Biochemical Pharmacology, Vol. 19, pp. 2251–2259. Pergamon Press. 1970. Printed in Great Britain

INTERACTIONS OF THE K-REGION EPOXIDES OF PHENANTHRENE AND DIBENZ[a,h]ANTHRACENE WITH NUCLEIC ACIDS AND HISTONE

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(*Received* 14 *July* 1969: *accepted* 10 *February* 1970)

Abstract—In neutral solution at 37°, the K-region epoxides of phenanthrene and dibenz[a,h]anthracene (phenanthrene 9, 10-oxide and dibenz[a,h]anthracene 5, 6-oxide) are more reactive towards *p*-nitrobenzylpyridine than methyl methanesulphonate, ethyl methanesulphonate and phenanthrene, dibenz[a,h]anthracene and their corresponding K-region dihydrodiols did not react.

Tritiated K-region epoxides of phenanthrene and dibenz[a,h]anthracene reacted with DNA, RNA and histone on incubation at 37°. The parent hydrocarbons and their respective K-region dihydrodiols did not react.

The reaction of the K-region epoxide of phenanthrene with DNA was reduced by the addition of rat-liver microsomes but the reaction of the K-region epoxide of dibenz[a,h]anthracene with DNA was not affected.

The possible implications of the reactions of hydrocarbon epoxides with cellular constituents is discussed in relation to chemical carcinogenesis.

THE MECHANISMS of action of the carcinogenic polycyclic hydrocarbons are not yet fully understood. Covalent binding of these compounds to cellular constituents occurs *in vivo*[1] and correlations have been drawn between the carcinogenicity of the hydrocarbons and the extent of their interactions with the nucleic acids of mouse skin.[2,3] It seems probable that metabolic activation of polycyclic hydrocarbons precedes their reaction with tissue components and results obtained with an *in vitro* system, involving the microsomal metabolism of polycyclic hydrocarbons in the presence of cellular macromolecules,[4] confirm this view. A similar explanation of the mechanism of action of the carcinogenic aromatic amines has been followed by the identification of derivatives that are more potent carcinogens than the parent amines,[5,6] and that react chemically with cell constituents.[5] The types of reactive intermediates that could be involved in the induction of cancer by polycyclic hydrocarbons include free radicals,[7] carbonium ions,[8] and epoxides.[9] Detailed investigations of the metabolism of hydrocarbons and their related K-region epoxides[10,11] support the original suggestion[12] that epoxides are formed in the microsomal oxidation of aromatic hydrocarbons. The 'K-region' epoxides of some polycyclic hydrocarbons have been prepared synthetically, but, when tested in animals, proved to be less active carcinogens than the parent hydrocarbons:[13–15] these results raised doubts about the role of hydrocarbon epoxides in carcinogenesis.

In earlier work on the *in vitro* metabolism of 7,12-dimethylbenz[a]anthracene by rat liver homogenates,[16] the product expected to result from the microsomal oxidation of the K-region, 5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene, was not detected: it was formed, however, if washed rat liver microsomes were used in place of homogenate. Similarly, when benzo[a]pyrene and chrysene were metabolized by rat liver preparations, none of the expected K-region products were detected,[17,18] although the 4,5 and 11,12 bonds of benzo[a]pyrene and the 5,6 bond of chrysene are susceptible to chemical oxidation. These results suggested that active intermediates were formed in the oxidative metabolism of the K-regions of these hydrocarbons that reacted with receptor molecules in the liver preparations and consequently were not available for further metabolism to the corresponding dihydrodihydroxy derivatives. It is possible that the active intermediates involved are the K-region epoxides of these hydrocarbons and an examination of the reactivity of compounds of this type towards cellular macromolecules seemed justified. For purposes of comparison with other alkylating agents the reactivity of epoxides towards 4-(*p*-nitrobenzyl)pyridine was also investigated; this paper presents some results obtained from experiments using the K-region epoxides of phenanthrene and dibenz[a,h]anthracene (phenanthrene 9,10-oxide and dibenz[a,h]anthracene 5,6-oxide).

