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SOME PROPERTIES OF "K-REGION" EPOXIDES OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract—Comparative studies of some of the properties of polycyclic hydrocarbon epoxides have been carried out using the "K-region" epoxides derived from phenanthrene, benz[a]anthracene, 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene, 3-methylcholanthrene and dibenz[a,h]anthracene. The results show that, within this series of epoxides, there are appreciable differences in the rates at which the epoxides, (a) rearrange in neutral solution to the corresponding phenols; (b) are metabolized by rat-liver microsomal fractions that contain the enzyme "epoxide hydrase" and; (c) alkylate 4-(*p*-nitrobenzyl)pyridine. The rearrangement of "K-region" epoxides to their isomeric phenols was not catalysed by protein.

IT IS now accepted that the formation of an epoxide is the initial step in the oxidation of a double bond of an aromatic polycyclic hydrocarbon by mammalian microsomal enzyme systems,[1,2] and the formation of a number of "K-region" epoxides from their parent hydrocarbons in these systems has been demonstrated,[2–5] the "K-region" being the region of high-electron density similar to the 9,10-bond of phenanthrene. Synthetic "K-region" epoxides alkylate nucleic acids and proteins[6,7] and, although they have lower carcinogenic activities than the corresponding hydrocarbons in whole animals,[8–10] they are, in general, more active than the related hydrocarbons, "K-region" dihydrodiols and "K-region" phenols in causing malignant transformations[11–13] and mutations[14] in mammalian cells in culture. "K-region" epoxides are also mutagenic in bacteriophage[15] and in bacteria[16] under conditions where the corresponding hydrocarbons, dihydrodiols and phenols are inactive. Since both the carcinogenic and the inactive polycyclic hydrocarbons appear to be hydroxylated by a process that involves the formation of epoxide intermediates, differences in the rates of the formation and further metabolism, in the stability and in the reactivity of these epoxides may account for the variation in the biological activities shown by the two types of hydrocarbons.

This paper describes some comparative studies on the rates at which members of a series of "K-region" epoxides rearrange to the corresponding phenols, on the rates at which they alkylate 4-(*p*-nitrobenzyl)pyridine and on the rates at which they are removed from rat-liver microsomal preparations, either by metabolism, by rearrangement or by reaction with cellular constituents.

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EXPERIMENTAL

Materials. Phenanthrene was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks; benz[a]anthracene from R. N. Emanuel Ltd., Wembley, Middlesex; 3-methylcholanthrene, dibenz[a, h]anthracene and 7,12-dimethylbenz[a]anthracene from Eastman Ltd., Rochester, New York; 4-(*p*-nitrobenzyl)pyridine from Hopkin and Williams, Ltd., Chadwell Heath, Essex and bovine serum albumin from Armour Pharmaceuticals, Eastbourne, Sussex.

7-Methylbenz[a]anthracene, phenanthrene 9,10-oxide, benz[a]anthracene 5,6-oxide, dibenz[a,h]anthracene 5,6-oxide, 7-methylbenz[a]anthracene 5,6-oxide, 3-methylcholanthrene 11,12-oxide and 7,12-dimethylbenz[a]anthracene 5,6-oxide were prepared by previously described methods.[17–21]

Estimation of phenolic products. "K-region" phenols were estimated fluorimetrically using an Aminco-Bowman spectrophotofluorimeter standardized with quinine sulphate in 0·05 M-H_2SO_4 (1 μg/ml). The fluorescence intensities of solutions obtained in the experiments below were measured at the following activation and fluorescence maxima: 9-hydroxyphenanthrene, activation 315 nm, fluorescence 380 nm; 5-hydroxybenz[a]anthracene, activation 340 nm, fluorescence 425 nm; 5-hydroxydibenz[a,h]anthracene, activation 353 nm, fluorescence 420 nm; 5-hydroxy-7,12-dimethylbenz[a]anthracene, activation 374 nm, fluorescence 450 nm; 5-hydroxy-7-methylbenz[a]anthracene, activation 365 nm, fluorescence 435 nm and 11-hydroxy-3-methylcholanthrene, activation 374 nm, fluorescence 422 nm. The concentrations of phenols were calculated from fluorescence calibration curves prepared from the authentic reference compounds.

