dihydro-5,6-dihydroxybenz[a]anthracene	191	*cis*-5,6-Dihydro-5,6-dihydroxybenz[a]anthracene	151	142	147
		Unidentified ethyl acetate-soluble product	0·72	1·85	3·72
		Water-soluble metabolites	0·07	0·02	0·02
Dibenz[a,h]anthracene	180	Dibenz[a,h]anthracene	53	57	51
		Phenolic metabolites	< 0·01	< 0·01	0·30
		Dihydrodiols	< 0·01	0·02	0·51
		Water-soluble metabolites	< 0·01	< 0·01	< 0·01
Dibenz[a,h]anthracene 5,6-oxide	170	5-Hydroxydibenz[a,h]anthracene	48	56	50
		trans-5,6-Dihydro-5,6-dihydroxydibenz[a,h]anthracene	4·37	5·80	8·40
		Water-soluble metabolites	0·41	0·52	2·30
5-Hydroxydibenz[a,h]anthracene	170	5-Hydroxydibenz[a,h]anthracene	48	35	41
		Unidentified ethyl acetate-soluble product	2·51	3·60	5·21
		Water-soluble metabolites	0·18	0·18	0·54
cis-5,6-Dihydro-5,6-dihydroxydibenz[a,h]anthracene	160	*cis*-5,6-Dihydro-5,6-dihydroxydibenz[a,h]anthracene	105	138	132
		Water-soluble metabolites	< 0·01	< 0·01	< 0·01

The metabolites were separated either on t.l.c. plates or on paper chromatograms and they were detected and estimated as described in the text.

by vol.). The chromatograms were cut, parallel to the base line, into thirty equal strips and the radioactivity present in each strip determined by liquid scintillation counting.

With all the aqueous fractions examined, considerable amounts of the material present on the developed chromatograms were located near to the solvent front. These areas were cut from the chromatograms and the absorbed material eluted with methanol. Examination by t.l.c. of the material thus eluted showed that it consisted of products with the chromatographic properties of the material present in the ethyl acetate extract of the same medium. In a few experiments, portions of the cell media were subjected to the chloroform–methanol extraction procedure of Diamond *et al.*[10] and the aqueous and organic phases examined by paper and thin layer chromatography as before and the results compared with those obtained in the first procedure. The amounts of the materials present near the solvent fronts on the paper chromatograms were much reduced but not completely eliminated, and there were corresponding increases in the amounts of products present on the t.l.c. plates. It was evident, therefore, that the complete extraction of the less polar metabolites from the cell media was difficult: allowances for the material remaining in the aqueous phases have been made in calculating the amounts of the ethyl acetate-soluble metabolites recorded in Table 1.

The term "water soluble metabolite" is defined in the present work as a metabolite that is not extractable from the medium into ethyl acetate and which has an R_f of 0·80 or less when subjected to paper chromatography as described above.

The radioactivity determined in the experiments with both the ethyl acetate-soluble and aqueous phases was corrected for quenching and the results in Table 1 are expressed in $\mu\mu$moles/ml of cell medium.

RESULTS

Incubations with benz[a]anthracene. The radioactive ethyl acetate-soluble products extracted from media in which hamster embryo cells were grown in the presence of the hydrocarbon were examined by t.l.c. with unlabelled *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene and *trans*-8,9-dihydro-8,9-dihydroxybenz[a]anthracene added as chromatographic standards. The positions on the chromatograms at which phenolic metabolites of benz[a]anthracene could be found were known from earlier work on the metabolism of the hydrocarbon by rat liver preparations.[8] The results in Table 1 show that there was a progressive increase in the amounts of the phenols and the dihydrodiols formed as the time of incubation of the cells with the hydrocarbon was increased. However, only about 20 per cent of the hydrocarbon added at the beginning of the incubations was recovered and only about 2 per cent of the added hydrocarbon was converted into ethyl acetate-soluble metabolites.

Two water-soluble metabolites, at R_f 0·19 and 0·61, were detected in the aqueous phase and the amounts formed increased as the time of incubation increased. They were not identified.