EXPERIMENTAL

Materials. DNA (ex salmon testes; Sigma Chemical Co., St. Louis, Mo., U.S.A.) was purified by a detergent/salt procedure[19] to a residual protein content of less than 0·2 per cent. RNA (highly polymerized, from yeast) and 4-(*p*-nitrobenzyl)pyridine were purchased from B.D.H., Poole, Dorset and calf thymus whole histone was the gift of Dr. E. W. Johns. Phenanthrene (sp. act. 169 mc/m-mole) and dibenz[a,h] anthracene (sp. act. 33·3 mc/m-mole) (generally labelled with tritium) were supplied by Radiochemical Centre, Amersham, Bucks. Tritiated 9,10-dihydro-9,10-dihydroxy-phenanthrene (sp. act. 28·1 mc/m-mole) and 5,6-dihydro-5,6-dihydroxydibenz[a,h] anthracene (sp. act. 16·0 mc/m-mole) were obtained from the hydrocarbons by oxidation with osmium tetroxide[20] and tritiated phenanthrene 9,10-oxide (sp. act. 29·8 mc/m-mole) and dibenz[a,h]anthracene 5,6-oxide (sp. act. 22·4 mc/m-mole) were prepared as previously described.[21] Methyl methanesulphonate and ethyl methanesulphonate were purchased from Kodak Ltd., Kirkby, Liverpool. Washed rat-liver microsomes were prepared as previously described.[22]

METHODS

Reaction with 4-(p-*nitrobenzyl)pyridine.* The reactivities of phenanthrene and dibenz-[a,h]anthracene derivatives towards 4-(*p*-nitrobenzyl)pyridine were measured using a modification[23] of a method employed for the colorimetric determination of alkylating agents.[24] In the present experiments reactions were carried out at 37° in solutions buffered to pH 7·4 and comparisons were made using methyl methanesulphonate and ethyl methanesulphonate.

For the determinations 0·2 μmole of the test material dissolved in acetone (0·5 ml) was mixed with 0·2 M Tris-HCl buffer, pH 7·4, (1 ml) and 2% w/v 4-(*p*-nitrobenzyl)pyridine in ethylene glycol (2 ml) and the mixture incubated at 37°. The mixture was

cooled in ice-water, the colour developed by the addition of 50% (v/v) triethylamine in acetone (2·5 ml) and the optical density at 560mμ determined immediately.

Stabilities of epoxides. The rates of rearrangement, in aqueous solutions, of the K-region epoxides of phenanthrene and dibenz[a,h]anthracene to the corresponding phenols were determined by a fluorimetric method.

Acetone solutions (0·1 ml) of the epoxides (10–50 μg) were added to tubes containing 50% (v/v) aqueous ethanol (pH 7·4, 10 ml) and the mixtures incubated at 37°. The increases in fluorescence were measured, using an Aminco–Bowman spectrophotofluorimeter, at the appropriate excitation and emission maxima for the phenols (9-phenanthrol, activation 310 mμ, fluorescence 390 mμ; 5-hydroxydibenz[a,h] anthracene, activation 350 mμ, fluorescence 420 mμ) and were compared with the fluorescence produced when the remaining epoxides in the solutions were converted into phenols by the addition of acid (0·1 ml of H_2SO_4).

Reactivity towards nucleic acids and histone. Fifty mg samples of nucleic acid or histone were dissolved in distilled water (100 ml) adjusted to pH 7·4 with 0·1 N sodium hydroxide; the hydrocarbons or their derivatives were added in acetone (0·1 ml), and the mixtures incubated in air at 37° for the periods indicated below: at the end of this time, hydrocarbons and non-polar derivatives were removed by extraction with ether (3 × 100 ml). RNA was reprecipitated by adding acetate buffer (4·5 ml of 0·6M, pH 4·5) followed by acetone (2 vol.), DNA by adding NaCl (4 g) followed by ethanol (2 vol.) and histone by adding N HCl (2 ml) followed by acetone (6 vol.); the reprecipitated materials were washed in two changes of fresh acetone or ethanol for at least 36 hr and then dried. DNA (5 mg) was hydrolysed in deoxyribonuclease solution (0·5 ml) and RNA (5 mg) or histone (5 mg) was dissolved in tetraethylammonium hydroxide solution (0·5 ml) before the radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Spectrometer, Model 3375, with a counting efficiency of 49 per cent for tritium.