When the epoxides were also present in the solutions, they were estimated by measuring the increases in fluorescence, at the appropriate wavelengths, produced by the addition to the extracts of conc. HCl (0·1 ml), which causes epoxides to rearrange into the related phenols.

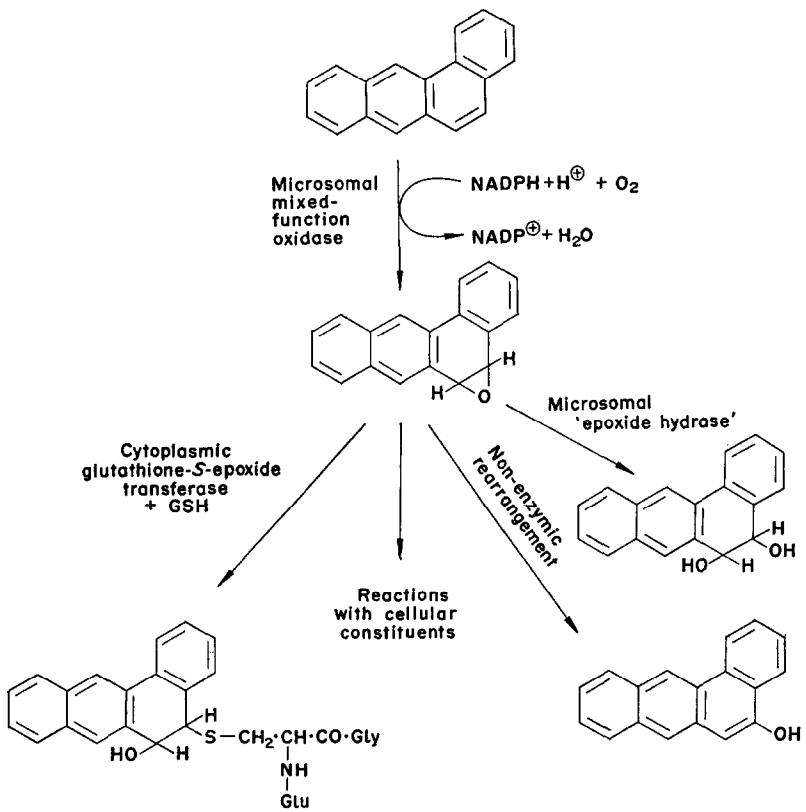
Rates of rearrangement of epoxides to phenols. Portions (0·5 ml) of solutions of epoxides in acetone (2 μmoles/ml) were added to 50% (v/v) aqueous-ethanol (10 ml) that had been buffered to pH 7·4 with Tris–HCl to a final concentration of 0·1 M Tris. The mixtures were incubated at 37° and samples (1 ml) were taken at intervals, diluted with ethanol (2 ml) and the phenols present in the solutions estimated as described above.

The effect of protein on the rates of rearrangement of epoxides to phenols was investigated in a similar manner except that bovine serum albumin (40 mg) was added to each 10 ml portion of buffered 50% aqueous-ethanol. The phenols produced were estimated as before.

In a study of the stabilities of arene oxides at a number of pH values, benz[a]anthracene 5,6-oxide or 7,12-dimethylbenz[a]anthracene 5,6-oxide (0·25 mg) in acetone (0·1 ml) was added to a buffer solution (10 ml) and the mixture incubated for 20 min at 37°. The buffers used were; 0·1 M citric acid–sodium citrate, pH 4, 5 and 6; 0·1 M-orthophosphate, pH 6, 7 and 8 and 0·1 M Tris–HCl, pH 8 and 9. The incubation mixtures were each extracted with ethyl acetate (7 ml), portions (1 ml) of this extract were diluted with ethanol (2 ml) and the phenols present estimated by their fluorescence intensities as described.

