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Biochemistry

Epoxidation reactions catalyzed by rat liver cytochromes P-450 and P-448 occur at different faces of the 8,9-double bond of 8-methylbenz[*a*]anthracene

(polycyclic aromatic hydrocarbon/HPLC/spectropolarimetry/epoxide hydrolase/stereoselective metabolism)

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ABSTRACT **8-Methylbenz[*a*]anthracene (8-MeB*a*A) *trans*-dihydrodiol metabolites were isolated by reversed-phase and normal-phase HPLCs from incubations of 8-MeB*a*A with liver microsomes or a reconstituted system containing purified cytochrome P-448 and epoxide hydrolase. Regardless of the enzyme source, the metabolically formed 8-MeB*a*A *trans*-3,4- and -5,6-dihydrodiols were found to be enriched in one enantiomeric isomer and differed only in the degree of optical purity. The 8-MeB*a*A *trans*-8,9-dihydrodiol formed by liver microsomes from either untreated or phenobarbital-treated rats was enriched with the (+)-enantiomer. In contrast, the 8-MeB*a*A *trans*-8,9-dihydrodiol formed either by liver microsomes from 3-methylcholanthrene-treated rats or by the reconstituted rat liver enzyme system containing cytochrome P-448 and epoxide hydrolase was enriched with the (−)-enantiomer. These results indicate that, in catalyzing the formation of 3,4- and 5,6-epoxide intermediates, the interaction with the unsubstituted 3,4- and 5,6-double bonds of 8-MeB*a*A by the different forms of cytochrome P-450 occur preferentially on the same face of the aromatic plane and they differ only in the degree of stereoselectivity. However, different forms of cytochrome P-450 may interact with different faces of the aromatic plane at the methyl-substituted 8,9-double bond of 8-MeB*a*A, resulting in the formation of *trans*-8,9-dihydrodiols enriched in different enantiomeric forms. This demonstrates that different forms of cytochrome P-450 may catalyze the epoxidation reaction preferentially at different sides of the methyl-substituted double bond of a planar polycyclic hydrocarbon molecule. These properties may be used to further classify and to understand the enzyme–substrate interactions of the different forms of cytochrome P-450 in the drug-metabolizing enzyme systems.**

Polycyclic aromatic hydrocarbons (PAHs) are known to be highly stereoselectively metabolized by the drug-metabolizing enzyme systems to form optically active *trans*-dihydrodiol metabolites of varying degrees of optical purity (1, 2). A parent PAH is stereoselectively metabolized by cytochrome P-450-containing mixed-function oxidases to form optically active epoxide intermediates (1, 2). The epoxides are converted *trans*-stereospecifically by microsomal epoxide hydrolase (EC 3.3.2.3, also known as epoxide hydrase or epoxide hydratase) to form optically active *trans*-dihydrodiols (1–3). Some dihydrodiols can be further converted by the mixed-function oxidases in a highly stereoselective manner to form optically active vicinal dihydrodiol epoxides. Vicinal and nonvicinal dihydrodiol epoxides of benzo[*a*]pyrene (B*a*P) and benz[*a*]anthracene (B*a*A) are known to differ in their mutagenic and carcinogenic activities, depending on their stereochemistry and absolute configuration (4–9). The optically active dihydrodiols formed from the metabolism of B*a*P and B*a*A that have been studied to date, regardless of the drug-metabolizing enzyme systems used, are enriched in either the (+)- or the (−)-enantiomer, differing only in the degree of optical purity (1, 2, 10). The results reported to date indicate that the enzymatic epoxidation reaction occurs preferentially toward a specific face of the aromatic plane of the PAH molecules (1, 2, 10).