RESULTS

Reaction with 4-(p-nitrobenzyl)pyridine. Phenanthrene 9,10-oxide and dibenz[a,h] anthracene 5,6-oxide both reacted with 4-(*p*-nitrobenzyl) pyridine when incubated in neutral solution at 37°, as did methyl methanesulphonate (Fig. 1). The K-region epoxides of some other polycyclic hydrocarbons also react with this reagent[25] but, when ethyl methanesulphonate, phenanthrene, 9,10-dihydro-9,10-dihydroxyphenanthrene, dibenz[a,h]anthracene or 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene were used, no detectable reaction occurred.

Stability of epoxides in aqueous solution. Fluorimetric estimations showed that incubation of phenanthrene 9,10-oxide or dibenz[a,h]anthracene 5,6-oxide in aqueous ethanol at 37° for 24 hr resulted in the rearrangement of 3–5 per cent of the added epoxide to the corresponding phenol. More than 90 per cent of each epoxide remained unchanged and could be converted into phenol by treatment with acid.

Reactivity of hydrocarbon epoxides towards nucleic acids and histone. Interaction of the K-region epoxides of phenanthrene or dibenz[a,h]anthracene with nucleic acids and histone occurred during incubation at 37° for 2 hr and the levels of reaction observed, measured by the amount of radioactivity remaining bound to the reisolated macromolecules, are shown in Table 1. The bound radioactivity is probably the result of covalent reactions between the epoxides and the nucleic acids and histone since this

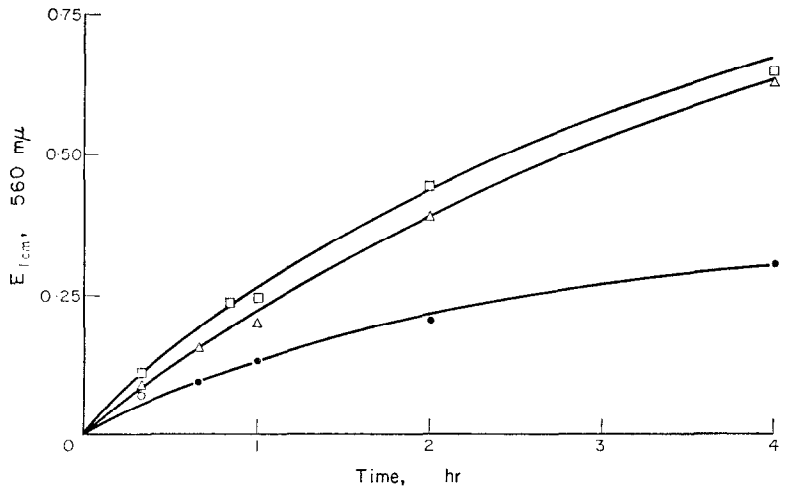

FIG. 1. Increase in colour formation with time of incubation at 37° resulting from the reaction of 0·2 μmoles of methyl methanesulphonate (●—●), phenanthrene 9, 10-oxide (△—△), and dibenz[a,h]-anthracene 5, 6-oxide (□—□) with *p*-nitrobenzylpyridine at pH 7·4.

TABLE 1. THE REACTION OF THE TRITIATED K-REGION EPOXIDES OF PHENANTHRENE AND DIBENZ[a,h]ANTHRACENE WITH NUCLEIC ACIDS AND HISTONE

	Epoxide bound to		
	RNA (μmoles/mole P)	DNA (μmoles/mole P)	Histone (μmoles/100 g)
Phenanthrene 9, 10-oxide	14·0	35·8	0·7
Dibenz[a,h]anthracene 5, 6-oxide	39·5	13·9	10·9

The tritiated epoxide (5 μg) was added to a solution containing 50 mg nucleic acid or histone in water (100 ml) and the mixtures incubated at 37° for 2 hr. The nucleic acids and protein were reisolated and the radioactivity determined by procedures described in the text.

radioactivity was not removed either during the solvent-washing procedures involved in the re-isolation of the macromolecules or by subsequent ether extractions of enzyme-hydrolysed samples of nucleic acid or histone.

