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Regioselectivity and Stereoselectivity in the Metabolism of *trans*-1,2-Dihydroxy-1,2-dihydrobenz[*a*]anthracene by Rat Liver Microsomes

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SUMMARY

Metabolism of [^{3}H]-(±)-*trans*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene by liver microsomes isolated from control, phenobarbital-treated, and 3-methylcholanthrene-treated Long-Evans rats and from 3-methylcholanthrene-treated Sprague-Dawley rats was examined. Liver microsomes from both control and phenobarbital-treated rats metabolized the dihydrodiol at a rate of 0.5 nmole/nmole of cytochrome P450 per minute, whereas prior treatment of rats with 3-methylcholanthrene stimulated the rate of metabolism by 4-fold. Prior treatment of the rats caused marked differences in the regio- and stereoselectivity of the metabolism of this pseudo-diaxial dihydrodiol. In each case, the major metabolites were three bis-dihydrodiols and a pair of diastereomeric 1,2-diol-3,4-epoxides in which the benzylic 1-hydroxyl group is either *cis* or *trans* to the epoxide oxygen (diol epoxides-*1* and -*2*, respectively). The presence of the diol epoxides in the incubation medium was inferred from the identification of their corresponding tetraols, which arise by hydrolysis of the diol epoxides on chromatography. Hepatic microsomes from control and phenobarbital-treated rats metabolized the 1,2-dihydrodiol predominantly to 1,2-diol-3,4-epoxides (68–85% of the total metabolites) whereas bis-dihydrodiols represented 28% and 13% of the total metabolites, respectively. In contrast, liver microsomes from 3-methylcholanthrene-treated rats of either strain metabolized the 1,2-dihydrodiol primarily to isomeric bis-dihydrodiols (51–56% of total metabolites), with diol epoxides accounting for only 36–38% of the total metabolites. Bis-dihydrodiol-*1* (32–35% of the total metabolites) was formed in greater amounts (2- to 4-fold) than either bis-dihydrodiols-*2* or -*3*, which were formed in about equal amounts and have identical absorption spectra. The ratio of the diastereomeric 1,2-diol-3,4-epoxides-*1* and -*2* was highly dependent upon the preparation used. For microsomes from control and phenobarbital-treated rats, this ratio was between 3:1 and 4:1 whereas microsomes from 3-methylcholanthrene-treated rats (>70% cytochrome P-450*c*) gave a ratio of between 1:1.5 and 1:2. The basis for this ratio in the latter case was explained by examination of the products formed from the (+)-(1*S*,2*S*)-and (−)-(1*R*,2*R*)-enantiomers of the dihydrodiol on metabolism by a highly purified system reconstituted with cytochrome P-450*c*. The (−)-isomer is a 3-fold better substrate than the (+)-isomer and forms only the diol epoxide-2 diastereomer, whereas the (+)-isomer forms much more diol epoxide-*1* than diol epoxide-*2* diastereomer.

INTRODUCTION

Cytochrome P-450-dependent epoxidation of benzo-ring dihydrodiols to diol epoxides in which the epoxide group forms part of a bay-region[3] of the hydrocarbon is now well established as the major pathway by which polycyclic aromatic hydrocarbons that contain bay-regions are metabolized to ultimate carcinogens (1–3). These non-bay-region dihydrodiols with bay-region double bonds generally prefer the pseudo-diequatorial conformation (4) in the absence of unusual steric or electronic factors (5–10), and epoxidation of their bay-region double bonds usually constitutes the major pathway of their metabolism, especially with liver microsomes from 3-methylcholanthrene-treated rats (38–95% for the dihydrodiols from B[*a*]P,[4] chrysene, and phenanthrene)

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[3] A bay-region of a polycyclic aromatic hydrocarbon is typified by the sterically hindered region between positions 4 and 5 in phenanthrene. See refs. 1 and 2 for the introduction of this terminology.

[4] The abbreviations used are: B[*a*]P, benzo[*a*]pyrene; B[*e*]P benzo[*e*]pyrene; DB[*a,h*]A, dibenz[*a,h*]anthracene; BA benz[*a*]an-

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(11-14). Thus far, the only such dihydrodiol substrate for which this specific regioselectivity has not been observed is BA 3,4-dihydrodiol, where bis-dihydrodiols have been identified as the predominant metabolites (15). These bis-dihydrodiols arise by the action of epoxide hydrolase (15) on dihydrodiol-arene oxide precursors. Although tetraols could arise from benzo-ring diol epoxides through the action of this enzyme, bay-regional diol epoxides have proved to be very poor substrates, if substrates at all (11, 16).