This paper reports that the major enantiomeric *trans*-dihydrodiol formed at the 3,4- and 5,6-double bonds of 8-methylbenz[*a*]anthracene (8-MeB*a*A), respectively, have the same absolute configuration regardless of the rat liver microsomal enzyme systems used in the *in vitro* incubation of 8-MeB*a*A. However, the major enantiomeric forms of the *trans*-8,9-dihydrodiol metabolite are different, depending on the cytochrome P-450 isozyme composition in the rat liver enzyme preparations. This indicates that a methyl substituent can drastically alter the stereoselective property of the drug-metabolizing enzyme systems toward a substrate molecule at the methyl-substituted aromatic double bond.

MATERIALS AND METHODS

8-MeB*a*A and 8-[*methyl*-^{3}H]MeB*a*A were synthesized as described (11). Liver microsomes were prepared from immature Long–Evans rats which were untreated or treated with 3-methylcholanthrene (MeC) in corn oil (40 mg/kg of body weight by intraperitoneal injection for 4 consecutive days) or with phenobarbital (PBar) in 0.9 M NaCl (25 mg/kg of body weight by intraperitoneal injection for 4 consecutive days). Rat liver cytochrome P-450 contents were 0.5 (untreated rats), 2.1 (MeC-treated), and 2.35 (PBar-treated) nmol/mg of protein, respectively. Cytochromes P-448 and P-450 from livers of MeC- and PBar-treated rats were purified by the method of West *et al.* (12). Epoxide hydrolase was purified from livers of PBar-treated rats as described by Lu *et al.* (13). One unit of epoxide hydrolase is defined as the amount of enzyme catalyzing the hydration of 1 nmol of styrene oxide per min at 37°C. NADPH-cytochrome *c* reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) was purified from PBar-treated rats by the method of Yasukochi and Masters (14). One unit of reductase is defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome *c* per min at 22°C. The extinction coefficients of 8-

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Abbreviations: B*a*A, benz[*a*]anthracene; 8-MeB*a*A, 8-methylbenz[*a*]anthracene; B*a*P, benzo[*a*]pyrene; 8-MeB*a*A *trans*-3,4-dihydrodiol, *trans*-3,4-dihydroxy-3,4-dihydro-8-methylbenz[*a*]anthracene; other dihydrodiols of B*a*A, 8-MeB*a*A, and B*a*P are similarly designated; CD, circular dichroism; PBar, phenobarbital; MeC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; Glc-6-*P*, glucose6-phosphate.

MeB*a*A dihydrodiols were determined from the HPLC-purified dihydrodiol metabolites of 8-[*methyl*-^{3}H]MeB*a*A of known specific activity.

Incubation of 8-MeB*a*A with Rat Liver Microsomes. 8-MeB*a*A was incubated with each of the three liver microsomal preparations in four 250-ml reaction mixtures (pH 7.5). Each ml of reaction mixture contained 80 nmol of 8-MeB*a*A (in 40 μl of acetone), 50 μmol of Tris·HCl, 3 μmol of $MgCl_2$, 0.1 unit of glucose 6-phosphate (Glc-6-*P*) dehydrogenase (type XII, Sigma), 0.13 μmol of $NADP^+$, 2.3 μmol Glc-6-*P*, and 1 mg of liver microsomal protein. Incubations were carried out in the dark at 37°C for 60 min. 8-MeB*a*A and its metabolites were extracted with 3 vol of acetone/ethyl acetate, 1:2 (vol/vol). The organic layer was dehydrated with anhydrous $MgSO_4$ and subsequently evaporated to dryness under reduced pressure. The residue was redissolved in tetrahydrofuran/methanol, 1:1 (vol/vol), for reversed-phase HPLC separation of metabolites.