Alkylation of 4-(p-*nitrobenzyl*)*pyridine by epoxides.* The rates of alkylation of this reagent by epoxides were determined using a modification[22] of a method employed

for the colorimetric determination of alkylating agents.[23] Epoxides (1·2 μmoles) in acetone (3 ml) were added to 0·1 M Tris–HCl buffer, pH 7·4, (6 ml) and a 2% (w/v) solution of 4-(*p*-nitrobenzyl)pyridine in ethylene glycol (12 ml) and the mixtures incubated at 37°. At intervals, samples (3 ml) of each incubation mixture were removed and cooled in ice. The colour was developed by the addition of a 50% (v/v) solution of triethylamine and acetone (2·5 ml) and the extinction at 560 nm measured immediately using a Unicam SP500 spectrophotometer.



SCHEME 1. Pathways involved in the metabolism of benz[a]anthracene at the "K-region".

Experiments with rat liver microsomal fractions. The livers of 8-week-old male Wistar rats were weighed and homogenized in 4 vol. of ice-cold 0·1 M phosphate buffer, pH 7·4, using a Potter–Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant thus obtained centrifuged at 30,000 *g* for 20 min. The mitochondrial pellet that was formed was discarded and the microsomal fraction sedimented by centrifuging the supernatant at 120,000 *g* for 45 min. The microsomal pellet was washed by resuspension in ice-cold phosphate buffer and resedimentation at 120,000 *g* for 45 min.

Solutions of an epoxide (1 μmole) in acetone (0·5 ml) were added to a number of flasks each containing 0·1 M phosphate buffer, pH 7·4, (10 ml) and a washed rat-liver microsomal fraction prepared from 2 g of liver. The flasks were incubated in a water

bath at 37° and at intervals a flask was removed from the bath and the contents extracted with ethyl acetate (7 ml). Portions (1 ml) of the extracts were diluted with ethanol (2 ml) and the phenol and epoxide present in the solutions estimated as described above.

Using benz[a]anthracene 5,6-oxide as substrate, the effect of pH on the conversion of an arene oxide into its related dihydrodiol by rat-liver microsomal fractions was investigated. The oxide (1 μmole) in acetone (0·5 ml) was added to a series of flasks, each containing one of the buffer solutions (10 ml) described above together with a microsomal fraction prepared from 2 g of liver. The flasks were incubated at 37° for 15 min and 5-hydroxybenz[a]anthracene and benz[a]anthracene 5,6-oxide present in the mixtures were extracted and estimated as described above.

In both these experiments, the results were calculated on the assumption that the difference between the amount of oxide added and the sum of the amount of oxide and phenol present at the end of the incubation represent the amount of dihydrodiol formed during the incubation. Some reaction of the oxides with microsomal proteins also takes place during the incubation, but experiments using the ^{3}H-labelled oxide have shown that, under the experimental conditions used, these reactions are small.*

RESULTS AND DISCUSSION

Among the polycyclic aromatic hydrocarbons, there are some that are highly active, some that are slightly active and some that are inactive as carcinogens. Epoxides are formed as intermediates in the metabolism of all these compounds and some of these intermediates may be involved in the biological effects attributed to the parent compounds. Thus differences in the stabilities and reactivities of the epoxides may

