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Metabolism of Chrysene and Phenanthrene to Bay-Region Diol Epoxides by Rat Liver Enzymes

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SUMMARY

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Liver microsomal metabolism of the weak carcinogen chrysene and the noncarcinogen phenanthrene are compared. In general, chrysene is a rather poor substrate whereas phenanthrene is a relatively good substrate for the cytochrome P-450-dependent monooxygenase system. With microsomes from 3-methylcholanthrene-treated rats, phenanthrene was metabolized at a rate of 7.6 nmoles/min/nmole of cytochrome P-450 as compared with a rate of only 1.5 with chrysene as substrate. Dihydrodiols were major metabolites for both substrates, accounting for 92%–96% of the metabolism of phenanthrene and 65%–76% of the metabolism of chrysene with microsomes from control and treated rats. The K-region 9,10-dihydrodiol is the major metabolite of phenanthrene, whereas the benzo-ring 1,2- and 3,4-dihydrodiols dominate the metabolism of chrysene from which very little K-region dihydrodiol was formed. For both hydrocarbons, microsomes from 3-methylcholanthrene-treated rats produced the (−)-[1R,2R]-dihydrodiol, which has a bay-region double bond, with ≥80% enantiomeric purity. With microsomes from phenobarbital-treated rats, the chrysene 1,2-dihydrodiol was only 10% enantiomerically pure. The (−)-[3R,4R]-dihydrodiols were the major enantiomers of phenanthrene and chrysene, whereas the (−)-[9S, 10S]-dihydriol predominated at the K-region of phenanthrene. Metabolism of the metabolically formed (−)-[1R,2R]-dihydrodiols of phenanthrene and chrysene by microsomes from 3-methylcholanthrene-treated rats resulted in the predominant formation in each case of a bay-region 1,2-diol-3,4-epoxide in which the benzylic hydroxyl group and oxirane oxygen are trans to each other (isomer-2). The respective 1,2-dihydrodiols were metabolized at the same rate as was chrysene. Thus, as had previously been observed for the metabolism of benzo[*a*]pyrene and benzo[*a*]anthracene, liver microsomal enzymes display high stereoselectivity in their formation of predominantly one of four possible stereoisomers of their respective bay-region diol epoxides. These diol epoxides from the four hydrocarbons are superimposable when their bay-regions are aligned.

INTRODUCTION

Bay-region diol epoxides on benzo-rings of polycyclic aromatic hydrocarbons are now well established as principal ultimate carcinogenic metabolites of the polycyclic aromatic hydrocarbon class of carcinogens (1). Results of biological studies on several different hydrocarbons have shown the predictions of the bay-region theory (2–4) to be essentially correct. One of the factors which the bay-region theory has made no attempt to take into account is the extent to which a particular hydrocarbon is metabolized to an ultimate carcinogenic bay-region diol epoxide. Thus, on the basis of the biological activity of its bay-region diol epoxides, an individual hydrocarbon could be more or less carcinogenic than expected, depending upon the extent to which these diol epoxides were formed from the parent hydrocarbon.

Three metabolic steps are presently thought to determine the extent to which a polycyclic aromatic hydrocarbon is converted into its bay-region diol epoxides: (a)

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the extent to which the cytochrome P-450 system forms the requisite non-bay-region arene oxide on an angular benzo-ring of the hydrocarbon relative to other sites of oxidation, (b) the effectiveness with which epoxide hydrolase converts such often highly unstable arene oxides into *trans*-dihydrodiols[3] before they isomerize to phenols, and (c) the rate and extent to which these dihydrodiols are converted into biologically active diol epoxides. Although enzymes such as epoxide hydrolase could detoxify diol epoxides, evidence has not been forthcoming to indicate that such is the case. In fact, bay-region and non-bay-region diol epoxides have been shown to be refractory to enzymatic hydration by epoxide hydrolase (5–9).

In previous studies (10, 11), we provided evidence that the first and third of the above metabolic factors were at least in part responsible for the fact that benzo[*a*]anthracene is a much weaker carcinogen than is dibenzo[*a*,*h*]anthracene (12), although there is reason to believe that the 3,4-diol-1,2-expoxides of benzo[*a*]anthracene could have higher biological activity than those of dibenzo[*a*,*h*]anthracene according to predicted chemical reactivity (2–4). Whereas only 1%–3% of the total metabolism of benzo[*a*]anthracene can be accounted for as the 3,4-dihydrodiol (13), as much as 24%–28% of the total metabolites of dibenzo[*a*,*h*]anthracene formed by liver enzymes consists of its 3,4-dihydrodiol (10). The present study examines the metabolism of the weak carcinogen chrysene and compares it with that of the noncarcinogen phenanthrene in order to establish how the above three factors control the formation of bay-region diol epoxides from these hydrocarbons. Earlier studies without the aid of authentic reference standards or modern analytic techniques had indicated that the 1,2-dihydrodiols of chrysene and phenanthrene were major and minor (but significant) metabolites of these hydrocarbons, respectively (14). A more recent, detailed study has confirmed the earlier results on the metabolism of phenanthrene (15), and mouse skin maintained in short-term organ culture has been shown to form substantial amounts of the 1,2-dihydrodiol of chrysene (16). In each case, the 1,2-dihydrodiol has a bay-region double bond and is a potential precursor for the formation of bay-region diol epoxides.

MATERIALS AND METHODS

Hydrocarbon substrates. The general procedure used to obtain tritiated chrysene and phenanthrene consisted of hydrogenolysis of 6-bromochrysene and 3-bromophenanthrene with tritium gas at New England Nuclear Corporation, Boston, Mass. 6-Bromochrysene was obtained by bromination of chrysene in chloroform (cf. ref. 17), and 3-bromophenanthrene was the generous gift of Dr. E. May at the National Institutes of Health. Reductions were carried out in the presence of 5% palladium on carbon and an excess of triethylamine in tetrahydrofuran under 1 atmos of hydrogen. Preliminary experiments established that reduction of the K-regions of either hydrocarbon did not occur to any significant extent under the conditions used. Both tritiated hydrocarbons were purified by HPLC on a DuPont Zorbax ODS column (0.62 × 25 cm) eluted with a 60%–100% methanol in water gradient at a rate of change of 1%/min and a flow rate of 1.2 ml/min. After dilution to the desired specific activities, the 3-[^{3}H]phenanthrene (53.4 μCi/μmole) and 6-[^{3}H]chrysene (61.9 μCi/μmole) were >98% radiochemically pure with <0.7% and <0.5% of the radioactivity emerging from the ODS column prior to each hydrocarbon, respectively.

Synthetic metabolite standards. Dihydrodiols (Fig. 1) of phenanthrene (18) and chrysene (19) were prepared as previously described, as was chrysene 5,6-quinone (17). Diastereomeric, bay-region diol epoxides of phenanthrene (Fig. 2) and chrysene in which the benzylic hydroxyl group is either *cis* (isomer-*1* series) or *trans* (isomer-*2* series) to the epoxide oxygen were prepared as described (20). The tetraols which result upon *cis* and *trans* hydrolysis of these diol epoxides by attack of water at the benzylic carbon of the epoxide were assigned relative stereochemistry based on the nuclear magnetic resonance spectra of their tetraacetates (20). Both 3- and 9-hydroxyphenanthrene were the generous gifts of Dr. E. May at the National Institutes of Health. MTPA was obtained from Aldrich Chemical Company, Madison, Wisc.

