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REACTIVITY OF THE K-REGION EPOXIDES OF SOME POLYCYCLIC HYDROCARBONS TOWARDS THE NUCLEIC ACIDS AND PROTEINS OF BHK 21 CELLS

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Abstract—Tritium-labelled samples of phenanthrene, benz[a]anthracene, 7-methylbenz-[a]anthracene or dibenz[a, h]anthracene were added to cultures of BHK 21/13 cells and to a polyoma virus-transformed line of these cells (PyY) and after 24 hr DNA, RNA and protein isolated from the cells. Similar experiments were carried out with tritiated K-region epoxides, dihydrodiols and phenols prepared from these hydrocarbons.

The four K-region epoxides were clearly much more reactive towards the constituents of both cell lines than either the parent hydrocarbons or the corresponding dihydrodiols and phenols. The reactivity of these epoxides in a biological system is discussed with reference to polycyclic hydrocarbon binding, cytotoxicity, and carcinogenesis.

POLYCYCLIC hydrocarbons can be induced to react with macromolecules by irradiation with u.v. light[1] or X-rays,[2] by incubation with a hydrogen peroxide-ferrous ion system[3] or with iodine,[4] or by incubation in the presence of a microsomal hydroxylating system.[5,6] The relative importance of activating mechanisms in polycyclic hydrocarbon carcinogenesis is unknown at present but, in studies with mammalian cells in culture, a correlation has been obtained between the extent of hydrocarbon metabolism and the amount of binding to cellular macromolecules.[7,8] These results, when considered in conjunction with those obtained with the *in vitro* microsomal systems,[5,6] strongly suggest that reactive intermediates are formed from polycyclic hydrocarbons by the action of the NADPH-requiring microsomal enzymes that oxidatively metabolize aromatic double bonds.

It was originally suggested that the dihydrodiols, phenols and mercapturic acids, which are the principle metabolites of aromatic hydrocarbons, all stem from common epoxide precursors[9] and this has now been confirmed by work on benzene[10] and on naphthalene.[11] In the latter experiments on the microsomal oxidation of ^{14}C-naphthalene, the use of carrier techniques has enabled the formation of radioactive naphthalene 1,2-oxide to be detected. Recent investigations using the K-region epoxides derived from phenanthrene and dibenz[a,h]anthracene have shown that these intermediates are alkylating agents that react chemically with nucleic acids and with histone: the parent hydrocarbons and the corresponding K-region dihydrodiols did not possess these properties.[12]

This paper concerns the reactivity of a group of polycyclic hydrocarbons and their K-region derivatives towards the nucleic acids and proteins of hamster kidney cells (BHK 21/13) and a polyoma virus-transformed line of these cells (PyY) grown in

tissue culture. The tritium-labelled hydrocarbons used were phenanthrene, benz[a]anthracene, 7-methylbenz[a]anthracene and dibenz[a,h]anthracene together with their respective K-region epoxides, dihydrodiols and phenols.

EXPERIMENTAL

Materials. Dodecyl sulphate, 8-hydroxyquinoline, sodium 4-aminosalicylate, dimethylsulphoxide (DMSO) and tetraethylammonium hydroxide solution (TEAH) were obtained from B.D.H., Poole, Dorset and deoxyribonuclease (Type I) and ribonuclease (Type 1A) from Sigma Chemical Co., London. Calf serum was purchased from Tissue Culture Services Ltd., Slough, Bucks. and Eagles medium from Wellcome Reagents Ltd., Beckenham, Kent. Phenol and *m*-cresol from commercial sources were redistilled before use. Phenanthrene (sp. act. 571 mc/m-mole), benz[a]anthracene (sp. act. 684 mc/m-mole), 7-methylbenz[a]anthracene (sp. act. 66 mc/m-mole) and dibenz[a,h]anthracene (sp. act. 536 mc/m-mole), were randomly labelled with tritium by exchange in aqueous solution by the Radiochemical Centre, Amersham, Bucks. The hydrocarbons were purified by column chromatography on alumina using 3 × 5 cm columns with light petroleum (boiling range 60–80°) as solvent, followed by recrystallization. The tritiated K-region dihydrodiols, 9,10-dihydro-9,10-dihydroxy phenanthrene (sp. act. 601 mc/m-mole), 5,6-dihydro-5,6-dihydroxybenz[a]anthracene (sp. act. 690 mc/m-mole), 5,6-dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene (sp. act. 61 mc/m-mole) and 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene (sp. act. 551 mc/m-mole) were obtained from these hydrocarbons by oxidation with osmium tetroxide.[13] The corresponding epoxides, phenanthrene 9,10-oxide (sp. act. 533 mc/m-mole), benz[a]anthracene 5,6-oxide (sp. act. 707 mc/m-mole), 7-methylbenz[a]anthracene 5,6-oxide (sp. act. 74 mc/m-mole) and dibenz[a,h]anthracene 5,6-oxide (sp. act. 498 mc/m-mole) and phenols, 9-phenanthrol (sp. act. 577 mc/m-mole), 5-hydroxybenz[a]anthracene (sp. act. 646 mc/m-mole), 5-hydroxy-7-methylbenz[a]anthracene (sp. act. 64 mc/m-mole) and 5-hydroxydibenz[a,h]anthracene (sp. act. 567 mc/m-mole) were prepared from the dihydrodiols by methods previously described for the corresponding unlabelled compounds.[14–16]