Incubation of 8-MeB*a*A in a Reconstituted Rat Liver Enzyme System. 8-MeB*a*A was incubated in a 50-ml reconstituted rat liver enzyme system. Each ml of reaction mixture (pH 7.4) contained 80 nmol of 8-MeB*a*A (in 40 μl of acetone), 0.1 mmol of potassium phosphate buffer, 2,648 units of NADPH-cytochrome *c* reductase, 3 μmol of $MgCl_2$, 0.02 mg of dilauroylphosphatidylcholine, 1.0 μmol of NADPH, 5.0 μmol of Glc-6-*P*, 14 units of Glc-6-*P* dehydrogenase, 1.2 nmol of cytochrome P-448 or 1.3 nmol of cytochrome P-450, and 8 units of epoxide hydrolase. The reaction mixtures were incubated at 37°C for 30 min (with cytochrome P-450) or for 60 min (with cytochrome P-448). 8-MeB*a*A and its metabolites were extracted and prepared for HPLC analysis as described above.

HPLC Isolation of 8-MeB*a*A Metabolites. A Spectra-Physics model 3500B liquid chromatograph was fitted with a DuPont 0.46 cm × 25 cm Zorbax ODS column. The column was eluted at ambient temperature with a 40-min linear gradient of methanol/water, 1:1 (vol/vol), to methanol at a solvent flow rate of 0.8 ml/min. Chromatographic peaks containing 8-MeB*a*A *trans*-3,4-, -5,6-, and -8,9-dihydrodiols were combined from repeated HPLC runs and were separated by an isocratic system on a DuPont 0.62 cm × 25 cm Zorbax SIL column with tetrahydrofuran/hexane, 2:3 (vol/vol), as the eluting solvent.

Identification of Metabolites. The identification of the *trans*-8,9-dihydrodiol as a metabolite of 8-MeB*a*A by UV absorption, fluorescence, and mass spectral analyses has been reported (11). Other dihydrodiol metabolites have been identified by similar physicochemical methods. Detailed evidence including ^{1}H-NMR spectral data of these dihydrodiols and other metabolites will be reported elsewhere. Circular dichroism (CD) spectra of 8-MeB*a*A dihydrodiols in methanol were measured in a cell of 1-cm path length at room temperature with a Jasco model 500A spectropolarimeter equipped with a Jasco model DP-500 data processor.

RESULTS AND DISCUSSION

The HPLC patterns of metabolites formed by incubation of 8-MeB*a*A in the reconstituted rat liver enzyme system containing highly purified epoxide hydrolase and cytochrome P-450 or P-448 (Fig. 1) are qualitatively similar to that formed by rat liver microsomes (11). In this study, the 1,2-, 3,4-, 5,6-, and 8,9-dihydrodiols of 8-MeB*a*A formed by liver microsomes from MeC-treated rats were purified for CD spectral determinations