Incubations. Liver microsomes were prepared from control and treated, immature (50–60 g) male rats of the Long-Evans strain and were assayed as previously described (10): specific contents of cytochromes P-450 (nanomoles per milligram of protein) were 0.77 for control, 2.00 for phenobarbital-treated, and 1.56 for 3-methylcholanthrene-treated rats. Components of the highly purified and reconstituted system with cytochrome P-450c (21), NADPH-cytochrome *c* reductase (22), and epoxide hydrolase (23) were as described. Cytochrome P-450c is the predominant isozyme of the cytochromes P-450 induced in liver by treatment of rats with 3-methylcholanthrene.

Incubations were carried out for 10 min at pH 7.4 and 37° as described (10) except where noted otherwise. Microsomal protein concentrations ranged from 0.063 to 0.25 mg of protein per milliliter for incubations with phenanthrene, from 0.50 to 1.50 mg of protein per milliliter for incubations with chrysene, from 0.125 to 1.0 mg of protein per milliliter for incubations with phenanthrene 1,2-dihydrodiol, and from 0.50 to 1.00 mg of protein per milliliter for incubations with chrysene 1,2-dihydrodiol. Substrates (160 nmoles of phenanthrene, 100 nmoles of chrysene, 100 nmoles of phenanthrene 1,2-dihydrodiol, or 50 nmoles of chrysene 1,2-dihydrodiol) were added in acetone such that the final concentration of the solvent in the incubations was 5%; final incubation volumes were adjusted to 2.0 ml (10), except in the case of incubations with chrysene 1,2-dihydrodiol where the final volume was adjusted to 1.0 ml. Substrate and metabolites were extracted as described (10) with the excep-

[3] The abbreviations used are: phenanthrene 1,2-dihydrodiol, *trans*-1,2-dihydroxy-1,2-dihydrophenanthrene; other dihydrodiols of phenanthrene and chrysene are similarly abbreviated: phenanthrene 1,2-diol-3,4-epoxide-*1*, (+)-1β,2α-dihydroxy-3β,4β-epoxy-1,2,3,4-tetrahydrophenanthrene; phenanthrene 1,2-diol-3,4-epoxide-*2*, (±)-1β,2α-dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrophenanthrene; diol epoxides of chrysene are similarly abbreviated; MTPA, (−)-α-methoxy-α-trifluoromethylphenylacetic acid. HPLC, high-pressure liquid chromatography. Unless otherwise designated, the term cytochrome P-450 refers to all forms of this class of enzymes.

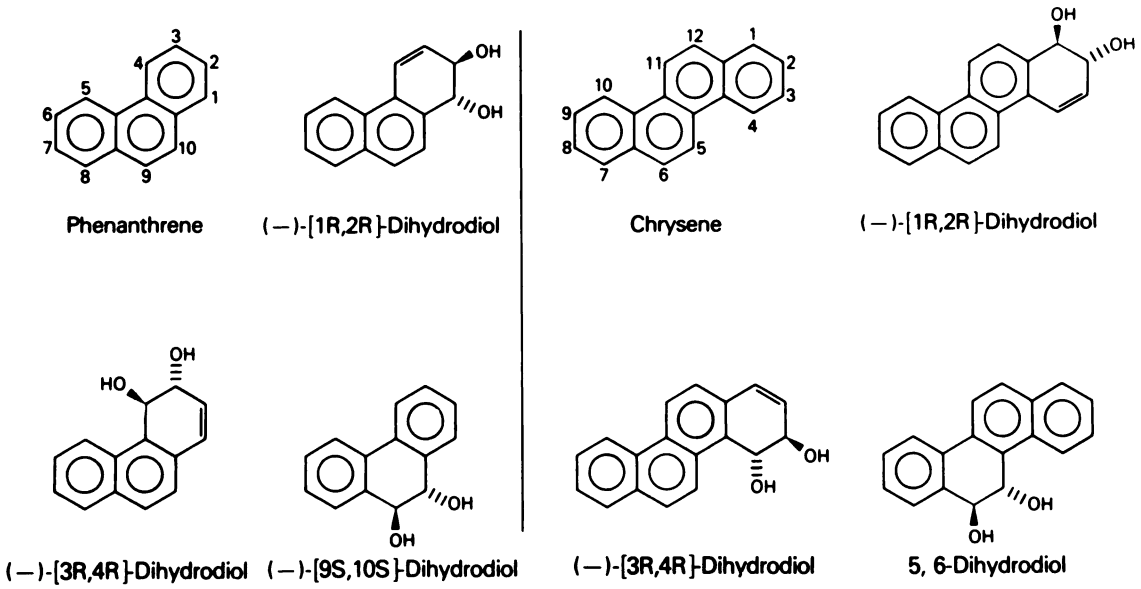

FIG. 1. *Structures of the metabolically possible trans dihydrodiols from phenanthrene and chrysene*

Where absolute stereochemistry is indicated, the enantiomer shown is the major enantiomer which is formed metabolically. Only relative stereochemistry is meant to be implied by the structure of the chrysene 5,6-dihydrodiol. Bay-regions consist of the sterically hindered areas between positions 9 and 10 of phenanthrene and positions 4 and 5 (10 and 11) of chrysene.

tion that incubation extracts in the experiments with phenanthrene were concentrated to a small volume rather than to dryness to prevent loss of phenanthrene, which is quite volatile. Incubations with the reconstituted system contained 0.05 to 0.10 nmole of cytochrome P-450c and either zero or 82 units of epoxide hydrolase and were run at pH 7.0 in 100 mM phosphate buffer in a final volume of 2.0 ml (10). All other components of the reconstituted system were as previously described (10). Controls consisted of zero time incubations.

Analysis of hydrocarbon metabolites. Concentrated extracts of incubated samples were dissolved in small volumes (<0.1 ml) of methanol, appropriate synthetic reference standards were added, and the samples were separated with a Spectra Physics high-pressure liquid chromatograph (Model 3500B). Conditions for individual separations are given in the figure legends. More efficient separations of the dihydrodiols were required than those previously reported (24). Use of a DuPont Zorbax CN column greatly improved the separation of the phenanthrene 1,2- and 9,10-dihydrodiols as compared with ODS columns, whereas a convex gradient (Spectra Physics, ramp 6) improved the separation of the chrysene 3,4- and 5,6-dihydrodiols on a DuPont Zorbax ODS column. Separation of tetraols was similar to that described (11, 20). Eluent from the chromatography columns was collected directly into scintillation vials (typically 0.36–0.60 ml/vial), and radioactivity was determined by scintillation spectrometry in Aquasol.