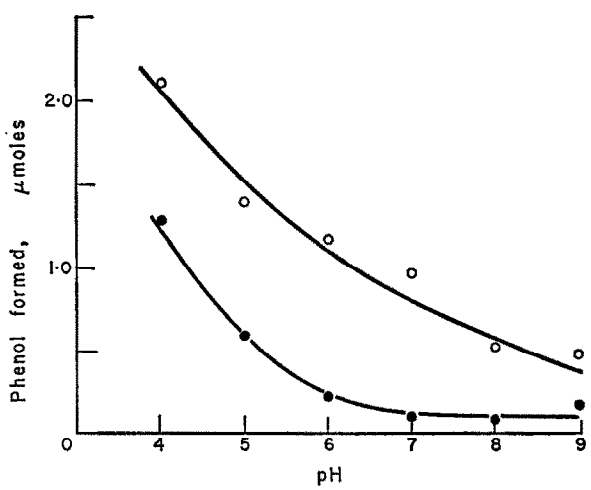


FIG. 1. The effect of pH on the rearrangement of "K-region" epoxides to phenols. Epoxides (0·25 mg) in acetone (0·1 ml) were added to buffer solutions (10 ml) and the mixtures incubated at 37° for 20 min. The reaction mixtures were extracted with ethyl acetate (7 ml) and the phenols, 5-hydroxybenz[a]-anthracene (●——●) and 5-hydroxy-7,12-dimethylbenz[a]anthracene (○——○) were determined fluorimetrically as described in the text.

* A. J. Swaisland, unpublished observations.

account for the observed differences in biological activities shown by the parent hydrocarbons. The results of the experiments described here show that there are appreciable differences in the rates at which "K-region" epoxides of polycyclic hydrocarbons are converted chemically and enzymically into phenols and dihydrodiols and in the rates at which they alkylate 4-(*p*-nitrobenzyl)pyridine.

Arene oxides rapidly rearrange to phenols in the presence of acid,[2,24] and the results shown in Fig. 1 indicate that benz[a]anthracene 5,6-oxide, which is derived from a virtually inactive hydrocarbon, and 7,12-dimethylbenz[a]anthracene 5,6-oxide,

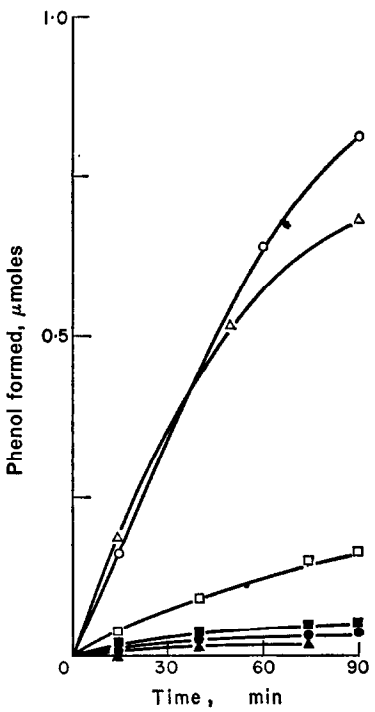


FIG. 2. Rates of rearrangement of epoxides to phenols at neutral pH. Epoxides (1 μmole) in acetone (0·5 ml) were added to 50% (v/v) aqueous-ethanol (10 ml) buffered to pH 7·4 with Tris–HCl and incubated at 37°. Samples (1 ml) were taken at various times, diluted with ethanol (2 ml) and the phenols 11-hydroxy-3-methylcholanthrene (△——△), 5-hydroxy-7,12-dimethylbenz[a]anthracene (○——○), 5-hydroxy-7-methylbenz[a]anthracene (□——□), 5-hydroxydibenz[a,h]anthracene (■——■), 5-hydroxybenz[a]anthracene (●——●) and 9-hydroxyphenanthrene (▲——▲) estimated fluorimetrically as described in the text.

which is derived from a carcinogenic hydrocarbon, both become increasingly unstable below pH 6. The rates at which members of a series of epoxides rearrange at pH 7·4 into their related phenols are shown in Fig. 2. These rearrangements appear to be more rapid in those epoxides that possess methyl groups in positions near to the "K-regions". There is no correlation between the rates of rearrangements and the carcinogenicities of the parent hydrocarbons. It has been reported[1,2] that the presence of proteins, small peptides or acetamide increases the rates at which some arene oxides rearrange to phenols. These findings have not been confirmed by other workers[24]