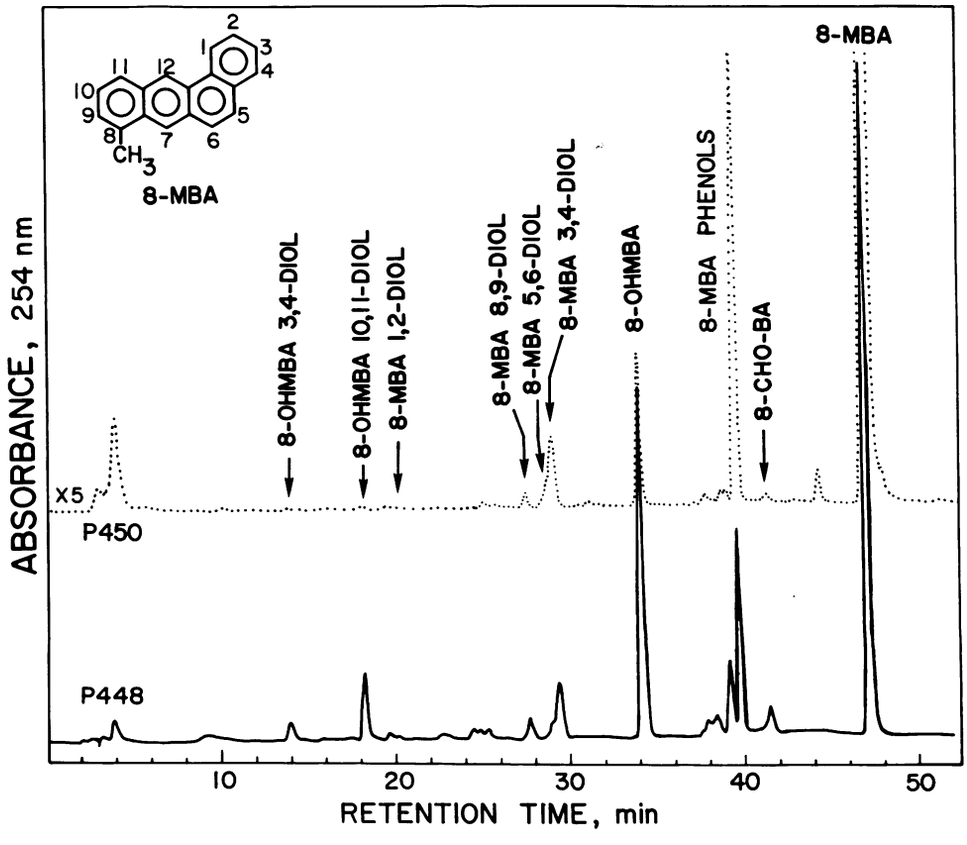

FIG. 1. Reversed-phase HPLC separation of metabolites formed by incubation of 8-MeB*a*A in a reconstituted rat liver enzyme system containing epoxide hydrolase and cytochrome P-450 (·····) or cytochrome P-448 (——). The detector sensitivity for the upper curve is set at 5 times higher than that for the lower curve. In the cytochrome P-448 system, ≈60 nmol of 8,9-dihydrodiol were formed, which accounted for 6% of the total metabolites. Experimental conditions were designed for maximal metabolite production, not for initial rate measurement. 8-MeB*a*A, methylbenz[*a*]anthracene; 8-OHMBA, 8-hydroxymethylbenz[*a*]anthracene; 8-CHO-BA, 8-formylbenz[*a*]anthracene.

whenever the dihydrodiol was obtainable in sufficient quantity. For the purpose of comparing the CD spectra, only the 8,9-dihydrodiol was purified from the incubation of 8-MeBaA in the reconstituted rat liver enzyme system containing cytochrome P-448 and epoxide hydrolase. No 8,9-dihydrodiol or other dihydrodiols was formed in the reconstituted system when epoxide hydrolase was omitted from the incubation. Although the 8,9-dihydrodiol metabolite was also formed in the reconstituted enzyme system containing cytochrome P-450 and epoxide hydrolase, it was not obtainable in sufficient quantity for CD spectral determinations. As shown in Fig. 1, the 8-MeBaA 5,6- and 3,4-dihydrodiols were not completely separable by the reversed-phase HPLC system. However, these two dihydrodiol metabolites were resolved by normal-phase HPLC (retention time 6.5 and 9.5 min, respectively) with the tetrahydrofuran/hexane elution solvent at a flow rate of 2 ml/min. The CD spectra of *trans*-1,2-, -3,4-, and -5,6-dihydrodiols formed by incubation of 8-MeBaA with liver microsomes from MeC-treated rats are shown in Fig. 2. 8-MeBaA *trans*-10,11-dihydrodiol was not obtainable in sufficient quantity for CD spectral determination. The CD spectra of (−)-BaA *trans*-1,2-dihydrodiol, (−)-BaA *trans*-3*R*,4*R*-dihydrodiol (8, 15), and (+)-BaA *trans*-5*R*,6*R*-dihydrodiol (10, 15) are also shown in Fig. 2 for comparison. The characteristic Cotton effects of the *trans*-3,4- and *trans*-5,6-dihydrodiols formed by incubation of 8-MeBaA with liver microsomes from untreated, PBar-treated and MeC-treated rats, respectively, are shown in Table 1. The results indicate that the 3,4- and 5,6-double bonds of 8-MeBaA are stereoselectively metabolized to form the 3*R*,4*R*- and 5*R*,6*R*-dihydrodiols as the predominant enantiomers by all three rat liver microsomal preparations. The optical purity of the *trans*-3,4-dihydrodiol formed by liver microsomes from PBar-treated rats is higher than those formed by liver microsomes from untreated- and MeC-treated rats (Table 1). However, liver microsomes from MeC-treated rats show the highest stereoselectivity in catalyzing the formation of 5*R*,6*R*-dihydrodiol as the predominant enantiomer (Table 1).