Once it had been determined that phenols were relatively minor microsomal metabolites from both hydrocarbons (4%–17% of total metabolites), attempts to identify individual phenols were not pursued. Treatment of the chrysene 1,2- and 3,4-dihydrodiols with 6 N HCl in acetone gave in each case a mixture of two phenols which could be separated by the gradient system used to analyze the metabolites. The early-eluting as well as late-eluting members of each pair of phenols were co-chromatographic. On the basis of previous studies (24), the early-eluting phenols probably are 2- and 3-hydroxychrysene, and the late-eluting phenols probably are 1- and 4-hydroxychrysene. The 5- and/or 6-hydroxychrysenes, which arise on dehydration of chrysene 5,6-dihydrodiol, chromatographed as a single peak between the above two peaks (cf. Fig. 3C). Because of the very small differences in retention time between the phenols, a single phenol fraction was tabulated for studies on the metabolism of chrysene. For metabolism of phenanthrene, two metabolically formed phenol fractions, phenol-1 and phenol-2, were tabulated. These minor metabolite fractions were presumed to be phenanthrols since they were

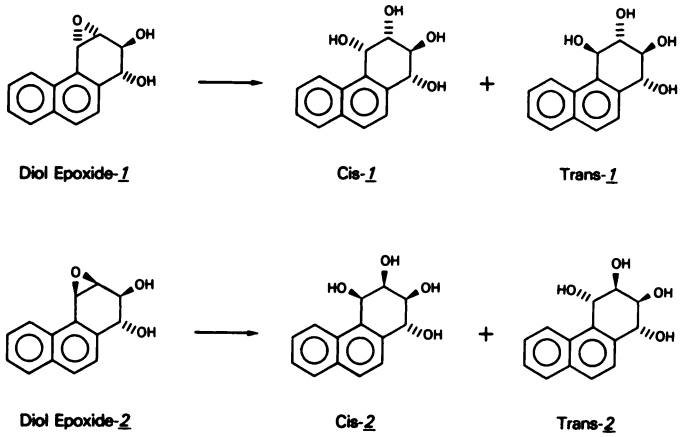

FIG. 2. *Bay-region diol epoxides of phenanthrene and the tetraols which result by cis and trans attack of water at the benzylic epoxide carbon atom*

Absolute stereochemistry is as shown for the diol epoxide-2 metabolite and its tetraols. A completely parallel set of structures is discussed for chrysene in the text.

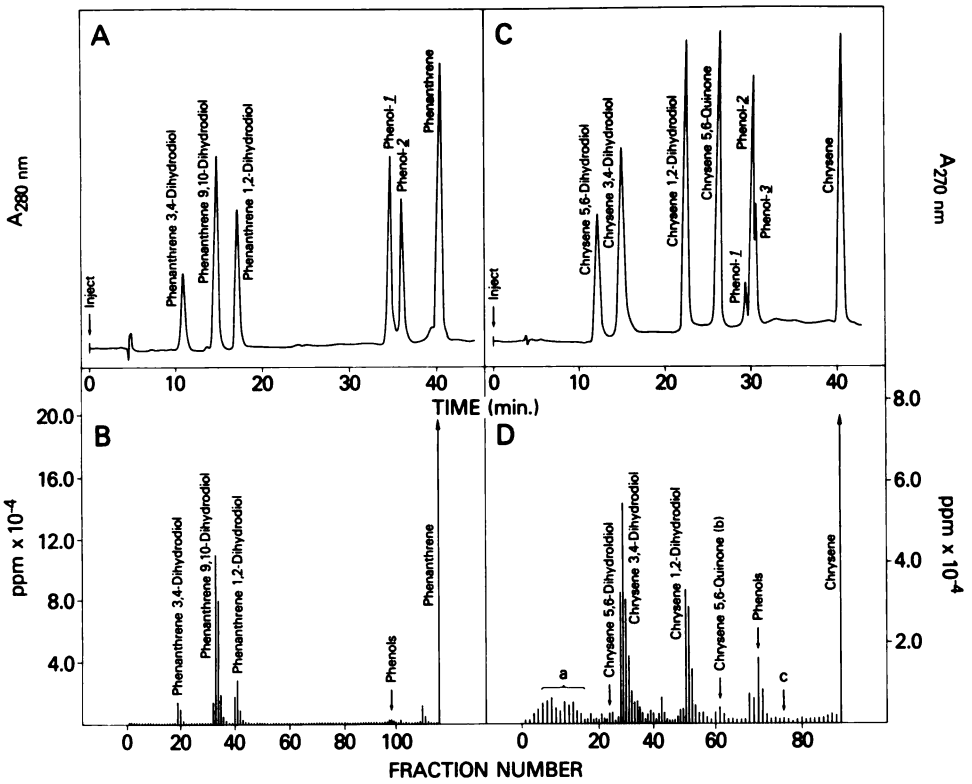

FIG. 3. *Chromatographic separation of selected synthetic standards and observed metabolites from phenanthrene and chrysene produced by liver microsomes from 3-methylcholanthrene-treated rats*

Protein concentrations were 1.00 mg/ml and 0.125 mg/ml, respectively. Synthetic standards (A) and metabolites (B) of phenanthrene were separated on a DuPont Zorbax CN column (0.62 × 25 cm) eluted with a linear gradient of 45%–65% methanol in water over a period of 30 min after an initial delay of 1 min. Synthetic standards (C) and metabolites (D) of chrysene were separated on a DuPont Zorbax ODS column (0.62 × 25 cm) eluted with a nonlinear gradient of 60%–100% menthanol in water over a period of 40 min after an initial 5-min delay. A convex gradient (Spectra Physics 3500B, ramp position 6) was found to improve the separation of the 3,4- and 5,6-dihydrodiols. Regions of elution of minor unknown metabolites *a*, *b*, and *c* are indicated in the radiochemical trace. The flow rate for each separation was 1.2 ml/min.

co-chromatographic with 3- and 9-hydroxyphenanthrene, respectively.

Isolation of dihydrodiols. In order to isolate sufficient amounts of the dihydrodiols from chrysene to determine their enantiomeric composition, the standard 2.0-ml incubations containing either 1.5 mg/ml of liver microsomal protein from control or phenobarbital-treated rats and 0.5 mg/ml of protein from 3-methylcholanthrene-treated rats were performed. Extracts from three or four flasks were pooled and separated under the conditions used to analyze for metabolites. Conversions of substrate were 10%, 16%, and 31%, respectively, for the three protein preparations used. A large-scale incubation was required to obtain sufficient metabolically formed, radioactive chrysene 1,2-dihydrodiol to study its further metabolism: five flasks each containing 100 mg of liver microsomal protein from 3-methylcholanthrene-treated rats in a final volume of 50 ml were incubated for 15 min, and their concentrated extracts were pooled. Two dihydrodiol fractions were isolated on a DuPont Zorbax SIL column (0.62 × 25 cm) eluted at 7.0 ml/min with ethanol-dioxane-cyclohexane (1.5:15:83.5): $k' = 8.0$ for the mixture of 1,2- and 5,6-dihydrodiols and $k' = 12.0$ for the 3,4-dihydrodiol. The 1,2-dihydrodiol was further purified on the ODS column used to analyze metabolites in order to remove small amounts of the K-region 5,6-dihydrodiol. The 1,2-dihydrodiol used in subsequent metabolism studies had ~6% more polar radiochemical impurities which arise during work-up after chromatography and thus cannot be avoided.

Dihydrodiols from phenanthrene, both for determination of their enantiomeric purity and for secondary oxidative metabolism studies on the 1,2-dihydrodiol, were obtained from five 50-ml incubations, each of which contained 50 mg of liver microsomal protein from 3-methylcholanthrene-treated rats. The flasks were incubated for 15 min to achieve 45% conversion of the substrate. The three phenanthrene dihydrodiols were isolated with a DuPont Zorbax SIL column under the above conditions used for the chrysene dihydrodiols. At a flow rate of 5.0 ml/min, the phenanthrene 9,10-, 1,2-, and 3,4-dihydrodiols had values of k' of 5, 8, and 13, respectively. The dihydrodiols were 94%–97% radiochemically pure on the ODS column.