TABLE 1. EFFECT OF PROTEIN ON THE RATES OF REARRANGEMENT OF EPOXIDES TO PHENOLS

"K-region" epoxide	Rates of rearrangement of epoxides to phenols (nmoles/min)	
	in Tris buffer	in Tris buffer containing bovine serum albumin
3-Methylcholanthrene 11,12-oxide	14·0	16·7
7,12-Dimethylbenz[a]anthracene 5,6-oxide	10·7	12·0
7-Methylbenz[a]anthracene 5,6-oxide	2·2	2·8
Dibenz[a,h]anthracene 5,6-oxide	0·7	0·5
Benz[a]anthracene 5,6-oxide	0·7	0·6
Phenanthrene 9,10-oxide	0·3	0·3

Epoxides (1 μmole) in acetone (0·5 ml) were added to Tris–HCl buffered 50% (v/v) aqueous-ethanol (pH 7·4, 10 ml) or buffered aqueous ethanol containing bovine serum albumin (40 mg) and incubated at 37°. Samples (1 ml) were taken at various times, diluted with ethanol (2 ml) and phenols estimated fluorimetrically as described.

and the results in Table 1 show that the effect of protein on the rearrangement of "K-region" epoxides is very small and that protein cannot be regarded as a catalyst in these rearrangements.

The results obtained in the reactions of a series of "K-region" epoxides with 4-(*p*-nitrobenzyl)pyridine are shown in Fig. 3. The estimation of the alkylating ability of these epoxides using 4-(*p*-nitrobenzyl)pyridine relies on the formation of alkylated

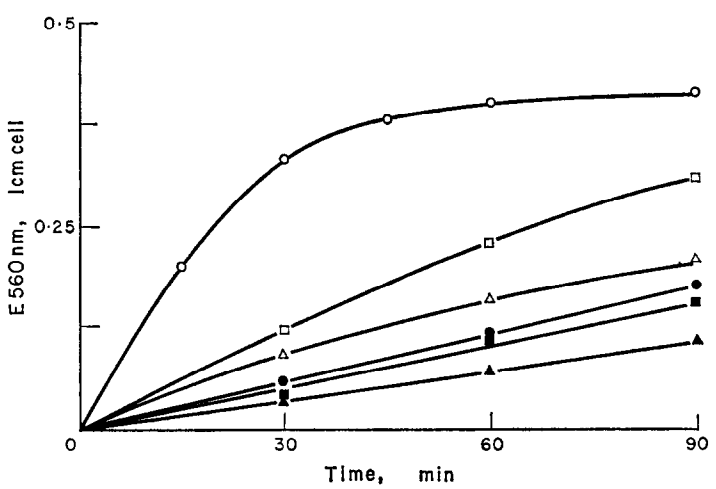


FIG. 3. Alkylation of 4-(*p*-nitrobenzyl)pyridine by epoxides. Epoxides (1·2 μmole) in acetone (3 ml) were incubated with 4-(*p*-nitrobenzyl)pyridine (0·24 g) in ethylene glycol (12 ml) and 0·1 M Tris–HC buffer, pH 7·4, (6 ml) at 37°. Samples (3 ml) were taken at various times, cooled on ice and the extinction at 560 nm measured immediately after the addition of 50% (v/v) triethylamine and acetone (2·5 ml). The increases in the extinction of the 4-(*p*-nitrobenzyl)pyridine derivatives of 7,12-dimethylbenz[a]anthracene 5,6-oxide (○——○), 7-methylbenz[a]anthracene 5,6-oxide (□——□), benz[a]anthracene 5,6-oxide (●——●), 3-methylcholanthrene 11,12-oxide (△——△), dibenz[a,h]anthracene 5,6-oxide (■—■) and phenanthrene 9,10-oxide (▲——▲) with the time of incubation are shown.