The CD spectra of the *trans*-8,9-dihydrodiols formed from the metabolism of 8-MeBaA by liver microsomes from untreated- and PBar-treated rats, respectively, have Cotton effects that are opposite in sign to those of the *trans*-8,9-dihydrodiol formed by either liver microsomes from MeC-treated rats or by the reconstituted enzyme system containing highly purified cytochrome P-448 and epoxide hydrolase (Fig. 3). We have established (11) that the *trans*-8,9-dihydrodiol formed from the metabolism of 8-MeBaA by liver microsomes from MeC-treated rats contains predominantly the (−)-enantiomer. Thus, the CD spectrum of (−)-8-MeBaA *trans*-8,9-dihydrodiol has Cotton effects similar to those of the (−)-BaA *trans*-8*R*,9*R*-dihydrodiol (Fig. 3). Although the absolute configuration of the (−)-8-MeBaA *trans*-8,9-dihydrodiol is not known, mirror images of the CD spectra of the 8-MeBaA *trans*-dihydrodiols indicated that the stereoselective properties of the enzyme systems in liver microsomes from untreated and PBar-treated rats are opposite from those of the liver microsomes from MeC-treated rats and that of the reconstituted liver enzyme system containing highly purified cytochrome P-448 and epoxide hydrolase. The stereoselective property of liver microsomes from untreated rats is closer to that of the liver microsomes from PBar-treated rats than to that from MeC-treated rats (Table 1).

There are three possible mechanisms by which different enantiomeric *trans*-8,9-dihydrodiols may be formed predominantly from the metabolism of 8-MeBaA by liver microsomes from MeC- and PBar-treated rats.

(*i*) Epoxidation reactions catalyzed by cytochromes P-450 and P-448 enzyme systems occur on different faces of the 8,9-double bond of 8-MeBaA, followed by cleavage of C9—O bonds of the 8,9-epoxide intermediates catalyzed by liver microsomal epoxide hydrolase from both MeC- and PBar-treated rats.