Benzo-ring dihydrodiols in which the diol group forms part of a bay-region prefer the pseudo-diaxial conformation due to steric hindrance caused by the proximate aromatic ring (cf. refs. 4 and 17). The first such pseudo-diaxial dihydrodiol whose metabolism was examined was B[*a*]P 9,10-dihydrodiol. Although the authors of the initial study had concluded that the dihydrodiol was oxidized to the corresponding catechol by liver microsomes (18), results of a subsequent study established that this conclusion was incorrect and that a phenolic dihydrodiol constituted ~75% of the total metabolites (19). We had thus speculated that the increased bulk and polarity of the pseudo-diaxial diol group inhibited metabolism at the proximate 7,8-double bond, since liver microsomes from 3-methylcholanthrene-treated rats converted the pseudo-diequatorial B[*a*]P 7,8-dihydrodiol almost entirely to 7,8-diol-9,10-epoxides (11). Although subsequent studies of the metabolism of the pseudo-diaxial B[*e*]P 9,10-dihydrodiol with rat liver microsomes did fail to provide evidence for the formation of benzo-ring diol epoxides (20), addition of α-naphthoflavone to the incubation medium altered the profile of metabolites such that trace amounts of tetraols resulting from B[*e*]P 9,10-diol-11,12-epoxides were detectable (21).

Sources of cytochrome P-450 other than rat liver microsomes have been used to study the metabolism of these pseudo-diaxial benzopyrene dihydrodiols. For example, hamster liver microsomes form a substantial amount of 9,10-diol-11,12-epoxides from B[*e*]P 9,10-dihydrodiol. Formation of diol epoxides from this substrate is stimulated when α-naphthoflavone is included in incubations with human and rabbit liver microsomes. In the presence of α-naphthoflavone, human liver microsomes form a considerable amount of 9,10-diol-7,8-epoxides from B[*a*]P 9,10-dihydrodiol (21). Formation of the B[*a*]P 9,10-diol-7,8-epoxides has also been observed with the filamentous fungus *Cunninghamella elegans* (22).

Metabolism of the bay-region pseudo-diaxial dihydrodiols from chrysene, BA and DB[*a,h*]A has been examined with rat liver microsomes. The amount of 3,4-diol-1,2-epoxides from (−)-chrysene (3R,4R)-dihydrodiol ranged from 66–90% of the total metabolites with liver microsomes from control and treated rats (23). Metabolism of racemic BA 1,2-dihydrodiol by liver microsomes from 3-methylcholanthrene-treated rats was reported to occur primarily at the 3,4-double bond to form predominantly tetraol metabolites derived from 1,2-diol-3,4-epoxides (24), although neither of these diol epoxides nor their tetraols appear to have been chemically characterized. Similar conclusions were reached by these same authors when the metabolism of racemic DB[*a,h*]A 1,2-dihydrodiol was examined (25). Unfortunately, both of these studies failed to provide quantitation for the metabolites, and thus little can be concluded about the importance of the diol epoxide pathway for these substrates.

From the above, it is quite apparent that the extent to which a pseudo-diaxial, bay-region dihydrodiol is metabolized to diol epoxides is dependent upon a number of factors; these include the species and prior treatment of the animal from which the liver microsomes are prepared, the presence of co-substrates such as α-naphthoflavone in the incubation medium, and the chemical structure of the dihydrodiol in question. The present study examines the metabolism of racemic BA 1,2-dihydrodiol and its enantiomers with the aid of radioactive substrate and well-characterized synthetic samples of the diol epoxides and tetraols such that a quantitative profile of metabolites could be established in the hope that a better understanding of the regio- and stereoselectivity of the cytochrome P-450 system toward polycyclic aromatic hydrocarbon substrates would result.

MATERIALS AND METHODS

Instrumentation. Ultraviolet spectra were recorded on a Hewlett-Packard 8450A UV-VIS spectrophotometer. Chemical ionization mass spectra with NO-N_2 as the ionizing gas were recorded on a Finnigan Model 1015 mass spectrometer. NMR spectra were recorded in $CDCl_3$ on a Jeol FX-100 NMR spectrometer. Chemical shifts are reported in parts per million (δ) relative to internal tetramethylsilane, and coupling constants (*J*) are reported in Hertz. A Spectra-Physics Model 3500B liquid chromatograph was used to separate metabolites on the columns indicated. Radioactivity was measured in Aquasol with an Intertechnique SL-4000 liquid scintillation spectrometer.