4-(*p*-nitrobenzylpyridine derivatives that are coloured in alkaline solution. Molar extinction coefficients for the compounds formed have not yet been determined, and it is possible that the observed differences in rates of reaction may be due to differences in these coefficients. Work on some other 4-(*p*-nitrobenzyl)pyridine derivatives of aromatic hydrocarbons, however, has shown that a change in the alkyl moiety from benzyl to 7-methylbenz[a]anthracenyl gives rise to a change in molar extinction of less than 1 per cent.[25] If, therefore, it can be assumed that there are only small differences in the extinction coefficients among the alkylated 4-(*p*-nitrobenzyl)pyridine derivatives studied here, then the differences in the observed rates of alkylation indicate that the presence of methyl substituents near to the "K-region" increases the reactivities of

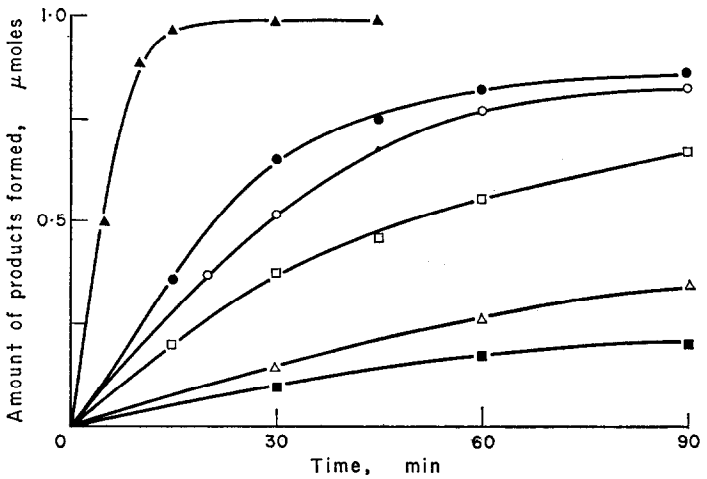


FIG. 4. Rates of formation of phenols and dihydrodiols from epoxides by rat-liver microsomal suspensions. Epoxides (1 μmole) in acetone (0·5 ml) were incubated with microsomal fractions (prepared from 2 g of liver) suspended in pH 7·4 phosphate buffer (10 ml). The mixtures were extracted with ethyl acetate (7 ml) and the amounts of the phenols present estimated fluorimetrically as described. The samples were then acidified with conc. HCl (0·1 ml) and amounts of the phenols present again measured. The concentrations of phenols and dihydrodiols in the solutions were calculated. The increases in the concentrations of these products derived from phenanthrene 9,10-oxide (▲——▲), benz[a]anthracene 5,6-oxide (●——●), 7,12-dimethylbenz[a]anthracene 5,6-oxide (○——○), 7-methylbenz[a]anthracene 5,6-oxide (□——□), 3-methylcholanthrene 11,12-oxide (△——△) and dibenz[a,h]anthracene 5,6-oxide (■——■) with the time of incubation are shown.

the epoxides. There appears to be no direct relationship between the ability of the epoxides to alkylate 4-(*p*-nitrobenzyl)pyridine and the carcinogenic activities of their parent hydrocarbons. Previous work[6] has shown that in the alkylation of 4-(*p*-nitrobenzyl)pyridine, phenanthrene 9,10-oxide and dibenz[a,h]anthracene 5,6-oxide are more reactive than methyl methanesulphonate, a compound that is known to alkylate DNA *in vivo*.[26]

The rates at which a series of "K-region" epoxides are converted by rat-liver microsomal fractions into mixtures of phenols and dihydrodiols are shown in Fig. 4. The data shown in this figure therefore give an indication of the speed at which each epoxide is inactivated through a combination of the two pathways. In general, epoxides, such as phenanthrene 9,10-oxide, which have low molecular weights are

inactivated more rapidly than those, such as dibenz[a,h]anthracene 5,6-oxide, with higher molecular weights. The rates at which the epoxides rearrange in aqueous ethanol at pH 7·4 to phenols and react with 4-(*p*-nitrobenzyl)pyridine do not appear to be related to the rates at which they are converted in microsomal systems into mixtures of phenols and dihydrodiols. The rearrangements of the epoxides to phenols occur to similar extents both in aqueous ethanol and in microsomal fractions. In the microsomal system, the amount of phenol formed from epoxides such as phenanthrene 9,10-oxide and benz[a]anthracene 5,6-oxide accounts for only a small