In similar comparative experiments, tritiated preparations of phenanthrene, 9,10-dihydro-9,10-dihydroxyphenanthrene, dibenz[a,h]anthracene and 5,6-dihydro-5,6-dihydroxydibenz[a,h]anthracene were incubated with nucleic acids and with histone. No significantly-raised levels of bound radioactivity resulted from incubations with the hydrocarbons. In one or two cases very low levels of bound radioactivity were found when the dihydrodiols were used but the figures were not consistent and may have been due to sample contamination.

The effects of varying the times of incubation on the levels of reactions of tritiated phenanthrene 9,10-oxide or dibenz[a,h]anthracene 5,6-oxide with DNA with histone

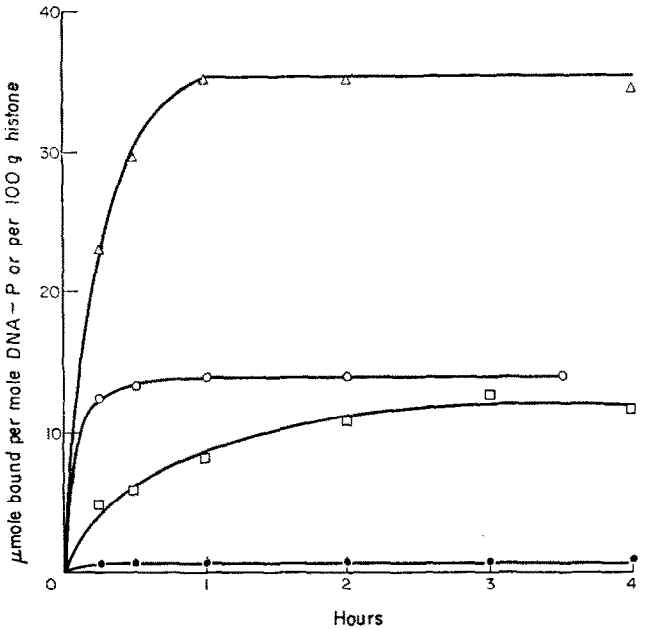

FIG. 2. Effect of time on the reactions at 37° between tritiated phenanthrene 9, 10-oxide (5 μg) and DNA (50 mg) (△—△) or histone (50 mg) (●—●) and between tritiated dibenz[a,h]anthracene 5, 6-oxide (5 μg) and DNA (50 mg) (○—○) or histone (50 mg) (□—□).

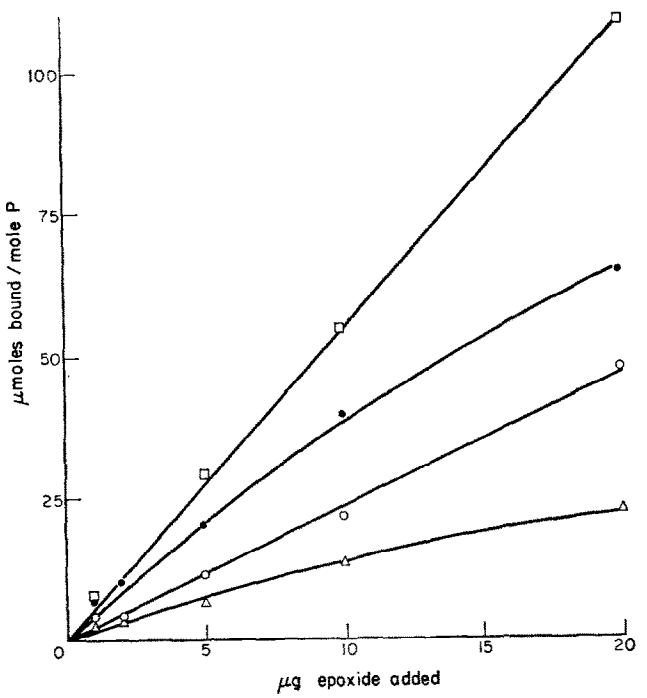

FIG. 3. Effect of epoxide concentration on reactions between tritiated phenanthrene 9, 10-oxide and DNA (□—□) or RNA (△—△) and between tritiated dibenz[a,h]anthracene 5, 6-oxide and DNA (○—○) or RNA (●—●). Reaction mixtures were incubated for 2 hr at 37°.

are shown in Fig. 2. The curves indicate that maximum reaction is achieved after 2 hr at 37° when 5 μg epoxide is incubated with 50 mg DNA or histone.