METHODS

Cell cultures. BHK 21 hamster fibroblasts and a polyoma virus transformed variant of them (PyY) were obtained from Dr. M. G. P. Stoker, Imperial Cancer Research Fund. Both lines of cells were grown as monolayer cultures in rotating 80 oz Winchester bottles using the Glasgow modification of Eagles medium[17] containing 10% calf serum and 10% tryptose phosphate broth. Cultures approaching confluence were chosen for experimental purposes when each bottle would contain about 10^8 cells.

Treatment with hydrocarbons. Labelled hydrocarbons and their derivatives were added as freshly prepared solutions in DMSO (1 ml) to the medium (200 ml) covering the cell monolayers to give a final concentration of 5 μg/ml. Cell cultures were then grown as described above for a further 24 hr. Two bottles of cells were used in each experiment.

Isolation of cellular constituents. The medium was decanted from the cell monolayers of each pair of Winchester bottles and deep frozen for metabolism studies. The

monolayers were treated with versene (2 mM) in phosphate-buffered saline (PBS; prepared from 9 vol. 0·146 M NaCl and 1 vol. 0·1 M phosphate buffer, pH 7·2) containing trypsin (0·25%) and the cells recovered by centrifugation, washed by resuspension in PBS and recentrifuged. Cell pellets were suspended in 5% sodium 4-aminosalicylate and DNA, RNA and protein isolated from them as described by Diamond *et al.*[18]

Assay for radioactivity. Portions of the isolated nucleic acids were hydrolysed with the appropriate nuclease and portions of the proteins were dissolved in TEAH solution so that the associated radioactivity in the macromolecules could be determined by liquid scintillation counting using a Packard TriCarb Spectrometer, Model 3375, with a counting efficiency for tritium of 49 per cent for both hydrolysates.

RESULTS AND DISCUSSION

The levels of reaction of the four hydrocarbons and their K-region derivatives with the DNA, RNA and protein isolated from the two cell lines used are given in Table 1.

TABLE 1. THE REACTION OF POLYCYCLIC HYDROCARBONS AND THEIR *K*-REGION EPOXIDES, DIHYDRODIOLS AND PHENOLS WITH THE NUCLEIC ACIDS AND PROTEIN OF CELLS GROWING IN CULTURE

Compound	Extent of reaction to cellular constituents					
	BHK 21, Clone 13			PyY		
	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/100 g)	DNA (μmoles/mole P)	RNA (μmoles/mole P)	Protein (μmoles/100 g)
Phenanthrene	0·35	0·18	0·48	0·54	0·07	0·27
Phenanthrene 9,10-oxide	4·10	2·38	28·2	3·31	2·34	39·1
9,10-Dihydro-9,10-dihydroxy phenanthrene	0·25	0·20	1·01	0·22	0·16	0·96
9-Phenanthrol	0·38	0·57	1·35	0·19	0·29	2·38
Benz[a]anthracene	0·61	1·18	2·15	0·90	0·79	0·68
Benz[a]anthracene 5,6-oxide	26·5	2·67	56·5	67·5	4·95	70·1
5,6-Dihydro-5,6-dihydroxy benz[a]anthracene	0·15	0·20	0·15	0·18	0·11	0·35
5-Hydroxybenz[a]anthracene	2·62	0·93	6·51	0·39	0·48	3·95
7-Methylbenz[a]anthracene	2·21	1·84	1·72	1·24	3·34	2·00
7-Methylbenz[a]anthracene 5,6-oxide	17·6	6·34	46·1	36·5	12·1	24·1
5,6-Dihydro-5,6-dihydroxy-7-methylbenz[a]anthracene	1·10	1·31	2·41	6·34	1·51	1·97
5-Hydroxy-7-methylbenz[a]anthracene	0·49	1·10	3·71	1·10	1·42	2·21
Dibenz[a,h]anthracene	0·68	0·35	3·22	0·35	0·26	1·69
Dibenz[a,h]anthracene 5,6-oxide	5·63	3·16	21·6	6·31	3·76	21·8
5,6-Dihydro-5,6-dihydroxy-dibenz[a,h]anthracene	0·85	0·45	2·22	0·51	0·29	1·99
5-Hydroxydibenz[a,h]anthracene	0·88	0·49	2·74	0·66	0·36	1·78

The tritiated compounds were added in DMSO (1 ml) to the medium (200 ml) covering the cell monolayers growing in rotating 80 oz Winchester bottles to give a concentration of 5 μg/ml. Twenty-four hr later the cells were harvested and the DNA, RNA and protein isolated. In all cases calculation of the extent of reaction has been based on the specific activity of the corresponding parent hydrocarbons.