Enantiomeric purity of dihydrodiols. Enantiomeric composition of the dihydrodiols from both hydrocarbons was determined by a previously described method (25). Each of the radioactive, metabolically formed dihydrodiols was diluted more than 10-fold with racemic dihydrodiol and was converted into a diastereomeric mixture of diesters with the acid chloride of MTPA. The diastereomers were separated chromatographically and their

TABLE 1

Effect of inducing agents on the metabolism of phenanthrene by rat liver microsomes

Numbers in the upper rows represent the percentage of each metabolite as compared with the total discrete metabolite peaks, whereas the numbers in the lower rows represent nanomoles of metabolites formed per nanomole of cytochrome P-450 per minute. Protein concentrations were 0.125 mg/ml of incubation volume.

Treatment	Phenanthrene 1,2-dihydrodiol	Phenanthrene 3,4-dihydrodiol	Phenanthrene 9,10-dihydrodiol	Phenol-1	Phenol-2	Total[a] conversion	Recovery[b] (%)
Control	13.8	1.7	76.6	1.7	6.2	5.1	
	0.52	0.065	2.88	0.063	0.24	4.2	89
Phenobarbital	10.6	1.2	83.7	0.9	3.6	12.9	
	0.41	0.047	3.23	0.034	0.14	4.1	93
3-Methylcholanthrene	17.9	9.3	67.1	2.1	1.6	18.6	
	1.22	0.62	4.65	0.14	0.11	7.6	88

[a] Total metabolism denotes the percentage conversion of substrate, i.e., total radioactivity above blank which emerges from the column before the substrate. Total metabolism does not take into account traces of unextracted metabolites, highly polar and nonextracted metabolites, or covalently bound metabolites which remain in the aqueous phase after extraction, since the total of these amounted to less than 8% of the total metabolism for each of the three incubations.

[b] Recovery represents the percentage of the total radioactivity due to metabolism emerging from the column before the substrate in discrete metabolite peaks as compared with total radioactivity due to metabolism.

radioactivity was determined. Conditions of the separations are given in Tables 3 and 4. Because of the low amount of metabolically formed chrysene 5,6-dihydrodiol, no attempt was made to establish its enantiomeric composition.

Analysis of metabolites of phenanthrene 1,2-dihydrodiol and chrysene 1,2-dihydrodiol. The bay-region 1,2-diol-3,4-epoxides of chrysene and phenanthrene are sufficiently stable that they largely survive the conditions of incubation and extraction. When the synthetic diol epoxides of phenanthrene and chrysene were analyzed on the Zorbax ODS column, they were found to be unstable during chromagraphy. Hence, to obtain accurate quantitation of diol epoxide metabolites, the dried extracts from incubated samples were hydrolyzed to tetraols with dilute perchloric acid in 10% dioxane-water and 50% dioxane-water (final observed pH ~ 2) for the phenanthrene and chrysene metabolites, repspectively, for 18 hr. The higher dioxane content for acid-treatment of the chrysene metabolites was used to ensure good solubility. Some decomposition of the dihydrodiols (~20%) occurred during the acid treatment. The resultant tetraols were analyzed by HPLC as described in the legend to Fig. 4.

RESULTS

Effect of inducers on the liver microsomal metabolism of chrysene and phenanthrene. Chromatographic mobilities of the synthetic standards as well as radiochemical histograms of the metabolites produced from both hydrocarbons by liver microsomes from 3-methylcholanthrene-treated rats are shown in Fig. 3. Quantitative comparison of the metabolism of the two hydrocarbons by liver enzymes prepared from the variously treated rats is given in Tables 1 and 2. For both hydrocarbons, metabolism was linear with protein at all concentrations studied except for the metabolism of chrysene by microsomes from 3-methylcholanthrene-treated rats, where linearity was lost above 1.0 mg of protein per milliliter. The most striking difference between the two hydrocar-

TABLE 2

Metabolites of chrysene formed by rat liver microsomes and by a highly purified and reconstituted monoxygenase system with and without epoxide hydrolase

Definitions are as described in Table 1. Microsomal incubations contained 1.0 mg of protein.

Protein	Chrysene 1,2-dihydrodiol	Chrysene 3,4-dihydrodiol	Chrysene 5,6-dihydrodiol	Phenols	Unknown			Total[a] conversion	Recovery (%)
					a	*b*	*c*		
Microsomes (control)	33.5	34.5	2.0	17.2	2.5	6.7	3.6	3.0	95
	0.12	0.12	0.007	0.063	0.009	0.025	0.013	0.39	
Microsomes (phenobarbital)	28.5	30.4	5.7	14.9	4.1	10.7	5.7	4.2	75
	0.046	0.049	0.009	0.024	0.007	0.017	0.009	0.21	
Microsomes (3-methylcholanthrene)	26.1	46.8	3.5	12.9	6.9	2.6	1.0	24.1	87
	0.35	0.63	0.047	0.17	0.092	0.035	0.016	1.54	
Cytochrome P-450c (0.05 nmole)	6.7	3.0	1.4	68.1	5.1	3.4	12.3	4.1	79
	0.43	0.19	0.09	4.39	0.33	0.22	0.79	8.1	
Cytochrome P-450c (0.05 nmole) + epoxide hydrolase (82 units)	26.8	59.8	4.7	4.0	4.7	—	—	7.8	98
	4.1	9.2	0.72	0.61	0.72	—	—	15.6	

[a] Total metabolism does not take into account nonextracted metabolites (cf. Table 1, footnote *a*), as these amounted to only 7%, 10%, and 14% of the total metabolites, respectively, for the microsomal incubations. For the reconstituted system in the absence of epoxide hydrolase, the nonextracted metabolites were 19% and were reduced to 11% when epoxide hydrolase was added.

bons is that liver microsomes are 5- to 20-fold less active with chrysene as the substrate as compared with phenanthrene. The low rate of metabolism of chrysene (0.2–1.5 nmoles of product/nmole of cytochrome P-450/min) is similar to that of another very insoluble hydrocarbon, dibenzo[*a,h*]anthracene (0.6–1.5 nmoles of product/nmole of cytochrome P-450/min) (cf. ref. 10), whereas phenanthrene (4.1–7.6 nmoles of product/nmole of cytochrome P-450/min) is one of the best substrates which we have yet studied. In parallel with studies on three other hydrocarbons, the rate of liver microsomal metabolism of chrysene is somewhat decreased (approximately 50%) by treatment of the animals with phenobarbital, whereas treatment with 3-methylcholanthrene results in a marked (approximately 4-fold) increase in the rate of its metabolism (cf. ref. 10) when the data are expressed per nanomole of cytochrome P-450. The rate of metabolism of phenanthrene, on the other hand, starts out quite high with microsomes from control animals, is little affected by phenobarbital treatment, and is less than doubled by 3-methylcholanthrene-treatment.