Figure 3 shows the increasing reactions that occur when the amounts of epoxide added to solutions of RNA or DNA are increased in the range 2–20 μg.

The effect of rat liver microsomes on the reaction of epoxides with DNA. The results of adding varying amounts of a preparation of washed rat liver microsomes to reaction mixtures containing DNA and either phenanthrene 9,10-oxide or dibenz[a,h]anthracene 5,6-oxide are given in Fig. 4. This shows that the level of interaction of the K-region epoxide of phenanthrene with DNA is progressively reduced by the addition of increasing amounts of microsomes, but that the level of reaction of the K-region epoxide of dibenz[a,h]anthracene is not affected by such additions.

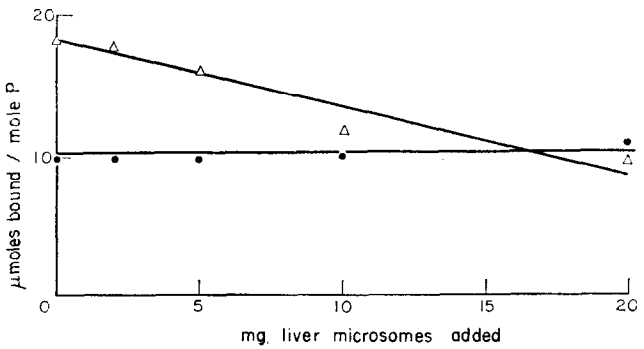

FIG. 4. The effect on the reactions between DNA (50 mg) and tritiated phenanthrene 9, 10-oxide (5 μg) (△—△) or tritiated dibenz[a,h]anthracene 5, 6-oxide (5 μg) (●—●) of adding increasing amounts of a rat liver microsomal preparation. Reaction mixtures were incubated for 2 hr at 37°.

DISCUSSION

Collectively, the results reported here indicate that K-region epoxides may be the active intermediates involved in the binding of polycyclic hydrocarbons to cellular macromolecules.

At physiological temperature and pH, phenanthrene 9,10-oxide and dibenz[a,h] anthracene 5,6-oxide were more reactive, on an equimolar basis, as measured by the alklyation of the pyridine nitrogen of 4-(*p*-nitrobenzyl) pyridine, than either methyl methanesulphonate or ethyl methanesulphonate. Both methyl and ethyl methanesulphonates cause dominant lethal mutations in rat spermatozoa,[26] and methyl methanesulphonate reacts *in vivo* with the guanine moieties of RNA and DNA or rat testes.[27] Consequently, the K-region epoxides of both phenanthrene and dibenz[a,h]-anthracene, and those of other polycyclic hydrocarbons, must be considered to be sufficiently reactive to effect the alkylation of nucleic acids *in vivo*.

Phenanthrene, dibenz[a,h]anthracene and the K-region dihydrodiols derived from these hydrocarbons did not react either with *p*-nitrobenzylpyridine or with nucleic acids or histone when incubated *in vitro*. This lack of reactivity of polycyclic hydrocarbons themselves is in accord with earlier results[4] which showed that microsomal metabolism of these compounds was necessary before interaction with DNA or protein occurred.

The amounts of reaction with nucleic acids observed following incubation with the K-region epoxides of phenanthrene and dibenz[a,h]anthracene, is of the same order as that which follows the treatment of mouse epidermis[3] or embryonic rodent cells in tissue culture[28] with 7,12-dimethylbenz[a]anthracene. However, higher levels of interaction of these epoxides with nucleic acids may be achieved by adjusting the conditions of reaction.[25]