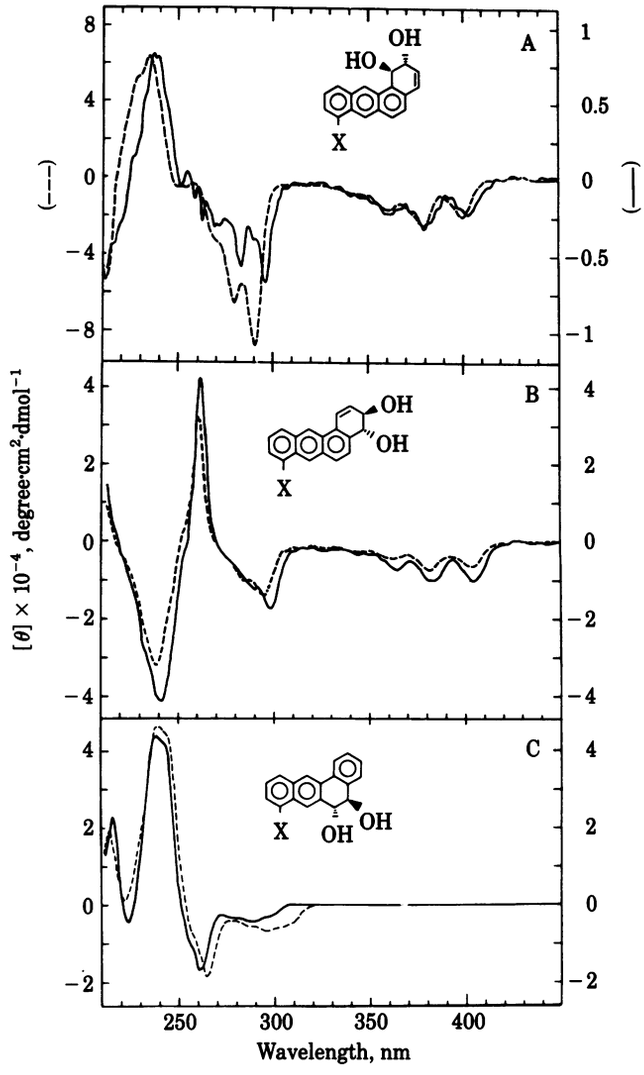

FIG. 2. CD spectra of products formed in the metabolism of 8-MeBaA (X = CH_3) by liver microsomes from MeC-treated rats compared with CD spectra of BaA (X = H) metabolites. (*A*) 8-MeBaA *trans*-1,2-dihydrodiol (——) and (−)-BaA *trans*-1,2-dihydrodiol (·····). (*B*) 8-MeBaA *trans*-3,4-dihydrodiol (——) and (−)-BaA *trans*-3*R*,4*R*-dihydrodiol (·····). (*C*) 8-MeBaA *trans*-5,6-dihydrodiol (——) and (+)-BaA *trans*-5*R*,6*R*-dihydrodiol (·····). The absolute configuration of (−)-BaA *trans*-1,2-dihydrodiol is still unknown.

(*ii*) Epoxidation reactions catalyzed by cytochromes P-450 and P-448 enzyme systems occur on different faces of the 8,9-double bond of 8-MeBaA, followed by cleavage of C8—O bonds of the 8,9-epoxide intermediates catalyzed by liver microsomal epoxide hydrolase from both MeC- and PBar-treated rats.

(*iii*) Epoxidation reactions catalyzed by cytochrome P-450 and P-448 enzyme systems occur on the same face of the 8,9-double bond of 8-MeBaA, followed by cleavage of C9—O (or C8—O) bond of the 8,9-epoxide intermediate catalyzed by liver microsomal epoxide hydrolase from MeC-treated rats and by cleavage of C8—O (or C9—O) bond catalyzed by liver microsomal epoxide hydrolase from PBar-treated rats, respectively.

Mechanism *iii* requires that the liver microsomal epoxide hydrolases from MeC- and PBar-treated rats have different mechanisms of action. Currently available evidence (17) indi-

Table 1. Comparison of the characteristic Cotton effects in the CD spectra of the dihydrodiol metabolites of BaA and 8-MeBaA

Dihydrodiol	Source of dihydrodiol*	ε,† $M^{-1}\cdot cm^{-1}$	$[\theta]\times10^{-4}$,‡ degree·cm^2·$dmol^{-1}$
8-MeBaA *trans*-1,2-dihydrodiol	LE-MeC	54,600 (263)	−0.69 (295), −0.23 (401)
8-MeBaA *trans*-3,4-dihydrodiol	LE-untreated	158,200 (262)	−3.18 (242), +3.94 (262)
	LE-PBar		−4.45 (242), +4.70 (262)
	LE-MeC		−4.10 (242), +4.26 (262)
8-MeBaA *trans*-5,6-dihydrodiol	LE-untreated	67,700 (269)	+7.61 (241), −3.02 (268)
	LE-PBar		+8.50 (241), −3.12 (268)
	LE-MeC		+9.97 (241), −3.92 (268)
8-MeBaA *trans*-8,9-dihydrodiol	LE-untreated	51,200 (264)	+1.16 (263), +0.96 (318)
	LE-PBar		+1.91 (263), +1.86 (318)
	LE-MeC		−1.65 (263), −1.71 (318)
	P-448§		−3.15 (263), −2.80 (318)
(−) BaA *trans*-1,2-dihydrodiol¶	Optically pure¶	69,238 (262)	−9.70 (290), −2.00 (400)
(−) BaA 3*R*,4*R*-dihydrodiol¶	Optically pure¶	104,450 (261)	−3.23 (238), +3.21 (260)
(+) BaA 5*R*,6*R*-dihydrodiol‖	SD-MeC‖	41,800 (266)	+4.65 (239), −1.85 (265)
(−) BaA 8*R*,9*R*-dihydrodiol‖	SD-MeC‖	71,950 (265)	−4.26 (263), −5.52 (317)
(−) BaA 10*R*,11*R*-dihydrodiol‖	SD-MeC‖	67,280 (274)	−1.49 (271), −1.24 (306)