Incubations with benz[a]anthracene 5,6-oxide. Unlabelled *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene and 5-hydroxybenz[a]anthracene were added to the ethyl acetate-soluble fraction to act as chromatographic markers. The results in Table 1 show that only about 35 per cent of the radioactivity added at the start of the experiment was recovered from the cell media and that, of this, most of the radioactivity

was associated with the band containing *trans*-5,6-dihydro-5,6-dihydroxybenz[a]-anthracene. 5-Hydroxybenz[a]anthracene was not detected. One water-soluble metabolite, of R_f 0·64, was detected, but was not identified. There was no increase in the amounts of any of the metabolites formed when the incubations were carried out for periods longer than 3 hr.

Incubations with 5-hydroxybenz[a]anthracene. 5-Hydroxybenz[a]anthracene was added to the ethyl acetate-soluble fractions to act as a chromatographic marker. The results show that 60–70 per cent of the phenol present at the start of the incubations remained unchanged for periods up to 24 hr. A small amount of an unidentified product, of R_f 0·53, was formed, the amounts of which increased as the time of incubation was increased.

One metabolite, of R_f 0·19, was present in the aqueous phase: the amounts of this product increased as the time of incubation was increased.

Incubations with cis-5,6-*dihydro*-5,6-*dihydroxybenz[a]anthracene.* The *cis*-dihydrodiol was added to the ethyl acetate-soluble fraction. The substrate was recovered unchanged from the cell media in high yield even after 24 hr incubation. One minor product was detected in amounts of less than 2 per cent of the amounts of the substrate added at the start of the incubation. The metabolite was not identified, but it could have resulted from the hydroxylation of the dihydrodiol because its R_f, at 0·22, was less than that, at 0·35, of the *cis*-dihydrodiol and because the amounts of the metabolite present in the media increased as the times of incubation increased. No water-soluble metabolites were detected.

Incubations with dibenz[a,h]anthracene. Table 1 shows that less than 30 per cent of the hydrocarbon added to the media at the beginning of the incubation could be recovered, even after an incubation time of 3 hr. Examination of the fraction from t.l.c. plates in which the phenols and dihydrodiol formed in the metabolism of the hydrocarbon could be expected[8] showed that only small amounts of these metabolites were present in the cell media, even after incubations of 24 hr. No water-soluble metabolites appeared to be formed.

Incubations with dibenz[a,h]anthracene 5,6-*oxide.* 5-Hydroxydibenz[a,h]anthracene and *trans*-5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene were added to the ethyl acetate-soluble fractions to act as chromatographic markers. The results in Table 1 show that only about 30 per cent of the oxide added at the start of the incubation could be accounted for as products in the cell media. The major product present in these media was 5-hydroxydibenz[a,h]anthracene and only relatively small amounts of the dihydrodiol were formed. The aqueous phase contained four unidentified water-soluble metabolites, of R_f 0·05, 0·18, 0·30 and 0·57, and of these, the metabolite of R_f 0·30 was a relatively minor product.

Incubations with 5-hydroxydibenz[a,h]anthracene. Between 20 and 30 per cent of the phenol added at the start of the incubations was recovered unchanged after 24 hr. One minor unidentified metabolite, of R_f 0·38, was formed in these incubations and its amount increased as the time of incubation was increased. Two unidentified products, of R_f 0·05 and 0·31, were detected in the aqueous fraction.

Incubations with cis-5,6-*dihydro*-5,6-*dihydroxydibenz[a,h]anthracene.* Over 80 per cent of the *cis*-dihydrodiol added at the start of the incubations was present unchanged in the incubation media, even after 24 hr. No metabolites were detected in either the ethyl acetate-soluble or the aqueous fraction.

DISCUSSION

Benz[a]anthracene was metabolized by hamster embryo cells into products with the chromatographic properties of dihydrodiols and phenols, similar to those formed from the hydrocarbon by rat-liver homogenates.[8,11] Dibenz[a,h]anthracene is similarly metabolized to dihydrodiols and phenols by rat-liver homogenates,[8,11] and these are presumably the same products that are formed by the cells. In a separate study on the metabolism of dibenz[a,h]anthracene by several types of rodent cell culture, Huberman, Selkirk and Heidelberger[12] found that the hydrocarbon was similarly metabolized to dihydrodiols and phenols and that, as in the present work, the percentages of the added hydrocarbon metabolized were small. Huberman *et al.*[12] also found that high recoveries of the added hydrocarbon were obtained only if the cells themselves were solubilized and extracted and the extracts added to the extracts of the cell media: it was not possible to carry out this procedure in the present work because the cells were used in the binding studies already reported,[7] but its omission may account for the low recoveries obtained.