The two hydrocarbons vary greatly in the region at which they are attacked by cytochrome P-450. Although 67%–84% of the total metabolism of phenanthrene is represented by its K-region 9,10-dihydrodiol, only 2%–6% of the K-region, 5,6-dihydrodiol is formed from chrysene whose major metabolites are the benzo-ring 1,2- and 3,4-dihydrodiols (26%–34% and 30%–47% of the total metabolites, respectively). Treatment of rats with 3-methylcholanthrene had the specific effect of increasing the percentage of the bay-region 3,4-dihydrodiols as metabolites from both hydrocarbons. The 1,2-dihydrodiol with a bay-region 3,4-double bond was formed about twice as extensively from chrysene (26%–34% of the total metabolites) as it was from phenanthrene (11%–18%). Phenols constituted 4%–8% of the metabolites of phenanthrene and 13%–17% of the metabolites of chrysene. Several unknown metabolite fractions (Fig. 3A–C) constituted a total of ~10%–20% of the total metabolites from chrysene. Although unknown *b* was co-chromatographic with chrysene 5,6-quinone, no attempt was made to characterize further this minor metabolite. Metabolism of chrysene by a highly purified system reconstituted with cytochrome P-450c in the presence or absence of epoxide hydrolase indicated that the dihydrodiols had arene oxide precursors but gave no particular insight into the origin of the unknown metabolites (Table 2). The addition of epoxide hydrolase had an apparent stimulatory effect which was most likely due to removal of the inhibitory effects of phenols (10, 13) or quinones (26) found in the absence of epoxide hydrolase. In summary, the metabolism of phenanthrene closely resembles that of benzo[*a*]anthracene (13) in that phenols are minor metabolites and the K-region diols are major products, whereas the metabolism of chrysene is quite similar to that of dibenzo[*a,h*]anthracene (10) in that benzo-ring dihydrodiols and phenols constitute the majority of the metabolites. With both of the latter two hydrocarbons, unidentified secondary oxidative metabolites and/or autoxidation products constitute a significant proportion (~10%–30%) of the total metabolites.

Enantiomeric purity of dihydrodiols from chrysene and phenanthrene. Enantiomeric purity of a dihydrodiol is the result of the combined specificity of the cytochrome P-450 system and epoxide hydrolase. Although previous studies on benzo[*a*]pyrene (27) and benzo[*a*]anthracene (25) indicated that treatment of rats with inducing agents had relatively little effect on the enantiomeric purity of the dihydrodiols, the results shown in Table 3 indicate that the enantiomeric purity of the 1,2-dihydrodiol of chrysene is rather sensitive to prior treatment of the rats with inducing agents. Its enantiomeric purity ranged from a low of 10% after phenobarbital-treatment to a high of 80% after 3-methylcholanthrene-treatment. This is in contrast to chrysene 3,4-dihydrodiol, the enantiomeric purity of which ranged from 90% to 97%. Insufficient 5,6-dihydrodiol was obtained to permit a determination.

With liver microsomes from 3-methylcholanthrene treated rats, the 1,2- and 3,4-dihydrodiols formed from phenanthrene were 93% and 97% enantiomerically pure (Table 4). This is in contrast to the low enantiomeric

TABLE 3

Effect of inducing agents on the enantiomeric composition of the 1,2- and 3,4-dihydrodiols formed from chrysene by liver microsomes

Dihydrodiol enantiomers were separated as their diastereomeric bis-esters with (−)-α-methoxy-α-trifluoromethylphenylacetic acid. The data represent the average of two separate experiments with different microsomal preparations. Enantiomer compositions varied only from 1% to 3% in the two experiments. The method used to assign absolute stereochemistry is discribed under Results. Baseline separation was achieved for the two diastereomeric dihydrodiol diester peaks (retention times in parentheses). Percentages refer to the amount of radioactivity in each peak. Chromatographic conditions consisted of isocratic elution of a DuPont Zorbax ODS column at 2.0 ml/min with 87% methanol in water. Percentage enantiomeric purity is defined as 100 (moles of major enantiomer minus moles of minor enantiomer) divided by (total moles of both enantiomers).

Treatment	1,2-Dihydrodiol			3,4-Dihydrodiol[a]		
	Enantiomer Composition		Enantiomeric purity (%)	Enantiomer Composition		Enantiomeric purity (%)
	(−)-[1R,2R] (19.5 min)	(+)-[1S,2S] (21.0 min)		(+)-[3S,4S] (12.0 min)	(−)-[3R,4R] (14.0 min)	
Control	76%	24%	52	3%	97%	94
Phenobarbital	55%	45%	10	5%	95%	90
3-Methylcholanthrene	90%	10%	80	1.5%	98.5%	97

[a] The bis-MTPA esters of (−)-*trans*-[3R,4R]- and (+)-*trans*-[3S,4S]-dihydroxy-1,2,3,4-tetrahydrochrysene had retention times of 19.0 min and 18.2 min, respectively, when chromatographed on a DuPont Zorbax ODS column; 60% to 70% linear gradient (1% change/min) of tetrahydrofuran-acetonitrile (30:70) into water at a flow rate of 1.2 ml/min.

TABLE 4

Enantiomeric composition and absolute configuration of the dihydrodiols formed from phenanthrene by liver microsomes from 3-methylcholanthrene-treated rats

Enantiomer composition was determined by separation of bis esters with (−)-α-methoxy-α-trifluoromethylphenylacetic acid as in Table 3. Absolute sterechemistry of the dihydrodiol is given in brackets, and the retention time (minutes) of its bis ester is given in parentheses.

Compound	Enantiomeric purity (%)	Enantiomer composition		Chromatographic system[a]
		(−)-dihydrodiol	(+)-dihydrodiol	
Phenanthrene 1,2-dihydrodiol	93	96.5% [R,R] (12.2 min)	3.5% [S,S] (11.0 min)	A
		(16.0 min)	(18.0 min)	B
Phenanthrene 3,4-dihydrodiol[b]	97	98.4% [R,R] (15.0 min)	1.6% [S,S] (17.0 min)	A
		(19.5 min)	(18.5 min)	C
Phenanthrene 9,10-dihydrodiol	16	58.0% [S,S] (12.5 min)	42.0% [R,R] (11.4 min)	D

[a] A, DuPont Zorbax SIL column (0.62 × 25 cm) eluted with isopropanol-dioxane-hexane (0.2:6.0:93.8) at 3.0 ml/min. B, DuPont Zorbax ODS column (0.62 × 25 cm) eluted with a linear gradient (1%/min) of 80%–99% methanol in water at 2.4 ml/min. C, DuPont Zorbax ODS column (0.62 × 25 cm) eluted with acetonitrile-tetrahydrofuran-water (44:19:37) at 2.0 ml/min. D, Altex ultrasphere SIL column (0.46 × 25 cm) eluted with isopropanol-dioxane-hexane (0.06:0.70:99.24) at 2.0 ml/min.

[b] In chromatographic system C (footnote *a* above), the bis-MTPA esters of (−)-*trans*-[3R,4R]- and (+)-*trans*-[3S,4S]-dihydroxy-1,2,3,4-tetrahydrophenanthrene had retention times of 11.0 min and 10.3 min, respectively. Baseline separation of the two peaks was achieved despite the small differences in their retention times.

purity of the K-region 9,10-dihydrodiol (16%). Since phenanthrene 9,10-oxide has a plane of symmetry and cannot be optically active, the low enantiomeric purity of the 9,10-dihydrodiol is a reflection only of the low selectivity of epoxide hydrolase toward this substrate.