Synthesis of racemic [1,2-^{3}H]-BA 1,2-Dihydrodiol. Reduction of 10 mg of BA 1,2-quinone with 2 mg of tritiated potassium borohydride (~250 mCi/mmole; New England Nuclear Corporation, Boston, Mass.) for 2 days in 40 ml of isopropyl alcohol provided [^{3}H]-BA 1,2-dihydrodiol in 30% yield after purification of the product on a DuPont Zorbax ODS column (0.62 × 25 cm) eluted with 62% acetonitrile in water. The resultant dihydrodiol was chemically identical with that previously reported (26), was free of the *cis*-isomer, had a specific activity of 160 mCi/mmole, and was >97% radiochemically pure when analyzed under the conditions described later for the separation of its metabolites. Other applications of this general labeling procedure for dihydrodiols will be described separately.[5]

Resolution of BA 1,2-dihydrodiol. The above radioactive, racemic dihydrodiol was diluted to a specific activity of 17.6 mCi/mmole and converted into a pair of diastereomeric bis-esters with (−)-menthoxyacetyl chloride in pyridine by procedures similar to those previously

thracene; BA 1,2-dihydrodiol, *trans*-1,2-dihydroxy-1,2-dihydrobenz[*a*]anthracene (other dihydrodiols are similarly abbreviated); BA 1,2-diol-3,4-epoxide-*1*, the (±)-1β,2α-dihydroxy-3β,4β-epoxy-1,2,3,4,-tetrahydrobenz[*a*]anthracene diastereomer in which the benzylic hydroxyl group and epoxide oxygen are cis; BA 1,2-diol-3,4-epoxide-*2*, the (±)-1β,2α-dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrobenz[*a*]anthracene diastereomer in which the benzylic hydroxyl group and epoxide oxygen are trans (similar abbreviations are used for diol epoxides of other hydrocarbons); HPLC, high-performance liquid chromatography; THF, tetrahydrofuran.

[5] Generally, non-*K*-region quinones of polycyclic aromatic hydrocarbons are rapidly reduced to catechols by borohydrides with only very small amounts of dihydrodiols formed. In the presence of air, the catechols auto-oxidize back to quinones. The cyclic reduction-oxidation process allows accumulation of dihydrodiol with time (cf. ref. 15 for the first report of this process).