* LE, Long–Evans rats; SD, Sprague–Dawley rats. Liver microsomes were prepared from untreated rats or rats treated with either MeC or PBar.

† The extinction coefficients (ε) of 8-MeBaA dihydrodiols in methanol at the wavelengths (nm) shown in parentheses were determined from the HPLC-purified dihydrodiol metabolites of 8-[*methyl*-^{3}H]MeBaA of known specific activity. The extinction coefficients of BaA dihydrodiols were from refs. 8 and 16.

‡ Molar ellipticity at wavelengths (nm) shown in parentheses. See Figs. 2 and 3 for CD spectra.

§ A reconstituted rat liver enzyme system containing highly purified cytochrome P-448 and epoxide hydrolase.

¶ Optically pure enantiomers were obtained by resolution of their diastereomeric (−)-dimenthoxyacetate derivatives by HPLC (8, 15).

‖ The major *trans*-dihydrodiol enantiomers were obtained by incubation of BaA with liver microsomes from MeC-treated male Sprague–Dawley rats and were purified by HPLC (15). For the assignment of absolute configurations and CD spectra of these dihydrodiols, see refs. 8, 10, and 15.

cates that liver microsomal epoxide hydrolases of untreated, PBar-treated, and MeC-treated rats have identical mechanism of action in the hydration of arene oxides to *trans*-dihydrodiols. The optical activity of the *trans*-8,9-dihydrodiol formed from the metabolism of 8-MeBaA in the reconstituted rat liver enzyme system also ruled out mechanism *iii* as a possibility. The reconstituted enzyme system contains, in addition to other components, cytochrome P-448 purified from liver microsomes of MeC-treated rats and epoxide hydrolase purified from liver microsomes of PBar-treated rats. Because the optical activity of the major *trans*-8,9-dihydrodiol enantiomer formed in the reconstituted enzyme system is the same as that formed by liver microsomes from MeC-treated rats (Fig. 3), the liver microsomal epoxide hydrolases of both MeC- and PBar-treated rats must have identical mechanisms of action. The results shown in Fig. 3 and Table 1 thus ruled out the possibility of mechanism *iii*.

Mechanisms *i* and *ii* require that liver microsomal epoxide hydrolases of MeC- and PBar-treated rats have identical catalytic properties—i.e., they catalyze the opening of the 8,9-epoxide ring at either the C8—O bond or the C9—O bond.