Whilst comparisons between the four groups of compounds show variations, firstly in the levels of reaction of the different types of derivatives used and secondly, in the reactivity of these derivatives towards the three types of cellular macromolecules isolated, one general point emerges clearly. This is that the synthetically prepared K-region epoxide of each of the hydrocarbons used was more reactive towards the cell constituents isolated than either the parent hydrocarbon itself or the K-region diol and phenol into which this epoxide is metabolically converted.[14–16] Although the earlier report of the chemical reactivity of hydrocarbon epoxides towards macromolecules[12] has therefore been confirmed in a biological system, there are many factors to consider in attempting to use these results in explaining the mechanisms of hydrocarbon binding, toxicity and carcinogenesis. As far as binding is concerned, the relatively low levels that resulted from incubation with comparatively high concentrations of the parent hydrocarbons in the present study are in agreement with the view that such binding is linked to the metabolic activity of the cell line used. BHK 21 cells are known to be unable to metabolize benzo[a]pyrene and also to be resistant to the toxic effects of this compound.[19] This cell line was selected for the present experiments because it ought to be easier, with cells of this type, to distinguish between the reactivity of the epoxides themselves and the reactivity of active intermediates formed from the hydrocarbons and also because it was thought that the epoxides might be less rapidly metabolized to inactive products in such cells. This reasoning is supported by the results obtained (Table 1) and it would now seem to be worthwhile to compare the relative levels of binding resulting from treatment with a range of hydrocarbons and their respective epoxide derivatives in rodent embryo cells that are known to be able to metabolize this type of compound.[7,19,20]

Positive correlations between the benz[a]anthracene-inducible activity of microsomal hydroxylating enzymes and the cytotoxicity of benzo[a]pyrene have been obtained from experiments with several different mammalian cell lines grown in culture.[21] In addition, a phenolic metabolite, 3-hydroxybenzo[a]pyrene, was found to be cytotoxic to both the hydrocarbon sensitive and resistant cell lines. This result could be interpreted as indicating that phenolic metabolites, or conjugates formed from them by cells, might be reactive towards cell constituents. In the present experiments, whilst the four K-region phenols used were generally somewhat more reactive towards cellular proteins than the corresponding hydrocarbons, these phenols were much less reactive than the related epoxides. If cytotoxicity results from such interactions then, on the basis of the levels of reaction found in the experiments reported here, the hydrocarbon epoxides should prove to be potent cytotoxic agents.

Some of the hydrocarbons and derivatives that have been used in this work have been tested for carcinogenicity, but the results obtained do not show a positive correlation with the levels of interaction found to occur with BHK 21 cell constituents. In particular, the K-region epoxides have all been shown to be less potent carcinogens in mice than the parent hydrocarbons[22–24] although they are apparently much more reactive compounds.[12] Moreover, reference to Table 1 shows that in this BHK 21 cell system epoxide reactivity does not appear to correlate with the carcinogenicity of the hydrocarbon from which the epoxide is derived. The K-region epoxide of the potent carcinogen dibenz[a,h]anthracene reacts to a lesser extent with, for example, DNA than does the corresponding epoxide derived from the weakly carcinogenic benz[a]anthracene. However, this apparent lack of correlation is not surprising if the

complexities of intra-cellular events and of hydrocarbon metabolism are taken into consideration. It then seems very unlikely that the gross level of reaction between an epoxide, formed on one particular double-bond of a hydrocarbon, and the total DNA, RNA or total cellular protein of BHK 21 cells grown in culture would correlate with hydrocarbon carcinogenesis, which is a rare occurrence if one takes into consideration the numbers of cells that are usually exposed in an *in vivo* carcinogenicity test.

Selective reactions with, for example, constituents of cells at a particular stage of the cell cycle or with a particular nucleic acid or protein species are probably necessary to initiate malignancy. These selective interactions could not be detected in the type of experiment that has been performed to date in attempts to obtain correlations between hydrocarbon binding and carcinogenicity.[25,26] This problem is illustrated by the recently reported preferential alkylation of mitochondrial DNA by *N*-methyl-*N*-nitrosourea,[27] a finding that would be masked by chromosomal nucleic acid binding in most studies.

A more precise understanding of chemical carcinogenesis will probably come from the development of quantitative systems based on the *in vitro* transformation of embryonic or of continuously growing rodent cell lines:[28] the ability of hydrocarbons and reactive derivatives to effect transformation of such cells in culture is under investigation at present.

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