The amounts of water-soluble metabolites formed from benz[a]anthracene were small and no water-soluble metabolites were formed from dibenz[a,h]anthracene. In this, the present results differ from those of Duncan, Brookes and Dipple,[13] who found that mouse embryo cells convert benz[a]anthracene and dibenz[a,h]anthracene, in almost quantitative yields, into water-soluble metabolites. Similarly, Diamond *et al.*[10,14] found that 7,12-dimethylbenz[a]anthracene and benzo[a]pyrene are converted largely into water-soluble derivatives by any one of a number of cell lines. Huberman *et al.*[12] found small, but significant, amounts of water-soluble metabolites after their cells were grown in the presence of dibenz[a,h]anthracene. In all this earlier work, however, the levels of water-soluble metabolites were estimated by difference following extraction of non-polar metabolites rather than by chromatographic procedures.

In the experiments of Kuroki *et al.*[7] where the binding of the hydrocarbons to macromolecules was measured, low levels of binding of both hydrocarbons to protein, DNA and RNA was observed. These findings and the findings now reported of low levels of metabolism of the hydrocarbons together support the idea that the binding of these compounds to macromolecules requires their prior metabolic activation.

Several differences between the metabolism of benz[a]anthracene 5,6-oxide and that of dibenz[a,h]anthracene 5,6-oxide were observed. Benz[a]anthracene 5,6-oxide was quickly converted into *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene, presumably by the microsomal "epoxide hydrase"[15–17] and no increases in the amounts of dihydrodiol formed were found after 3 hr. Kuroki *et al.*[7] found that the maximum level of binding to cellular macromolecules had occurred within 3 hr. Presumably by this time the epoxide had been removed from the system, either by conversion into metabolites or by reaction with cellular constituents. The rapid removal of the epoxide from the system would also explain why 5-hydroxybenz[a]anthracene was not detected: the non-enzymic conversion of the epoxide into the phenol is a slow reaction with this hydrocarbon.* Dibenz[a,h]anthracene 5,6-oxide, on the other hand, was only slowly converted into *trans*-5,6-dihydro-5,6-dihydroxybenz[a,h]anthracene. This oxide is a poor substrate for the "epoxide hydrase".[18] Much of the 5-hydroxydibenz[a,h]-

* A. Swaisland and P. Sims, unpublished observation.

anthracene found in the cell media could well have been formed from the epoxide after the media had been separated from the cells.

Benz[a]anthracene 5,6-oxide yielded only one water-soluble metabolite. Although this has not been identified it clearly differs from the single water-soluble metabolite formed from 5-hydroxybenz[a]anthracene. The water-soluble metabolite, of R_f 0·61, formed from benz[a]anthracene had the same chromatographic characteristics as those of the water-soluble metabolite formed from the oxide, but it was not possible to show that they were identical.

Dibenz[a,h]anthracene 5,6-oxide yielded five water-soluble products and of these, two had R_f values similar to those of the water-soluble metabolites of 5-hydroxydibenz[a,h]anthracene.

In the comparative studies on the binding of dibenz[a,h]anthracene 5,6-oxide and 5-hydroxydibenz[a,h]anthracene,[7] the oxide gave higher levels of binding to protein than the phenol. On the other hand both compounds were bound in about equal amounts to DNA and RNA. These results are difficult to interpret in the light of the present metabolic studies, but it may be significant that the oxide and the phenol are equally toxic to mouse prostate cells in culture, whereas the oxide, but not the phenol will induce malignant transformations in these cells.[5]

Little metabolism of *cis*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene and *cis*-5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene appears to take place in hamster embryo cells. Similarly only low levels of binding to cellular macromolecules were found with these compounds.[7] Both these dihydrodiols were slightly active when tested for their ability to induce malignant transformation in hamster embryo cells,[3] but were inactive in mouse prostate cells.[5] It should be pointed out, however, that the dihydrodiols formed in the metabolism both of the hydrocarbons and of the oxides have the *trans*-configurations:[8] the *trans*-dihydrodiols were unable to effect malignant transformations when tested on rodent cells.[5,6]

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