However, mechanism *ii* requires the water molecule activated by the catalysis of microsomal epoxide hydrolase to undergo nucleophilic attack at the C8 carbon of the 8-MeBaA 8,9-epoxide intermediate. The hydration mechanism of arene oxides such as 8-MeBaA 8,9-epoxide by microsomal epoxide hydrolase have not been studied, although our study has shown that it is a substrate of microsomal epoxide hydrolase. The closest examples of methyl-substituted arene oxides that have been studied are 2,5-dimethylbenzene 1,2-oxide and 2-methylnaphthalene 1,2-oxide, but these were found not to be substrates of guinea pig liver microsomes (18). Recent studies on the hydration of monosubstituted and 1,1-disubstituted oxirane [^{18}O]oxides by rat liver microsomes indicated that epoxide hydrolase activates water molecules for nucleophilic attack at the less hindered oxirane carbon (19). Thus, based on the available evidence, mechanism *ii* is less likely to be responsible for the observed results presented in this paper. However, because both the hydration mechanism of 8-MeBaA 8,9-epoxide and the absolute configuration of the enantiomeric 8-MeBaA *trans*-8,9-dihydrodiol are unknown, it is not possible to determine if one or both mechanisms *i* and *ii* actually occurred in the metabolic conversion of 8-MeBaA to the (+)- and (−)-*trans*-8,9-dihydrodiols by rat liver microsomes.

Previous results indicated that BaA interacts with various forms of cytochrome P-450 in an orientation such that the epoxidation reaction occurs predominantly at only one of the two faces of the 3,4-, 5,6-, 8,9-, and 10,11-double bonds (2). The results of this report indicate that cytochromes P-448 and P-450, the major induced form of the cytochrome in the livers of MeC- and PBar-treated rats, respectively, interact with different faces of the 8,9-double bond of 8-MeBaA, due to the presence of a methyl substituent at C8. This is the first example indicating that a methyl substituent on a PAH molecule can drastically alter the stereoselective interaction of the substrate molecule with different forms of cytochrome P-450.

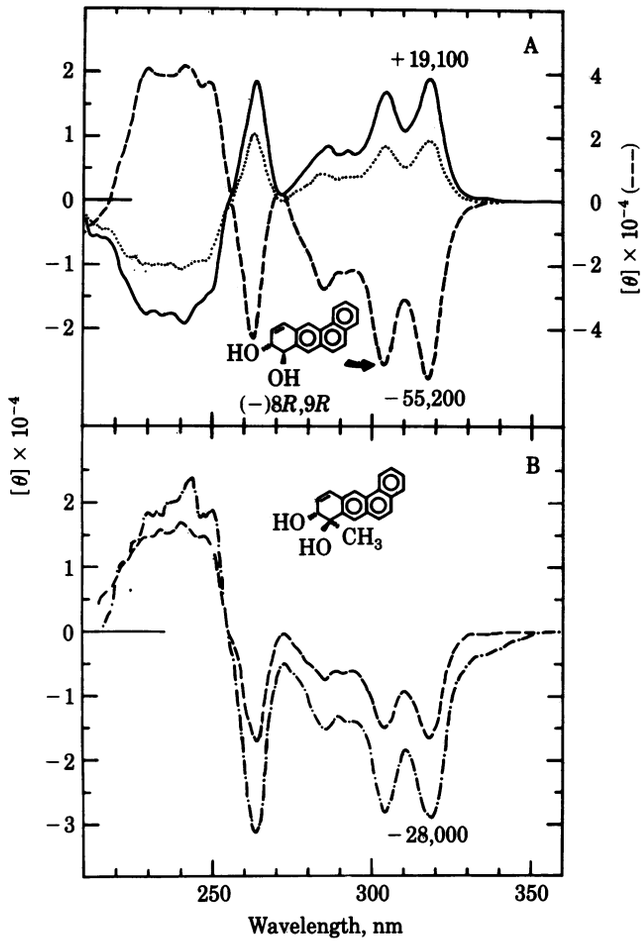

FIG. 3. The CD spectra of 8-MeBaA *trans*-8,9-dihydrodiol formed in the incubation of 8-MeBaA with liver microsomes. (*A*) Liver microsomes from untreated (·····) or PBar-treated (——) immature Long-Evans rats. (*B*) Liver microsomes from MeC-treated rats (---) or reconstituted rat liver enzyme systems containing cytochrome P-448 (-·-·-). The CD spectrum of (−)-BaA *trans*-8*R*,9*R*-dihydrodiol (---) is shown in *A* for comparison.

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