Virus-transformed cells in culture exhibit a smaller capacity for the metabolism of polycyclic hydrocarbons than normal cells[29] and lower levels of reactions with nucleic acids are found following treatment with 7,12-dimethylbenz[a]anthracene.[28] If epoxides are the active intermediates in the *in vivo* reactions of polycyclic hydrocarbons with macromolecules, it might be expected that the treatment of cells with tritiated K-region epoxides would give higher levels of nucleic acid and protein labelling than those that occur with the corresponding hydrocarbons. These differences should be more marked in virus-transformed cells, which are less active in the metabolism of these compounds. Experiments in which nucleic acids and proteins were isolated from polyoma-transformed hamster-kidney fibroblasts that had been treated in culture with tritiated phenanthrene, dibenz[a,h]anthracene or the corresponding K-region epoxides, confirmed this view. Consistently higher levels of radioactivity were associated with the nucleic acids and proteins obtained from cultures containing the epoxides than from cultures with the parent hydrocarbons.[30]

The stabilities of the K-region epoxides of phenanthrene and dibenz[a,h]anthracene in aqueous solution are such that the half-lives of these compounds *in vivo* are probably much longer than those of the carbonium ions and free radicals that have also been proposed as active intermediates involved in hydrocarbon carcinogenesis.[8–10] The relative stability of the active intermediates, produced by microsomal metabolism of hydrocarbons, may be important if it is necessary for them to enter the nucleus, before interactions with DNA occur. Conversely, reactivity rather than stability may be of more importance if reactions with genetic material occur when the nuclear membrane is absent in mitosis.

The maintenance of effective levels of an intermediate formed by metabolism may also be a factor if interactions with genetic material, at a particular stage in the cell cycle, are involved in chemical carcinogenesis. The initial microsomal oxidation of hydrocarbons to form epoxides[31] is probably followed by several competing reactions (Fig. 5); the epoxide may react enzymically with water to yield the dihydrodiol,[21,32] undergo a non-enzymic, protein-catalysed rearrangement to yield the related phenol,[32] conjugate enzymically with glutathione,[33] or react chemically with cytoplasmic proteins and RNA or with DNA. Variations in the rates of removal of epoxides by these competing reactions, could help to account for the wide range of carcinogenicities associated with various polycyclic hydrocarbons that are metabolized by similar pathways. Differences in the rates of enzymic hydration of the K-region epoxides of phenanthrene and dibenz[a,h]anthracene by rat-liver microsomes to yield the corresponding dihydrodiols, have been demonstrated *in vitro*;[21] the more rapid rate of microsomal hydration of phenanthrene 9,10-oxide probably accounts for the reductions in the amount of the reaction found when this epoxide was incubated with DNA in the presence of microsomal preparations (Fig. 4).

The *in vivo* interactions of dibenz[a,c]anthracene, which does not possess a K-region, with mouse epidermis[2,3] indicates that active intermediates, which may be

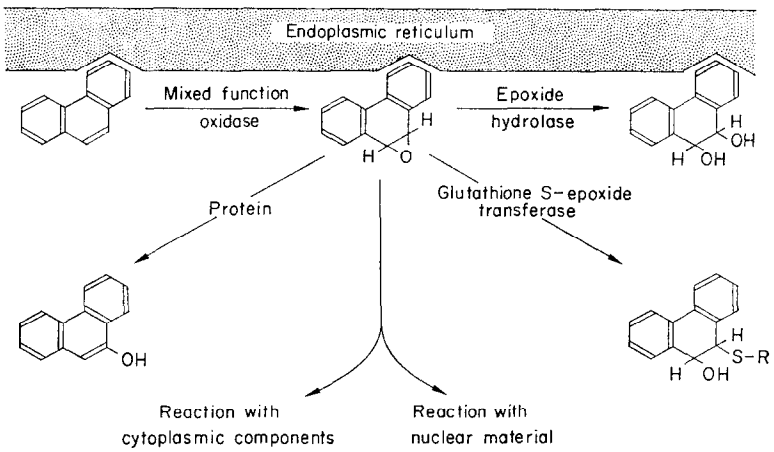

FIG. 5. Pathways involved in the metabolism of phenanthrene 9, 10-oxide.

epoxides, are probably formed on other bonds during the metabolism of polycyclic hydrocarbons. This possibility is under investigation at present, in addition to more obvious studies aimed at identifying the sites involved and the products formed, when hydrocarbon epoxides react with cellular constituents.

Acknowledgements—The authors thank Professor E. Boyland for encouraging this work and acknowledge the technical assistance of Mr. A. Hewer. 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 British Empire Cancer Campaign for Research.

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