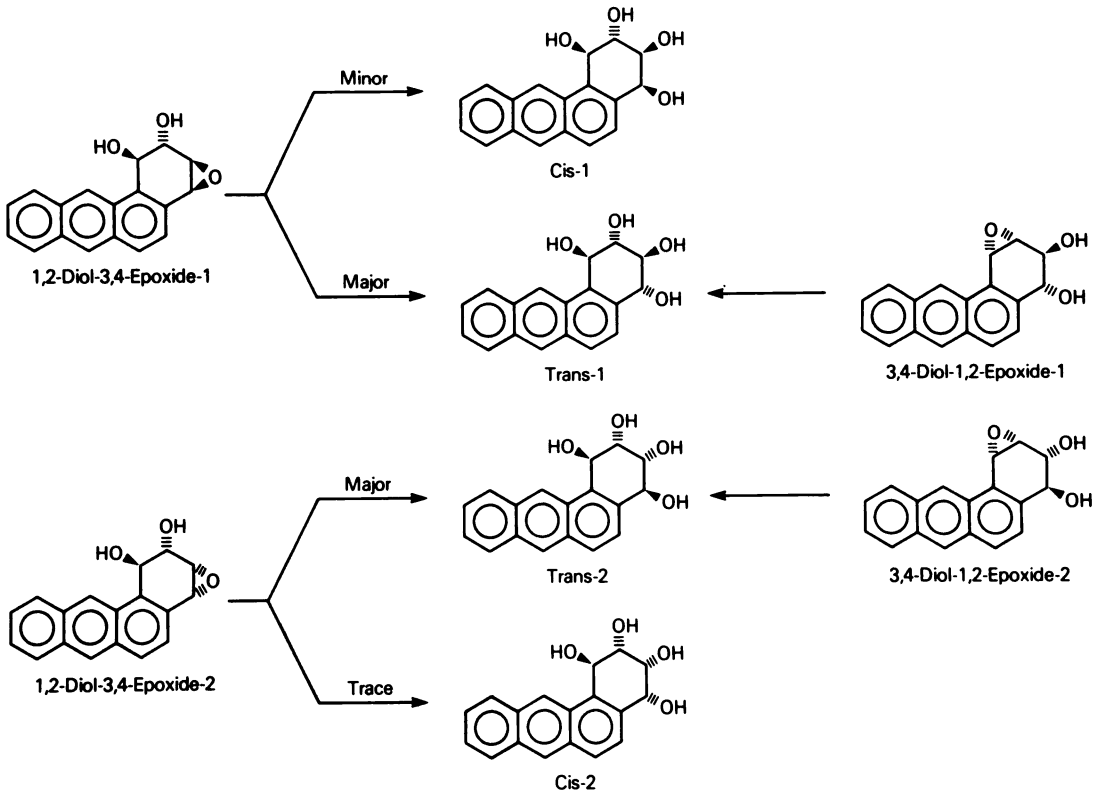

FIG. 1. *Hydrolysis products of the BA 1,2-diol-3,4-epoxides-1 and -2 in acid*

For isomer-*1*, the benzylic 1-hydroxyl group and the epoxide oxygen are cis, whereas these groups are trans in isomer-*2*. Only relative stereochemistry is implied. The tetraols *trans-1* and *trans-2* are known hydrolysis products of the BA 3,4-diol-1,2-epoxides-*1* and -*2*, respectively (15).

described (27). The diastereomers were separated by HPLC (α = 1.25) on a DuPont Zorbax SIL column (0.62 × 25 cm) eluted with 10% ether in cyclohexane; less polar (early eluting) diastereomer $[\alpha]_D$ −562° ($CHCl_3$), more polar diastereomer $[\alpha]_D$ +501° ($CHCl_3$). In both diastereomers, the methylene hydrogens of the —$OCOCH_2O$— groups are magnetically non-equivalent in their ^{1}H-NMR spectra (220 MHz, benzene-d_6). Each hydrogen appears as a doublet such that each —CH_2— appears as an AB-quartet; doublets centered at 3.94 and 4.04δ and at 3.99 and 4.09δ for the less polar isomer; doublets centered at 3.65 and 3.82δ and at 3.65 and 3.85δ for the more polar isomer. For all previous examples of bis-menthoxy esters of *trans* diols, the less polar diastereomer with the larger negative $[\alpha]_D$ shows the least magnetic non-equivalence for these hydrogens and has (*R,R*) absolute configuration (cf. ref. 27). Such is also the case in the present study. The diastereomers were solvolyzed with sodium methoxide in methanolic tetrahydrofuran, worked up by standard methods, and purified by HPLC on the above column eluted with 50% ethyl acetate in hexane. The less polar diastereomer gave the (−)-(1*R*,2*R*)-dihydrodiol with $[\alpha]_D$ −614° (THF), whereas the more polar diastereomer gave the (+)-(1S,2S)-dihydrodiol with $[\alpha]_D$ +595° (THF). Assignments of absolute configurations are based on chemical interrelationship with resolved and assigned 1,2,3,4-tetrahydro BA 1,2-epoxide. Details will be described separately.[6]

Synthesis of BA 1,2-Diol-3,4-Epoxides and Tetraols. Direct epoxidation of BA 1,2-dihydrodiol with *m*-chloroperoxybenzoic acid in tetrahydrofuran resulted in a 4:6 mixture of the two possible diastereomers in which the benzylic 1-hydroxyl group is either *cis* (diol epoxide-*1*) or *trans* (diol epoxide-*2*) to the epoxide oxygen (Fig. 1), as expected for a pseudo-diaxial dihydrodiol (cf. ref. 28). The diastereomeric mixture of 1,2-diol-3,4-epoxides-*1* and -*2* was separated by passage through a DuPont Zorbax SIL column (0.95 × 25 cm) eluted with 28% dioxane in hexane; k' = 6.2 and 7.0 for diol epoxides-*1* and -*2*, respectively. Complete details of the synthesis and kinetics of hydrolysis of these diol epoxides will be reported elsewhere.[7]