Absolute stereochemistry of the dihydrodiols formed from chrysene. At the outset of the present study, absolute stereochemistry had not been assigned for the three metabolically formed dihydrodiols from chrysene. In experiments to be described separately,[4] we have resolved *trans*-1,2-dihydroxy-1,2,3,4-tetrahydrochrysene by chromatographic separation of its diastereomeric bis esters with (−)-menthoxyacetic acid. The (−)-tetrahydrodiol ($[\alpha]_D$ − 77°, c = 6 mg/ml, tetrahydrofuran) was converted to its bis-*p*-dimethylaminobenzoate. Observation of a positive, long-wavelength Cotton effect in the circular dichroism spectrum of this molecule allowed assignment as (−)-*trans*-[1S,2S]-dihydroxy-1,2,3,4-tetrahydrochrysene (cf. ref. 25). Further synthetic transformation of the (−)-tetrahydrodiol provided (+)-chrysene [1S,2S]-dihydrodiol with $[\alpha]_D$ +109° (c = 5 mg/ml, tetrahydrofuran). Retention time of the bis-MTPA ester of (+)-1,2-dihydrodiol (Table 3) established it as the minor enantiomer produced from chrysene.

Also in experiments to be described separately,[4] racemic chrysene 3,4-dihydrodiol was resolved by chromatographic separation of its bis esters with (−)-MTPA; DuPont Zorbax SIL column (0.62 × 25 cm) eluted with 15% ether in cyclohexane at 2.0 ml/min. After hydrolysis of each diastereomer (k_1' = 2.20, k_2' = 2.53), the resultant dihydrodiols had $[\alpha]_D$ −345° and +313° (c = 0.7 mg/ml, tetrahydrofuran), respectively. The predominant metabolite enantiomer was the (−)-dihydrodiol when analyzed by the conditions described in Table 3. The (−)-3,4-dihydrodiol metabolite was assigned [3R,4R] absolute configuration by a method analogous to that described above for the 1,2-dihydrodiol.

Absolute stereochemistry of the dihydrodiols formed from phenanthrene. Absolute stereochemistry of the phenanthrene 9,10-dihydrodiol enantiomers are known from chemical degradation (28). Chromatography of the bis-MTPA esters of the 9,10-dihydrodiol formed from phenanthrene (Table 4) established the (−)-[9S,10S]-enantiomer [$[\alpha]_D$ −170° (c = 3 mg/ml, tetrahydrofuran)] as the major metabolite. As anticipated, the 9,10-dihydrodiol formed from phenanthrene [58% (−)-enantiomer] has essentially the same enantiomeric purity as the 9,10-dihydrodiol formed by the action of microsomal epoxide hydrolase [63% (−)-enantiomer] on phenanthrene 9,10-oxide (29).

Both chemical degradation and spectral methods had previously allowed assignment of absolute stereochemistry to (−)-*trans*-[1R,2R]-diacetoxy-1,2-dihydrophenanthrene (30). For the present study, racemic phenanthrene 1,2-dihydrodiol was completely resolved as its bis-MTPA esters (Table 4, chromatographic system A). After hydrolysis, the free dihydrodiols had $[\alpha]_D^{23}$ +343° and −312° (c = 2 mg/ml, tetrahydrofuran). Acetylation of the (−)-dihydrodiol provided the (−)-diacetate with $[\alpha]_D^{23}$ −894° (c = 1.5 mg/ml, tetrahydrofuran). Thus both the (−)-1,2-diacetate and the (−)-1,2-dihydrodiol have [1R,2R] absolute stereochemistry, as does the major enantiomer formed from phenanthrene (Table 4).

In order to assign absolute stereochemistry to the (+)- and (−)-enantiomers of phenanthrene 3,4-dihydrodiol, advantage was taken of the fact that absolute stereochemistry had been assigned to (−)-*trans*-[3R,4R]-dihydroxy-1,2,3,4-tetrahydrophenanthrene ($[\alpha]_D$ −25°, c = 33 mg/ml, dioxane) in earlier studies (31).[5] Reduction (45 min) of the radioactive 3,4-dihydrodiol metabolite in tetrahydrofuran with hydrogen (1 atmos) in the presence of platinum provided (−)-*trans*-[3R,4R]-dihydroxy-1,2,3,4-tetrahydrophenanthrene of 97%–98% enantiomeric purity (Table 4, chromatographic system C, footnote *c*). Preparative resolution (Table 4, chromato-

[4] Manuscript in preparation.

[5] D. R. Boyd, R. M. E. Greene, F. D. Neill, M. E. Stubbs, H. Yagi, and D. M. Jerina. Absolute stereochemistry of the 3,4-oxide, *trans*-3,4-dihydrodiol, and *cis*-3,4-dihydrodiol metabolites of phenanthrene, manuscript in preparation.

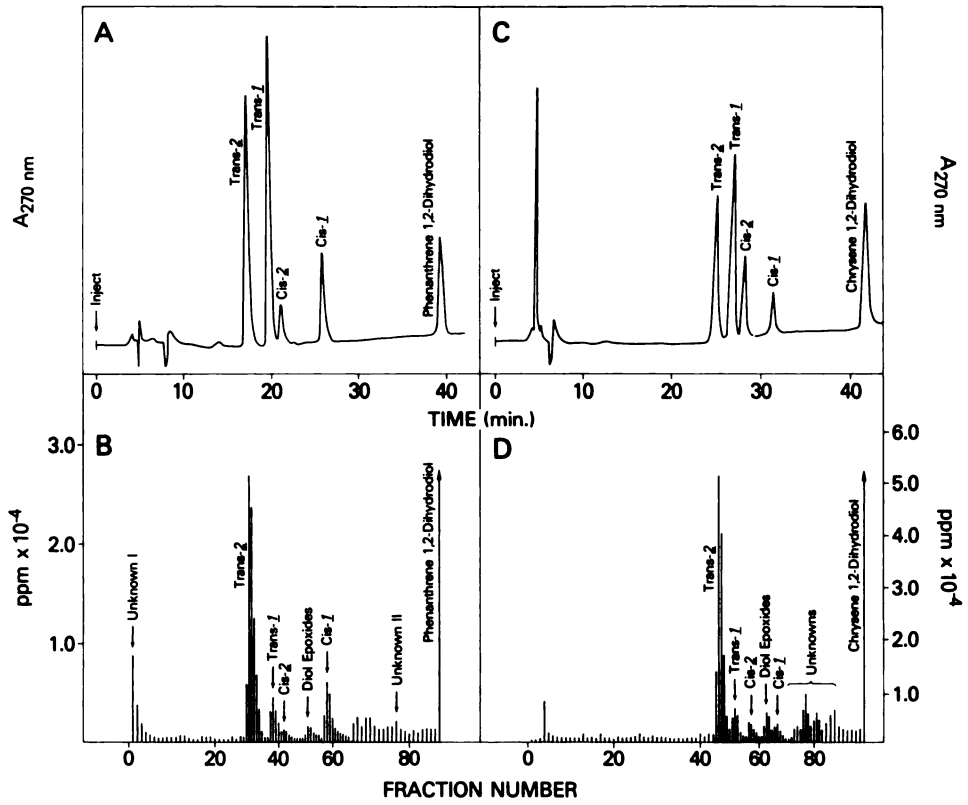

FIG. 4. *Chromatographic separation*

A, Synthetic tetraols (cf. Fig. 2) formed upon hydrolysis of phenanthrene 1,2-diol-3,4-epoxides *1* and *2*; B, acid-treated metabolites of [^{3}H]-phenanthrene [1R,2R]-dihydrodiol [96.5% (−)-enantiomer] formed by liver microsomes from 3-methylcholanthrene-treated rats; C, synthetic tetraols formed upon hydrolysis of the chrysene 1,2-diol-3,4-epoxides *1* and *2*; D, acid-treated metabolites of [^{3}H]chrysene [1R,2R]-dihydrodiol [90% (−)-enantiomer] formed by liver microsomes from 3-methylcholanthrene-treated rats. The radioactive dihydrodiols were obtained from preparative incubation of the parent hydrocarbons with liver microsomes from 3-methylcholanthrene-treated rats (cf. Tables 3 and 4). Phenanthrene derivatives were separated on a DuPont Zorbax ODS column (0.62 × 25 cm) eluted with a linear gradient of 30%–80% methanol in water at a rate of gradient change of 1%/min and a flow rate of 1.2 ml/min, whereas a linear gradient of 60%–90% methanol in water was used for the chrysene derivatives.

graphic system A) of the (+)- and (−)-enantiomers of the 3,4-dihydrodiol ([α]$_D$ +464° and −443° (c = 0.75 mg/ml, tetrahydrofuran)) as their bis-MTPA esters established that the predominant enantiomer of the metabolite was the (−)-isomer.