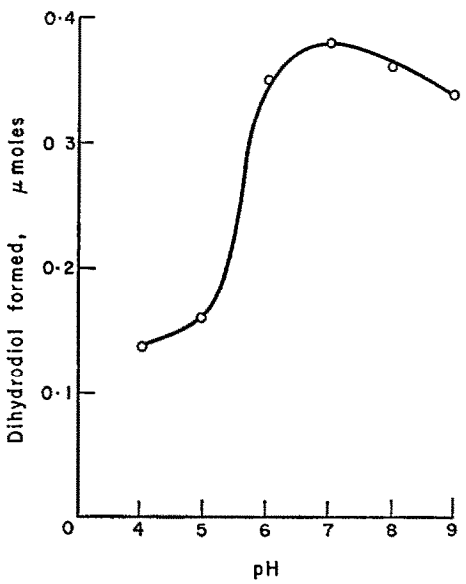


FIG. 5. The effect of pH on the formation of 5,6-dihydro-5,6-dihydroxybenz[a]anthracene from benz[a]-anthracene 5,6-oxide. The epoxide (1 μmole) in acetone (0·5 ml) was added to a number of buffer solutions of different pH (10 ml) containing the microsomal fraction derived from 2 g of liver and incubated at 37° for 15 min. The solutions were extracted with ethyl acetate (7 ml), samples (1 ml) of the extracts diluted with ethanol (2 ml) and the phenol, 5-hydroxybenz[a]anthracene, estimated by its fluorescence intensity. The sample was then acidified with conc. HCl (0·1 ml) and the amount of 5-hydroxybenz[a]anthracene present again measured. The concentration of 5,6-dihydro-5,6-dihydroxy-benz[a]anthracene was calculated from the fluorescence intensities as described.

percentage of the amount of epoxide originally present, whereas with 7,12-dimethyl-benz[a]anthracene 5,6-oxide over 50 per cent of the epoxide added is converted into phenol.

As shown in Fig. 5 the "epoxide hydrase" of rat-liver microsomal fractions converts benz[a]anthracene 5,6-oxide into *trans*-5,6-dihydro-5,6-dihydroxybenz[a]anthracene most efficiently at about pH 7 whereas a partially purified epoxide hydrase preparation from guinea pig liver converts styrene oxide into styrene glycol optimally at pH 9.[27] This difference may be due to species variation in the properties of the enzyme, or possibly to the presence of two or more "epoxide hydrases" with different substrate specificities.

The results now obtained show that although there are wide variations among a series of "K-region" epoxides in the rates at which they rearrange to phenols, alkylate

4-(*p*-nitrobenzyl)pyridine or are converted enzymically into dihydrodiols, none of these factors alone can be directly correlated with the carcinogenicity of the parent hydrocarbons. If the biological activities of the polycyclic hydrocarbons are mediated through epoxides, then the maintenance of effective levels of epoxide within the cell is probably important. Similarly, if reactions with cellular macromolecules are involved then the extent of these would presumably depend upon the concentration of epoxide which would, in turn, be dependent upon the rates, as yet unmeasured, at which the epoxides are formed from the hydrocarbons by the microsomal mixed-function oxidase as well as upon the rates at which the epoxides are removed by the many competing pathways shown in Scheme I. The reactivities of the epoxides may also, in part, determine the extent to which they react with the nucleic acids and proteins of the cell. Thus it is possible for an epoxide of low reactivity which is long-lived to be as potent in alkylating macromolecules as an epoxide which is short-lived but of high reactivity.

It has been suggested that the "K-region" of the hydrocarbon is essential for its carcinogenicity[28] but it is likely that "K-region" epoxides are not the only epoxides formed, since epoxidation of bonds other than those of the "K-region", is almost certainly involved in the metabolism of the hydrocarbons. Some non-"K-region" epoxides have now been synthesized[29–31] and a study of their properties and biological activities may provide more information relevant to polycyclic hydrocarbon carcinogenesis.

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