Metabolism of chrysene and phenanthrene 1,2-dihydrodiols. Since the metabolically formed, 1,2-dihydrodiols of both hydrocarbons consisted of 90%–97% of the (−)-[1R,2R]-enantiomers when formed by liver microsomes from 3-methylcholanthrene-treated rats, they may be considered as essentially homogeneous substrates. Chromatographic mobilities of the tetraols formed from the diastereomeric phenanthrene and chrysene 1,2-diol-3,4-epoxides upon *cis* and *trans* hydrolysis at C-4 are shown in Fig. 4A and 4C, respectively. The histograms of the radiochemical metabolites formed from [^{3}H]phenanthrene-1,2-dihydrodiol and [^{3}H]chrysene 1,2-dihydrodiol are shown in Fig. 4B and 4D, respectively. These histograms were obtained by chromatography of the metabolites after their treatment with acid (see Materials and Methods) in order to hydrolyze metabolically formed bay-region diol epoxides. Introduction of this step was essential because these diol epoxides largely but incompletely survive the conditions of incubation and chromatography owing to their low reactivity (11, 20). The acid treatment resulted in some decomposition of the dihydrodiols, and hence chromatographic runs performed before acid treatment (not shown) were used for quantitation of the over-all metabolism, whereas chromatographic runs performed after acid treatment were used for the estimation of relative amounts of diol epoxides-*1* and -*2* based on the ratios of stable tetraols produced. The data in Fig. 4 and Table 5 indicate that diol epoxide-*2* is a major metabolite of the (−)-enantiomers of phenanthrene 1,2-dihydrodiol and chrysene 1,2-dihydrodiol when liver microsomes from 3-methylcholanthrene-treated rats are used as source of enzymes, since the trans-*2* tetraols constituted 54% and 46% of total metabolites, respectively. *Cis-2*, *trans-1*, and *cis-1* tetraols are minor but significant metabolites of both dihydrodiols. Bay-region diol epoxides amount to 78% and 73% of the total metabolites of the phenanthrene 1,2- and chrysene 1,2-dihydrodiols, respectively. The diastereomeric diol epoxides -*1* and -*2* of phenanthrene are formed in the ratio of ~1 to 3, and the diol epoxides -*1* and -*2* of chrysene are formed in the ratio of ~1 to 3.5. Although no attempt has been made to determine the identity of the unknown metabolites (Fig. 4), the metabolite peaks attributed to tetraols were found to have the expected chromophores. Both 1,2-dihydrodiols are metabolized by

TABLE 5

Metabolites of phenanthrene 1,2-dihydrodiol and chrysene 1,2-dihydrodiol formed by hepatic microsomes from 3-methylcholanthrene-treated rats

[^{3}H]Phenanthrene 1,2-dihydrodiol and [^{3}H]chrysene 1,2-dihydrodiol were obtained as metabolites of [^{3}H]phenanthrene and [^{3}H]chrysene formed by hepatic microsomes from 3-methylcholanthrene-treated rats and were of 93% and 80% enantiomeric purity, respectively. The substrate concentration was 50 μM for each dihydrodiol. Protein concentration were 0.125 mg/ml for phenanthrene 1,2-dihydrodiol and 0.5 mg/ml for chrysene 1,2-dihydrodiol. Metabolites are expressed as the percentage of total metabolism emerging from the column in discrete peaks prior to the substrate. Metabolites were analyzed after treatment at pH ~2.0 for 18 hr to hydrolyze residual diol epoxide. The nature of the radioactivity which emerged at the retention time of intact diol epoxide was not investigated.

Substrate	Metabolites (%)							Total[a] conversion (%)	Recovery[b] (%)
	Trans-1	*Cis-1*	*Trans-2*	*Cis-2*	Diol epoxides	Unknown[c] I	Unknown[c] II		
Phenanthrene 1,2-dihydrodiol	8.2	11.3	54.3	3.0	1.4	9.2	12.6	4.8 (1.45)	87
Chrysene 1,2-dihydrodiol	8.8	5.3	46.1	5.4	7.5	26.9	—	19.2 (1.44)	75

[a] Total conversion of the substrate was calculated on the basis of non-acid-treated samples (see legend). For non-acid-treated samples, much of the radioactivity emerges from the column in the fraction labeled "diol epoxides" (see Fig. 4). Numbers in parentheses represent nanomoles of product formed per nanomole of hemoprotein per minute from each substrate. As in Tables 1 and 2, nonextracted metabolites were not entered into the calculations. These amounted to 15% and 3% of the total extracted metabolites for incubations of the phenanthrene and chrysene dihydrodiols, respectively.

[b] Recovery is the percentage of radioactivity which emerges from the column as discrete metabolite peaks as compared with the total metabolism as measured radiochemically.

[c] Unknown metabolites of phenanthrene 1,2-dihydrodiol and chrysene 1,2-dihydrodiol are indicated in Fig. 4B and D, respectively.

liver microsomes from 3-methylcholanthrene-treated rats at approximately the same rate (1.45 nmoles of product/nmole of cytochrome P-450/min) at which chrysene is metabolized. This rate is only 20% of the rate at which phenanthrene is metabolized. Conversions of both dihydrodiols were linear with protein concentrations up to 1.0 mg/ml.

DISCUSSION

In order to provide further insights into the virtual lack of tumorigenic activity of the hydrocarbon phenanthrene and the weak to moderate activity of chrysene, the present study has sought (a) to examine the rates and extent to which these hydrocarbons are metabolized to 1,2-dihydrodiols with bay-region double bonds, (b) to determine the enantiomeric purity and absolute stereochemistry of these 1,2-dihydrodiols, and (c) to establish the rate and extent to which these 1,2-dihydrodiols are metabolized to bay-region diol epoxides. Metabolism of chrysene is very similar to that of another very insoluble hydrocarbon, dibenzo[*a*,*h*]anthracene (10). Both of these hydrocarbons are metabolized to very little of their K-region 5,6-dihydrodiols, but are substantially converted to benzo-ring dihydrodiols—as much as 73% of the total metabolism in the case of chrysene. Like dibenzo[*a*,*h*]-anthracene (10), the dihydrodiol with the bay-region double bond is most extensively formed with microsomes from untreated animals and represents as much as 33% of the total metabolism in the case of chrysene. Presently, this represents the most extensive known conversion of a hydrocarbon to a precursor of a bay-region diol epoxide. In itself, this represents a significant factor which contributes to the carcinogenicity of chrysene. In contrast, the rather soluble hydrocarbons phenanthrene and benzo[*a*]anthracene (13) are extensively metabolized at their K-regions to form dihydrodiols—as much as 83% of the total metabolism in the case of phenanthrene. Substantially lower amounts (4%–27%) of dihydrodiols are formed on the angular benzo-ring of these hydrocarbons.

With liver microsomes from 3-methylcholanthrene-treated rats, metabolism of both hydrocarbons to benzo-ring dihydrodiols is associated with very high enantiomeric stereoselectivity. From 90% to 98% of the 1,2- and 3,4-dihydrodiols formed are represented by single enantiomers. Assignment of absolute stereochemistry in all four cases has established that the predominant enantiomers have the [R,R] configuration and have negative values of $[\alpha]_D$ in tetrahydrofuran. Similar high specificity to form [R,R] dihydrodiols on benzo-rings has also been noted in the metabolism of benzo[*a*]pyrene at the 7,8-position (32, 33) and of benzo[*a*]anthracene (25) at the 3,4-, 8,9- and 10,11-positions.[6] Although previous studies (25, 27) have found the enantiomeric composition of dihydrodiols to be relatively insensitive to the effects of inducing agents, this is not the case for the formation of chrysene 1,2-dihydrodiol. Microsomes from control rats and particularly phenobarbital-treated rats have far less enantiomeric specificity as compared with microsomes from 3-methylcholanthrene-treated rats. Enantiomeric purity of the (−)-[1R,2R]-dihydrodiol ranged from 10% to 80% as a function of treatment. Thus, the major metabolically formed enantiomer of all benzo-ring dihydrodiols studied thus far have [R,R] absolute configuration and neagtive values of $[\alpha]_D$ in tetrahydrofuran. The relative roles of epoxide hydrolase and the cytochromes P-450 in determining the stereochemical course of dihydrodiol formation can be quite complex and is an area of current study (34). In addition, Boyd *et al.* (31)[5] have recently shown that certain non-K-region arene oxides readily racemize, although the extent to which such racemization would occur in liver microsomes before the

[6] M. Koreeda and S. Neese at the Johns Hopkins University have established that (−)-*trans*-[8R,9R]-dihydroxy-8,9-dihydrobenzo[*a*]anthracene has $[\alpha]_D$ −237° (*c* = 0.4 mg/ml, tetrahydrofuran) as discussed in ref. 25.

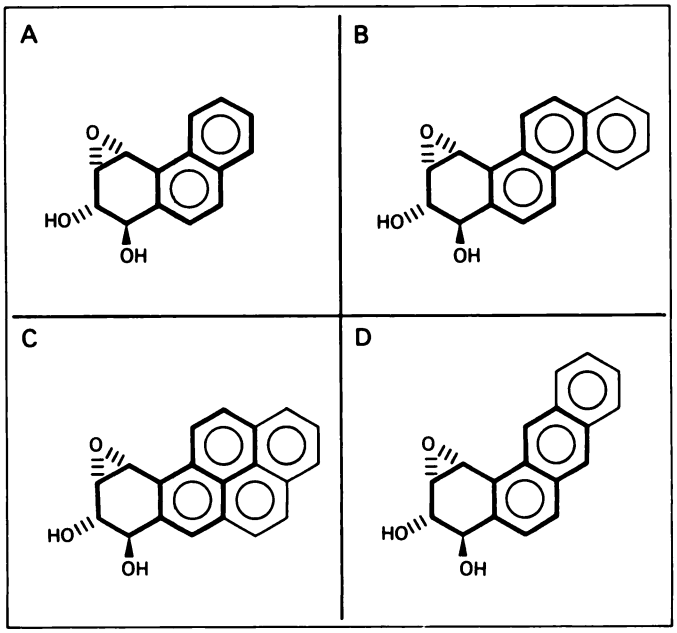

FIG. 5. *The metabolically predominant stereoisomers of the bay-region diol epoxides*

The stereoisomers formed from phenanthrene, chrysene, benzo[*a*]anthracene, and benzo[*a*]pyrene are superimposable and have [R,S]-diol-[S,R]-epoxide absolute stereochemistry when designated in a clockwise fashion (see Fig. 5) around the angular benzo-ring. All of their dihydrodiol precursors have [R,R] absolute stereochemistry.[7]

arene oxide was hydrated by epoxide hydrolase is presently unknown.

Studies of the configuration of the major enantiomers of the K-region dihydrodiols formed by liver microsomes at the 5,6-position of benzo[*a*]anthracene (25) and the 4,5-position of benzo[*a*]pyrene[8] have established that [R,R]-dihydrodiols with positive values of $[\alpha]_D$ in tetrahydrofuran predominate. Unlike the metabolism of benzo[*a*]pyrene (32, 33), the K-region dihydrodiols of both benzo[*a*]anthracene (25) and phenanthrene are formed with very low enantiomeric specificity. The 9,10-dihydrodiol of phenanthrene represents the first example of metabolism of a hydrocarbon by rat liver microsomes to a dihydrodiol in which the [S,S]-enantiomer (negative $[\alpha]_D$ in tetrahydrofuran) is in excess. Since the precursor phenanthrene 9,10-oxide has a plane of symmetry and thus cannot be optically active, all of this specificity must be due to epoxide hydrolase. Theoretical considerations (31) have suggested that K-region arene oxides are not subject to facile racemization.

Previous studies of the metabolism of benzo[*a*]pyrene 7,8-dihydrodiol to its diastereomeric, bay-region 7,8-diol-9,10-epoxides (27) have established that this oxidation is subject to a very high degree of stereochemical control. Mainly (+)-diol epoxide-*2* is formed from the predominant (−)-[7R,8R]-dihydrodiol, whereas mainly (+)-diol epoxide-*1* is formed from the minor (+)-[7S,8S]-dihydrodiol. The predominantly formed (−)-[1R,2R]-dihydrodiols of phenanthrene and chrysene are directly superimposable on the (−)-[7R,8R]-dihydrodiol of benzo[*a*]pyrene when the bay-regions are aligned (cf. Fig. 5), as is the case for the (−)-[3R,4R]-dihydrodiol of benzo[*a*]anthracene (35). The present study, as well as work in progress on benzo[*a*]anthracene, has established that each of these [R,R]-dihydrodiols is also predominantly epoxidized from the side of the dihydrodiol opposite to that of the benzylic hydroxyl group to form diol epoxide-*2* diastereomers. These diol epoxides are superimposable when their bay-regions are aligned (Fig. 5). Thus, for the three tumorigenic hydrocarbons as well as for phenanthrene, the over-all stereochemical consequence of three separate enzymatic steps is a highly selective formation of one of the four possible stereoisomers of the hydrocarbon's bay-region diol epoxides. For the three tumorigenic hydrocarbons, this is the isomer which is either known or suspected to be the most tumorigenic stereoisomer (27, 35–37). In conclusion, the lack of tumorigenicity for phenanthrene lies solely in the fact that its bay-region diol epoxides have extremely weak biological activity (7, 37), since metabolism of phenanthrene to bay-region diol epoxides closely parallels that of tumorigenic hydrocarbons.

[7] It should be noted that the change in designation of the nonbenzylic hydroxyl group from R in the dihydrodiol to S in the diol epoxide is a consequence of the sequence rules and does not represent a change in the spatial arrangement of the atoms about this carbon.

[8] B. Kedzierski, D. R. Thakker, R. N. Armstrong, and D. M. Jerina. Absolute configuration of the 4,5-dihydrodiols and 4,5-oxide of benzo[*a*]pyrene, manuscript in preparation.

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