Upon acid-catalyzed hydrolysis for greater than 10 half-lives (pH 3, 10% dioxane in water containing 0.1 M sodium perchlorate, 25°, 30 min), each diol epoxide formed a pair of tetraols (Fig. 1) resulting from *cis* and *trans* opening of the epoxide by attack of water at the benzylic 4-position. Each pair of *cis* and *trans* tetraols was separated by chromatography on a DuPont Zorbax ODS column (0.45 × 25 cm) eluted with 55% methanol in water at a flow rate of 1.2 ml/min; $k'_{trans\text{-}2}$ = 4.85, $k'_{trans\text{-}1} = k'_{cis\text{-}2}$ = 6.13, and $k'_{cis\text{-}1}$ = 9.23. All four tetraols had the characteristic ultraviolet spectrum (λ_{max} ~ 256 nm, methanol) of a 1,2,3,4-tetrahydrobenz[a]anthracene chromophore and gave the expected m/e 464 (M^+) as their tetraacetates (pyridine/acetic anhydride, 18 hr, 25°). The structures of the tetraols *trans-1* and *trans-2* were assigned based on their identity (HPLC retention times and NMR spectra of their tetraacetates) with the same two tetraols which arise by *trans* hydrolysis of the isomeric BA 3,4-diol-1,2-epoxides-*1* and -*2* (15).[7] The NMR spectrum of the tetraol *cis-1* as its tetraacetate had characteristic signals at δ 1.97 (*s*, 6*H*, 2 methyl), δ 2.05 (*s*, 3*H*, methyl), δ 2.07 (*s*, 3*H*, methyl), δ 5.29 (*q*, 1*H*, H_3, $J_{2,3}$ = 10.2), δ 5.87 (*q*, 1*H*, H_2, $J_{1,2}$ = 4.2), δ 6.29 (*d*, 1*H*, H_4, $J_{3,4}$ = 3.2), and δ 6.86 (*d*, 1*H*, H_1). These coupling constants are practically identical with those observed for the tetraacetate of the structurally related *cis-1* tetraol derived from B[*e*]P 9,10-diol-11,12-epoxide-*1* (28). Both tetraol tetraacetates are best described as skew-boat forms in which the benzylic acetates reside primarily in pseudo-axial conformations. Tetraol *cis-2* forms to a very small extent (<7%) under conditions of acid-catalyzed hydrolysis. Since the tetraol *cis-2* was such a minor product from BA 1,2-diol-3,4-epoxide-

[6] Manuscript in preparation.

[7] J. M. Sayer, D. L. Whalen, S. Friedeman, A. Paik, H. Yagi, K. P. Vyas, and D. M. Jerina. Conformational effects in the hydrolysis of benzo-ring diol epoxides that have a bay-region diol group; submitted for publication.

2, this tetraol was synthesized directly from *trans*-1,2-diacetoxy-1,2-dihydrobenz[*a*]anthracene (0.035 mmole) via the oxidation procedure of Woodward and Brutcher (29) using 0.036 mmole of iodine and excess silver acetate followed by heating at 80–100° (~20 hr) in wet acetic acid. The crude products, isolated after addition of ethyl acetate, washing with sodium bicarbonate, and solvent evaporation, were treated with saturated methanolic ammonia, and the resultant tetraol *cis-2* was purified by preparative HPLC on a DuPont Zorbax ODS column (2.12 × 25 cm) eluted with 60:40 methanol/water; $k' = 3.04$. This method was necessary for introduction of the *cis*-diol with the desired stereochemistry, since reaction of BA 1,2-dihydrodiol or its diacetate with osmium tetroxide in pyridine resulted in the predominant formation of *cis-1* and very little *cis-2*. The NMR spectrum of the tetraacetate of *cis-2* had signals at δ 2.10–2.17 δ (methyl protons), δ 5.70 (q, 1*H*, H_2, $J_{2,3} = \sim 2$), δ 5.77 (q, 1*H*, H_3, $J_{3,4} = 4.3$), δ 6.52 (d, 1*H*, H_4), and δ 6.95 (d, 1*H*, H_2, $J_{1,2} = 3.8$), which is consistent with a skew-chair conformation in which the acetoxy group at position −1 is pseudoaxial. Related *cis-2* tetraols have been isolated from B[*e*]P and triphenylene (28).

Preparation of liver enzymes. Male, mature (180–200 g) rats of the Long-Evans strain were treated with either phenobarbital (dissolved in saline, 75 mg/ml) at a dose of 75 mg/kg/day, i.p. for 3 days, or 3-methylcholanthrene (dissolved in corn oil, 10 mg/ml) at a dose of 25 mg/kg/day, i.p. for 4 days. Male, mature (180–200 g) rats of the Sprague-Dawley strain were similarly pretreated with 3-methylcholanthrene. Liver microsomes were prepared 24 hr after the last dose of inducer from control and treated rats as described (30). The microsomal protein and cytochrome P-450 concentrations were determined according to the methods of Lowry *et al.* (31) and Omura and Sato (32), respectively. The specific content of cytochrome P-450 (nanomoles of cytochrome P-450 per milligram of protein) was found to be 0.97 for control microsomes, 1.48 for microsomes from phenobarbital-treated rats, 1.08 for microsomes from 3-methylcholanthrene-treated Long-Evans rats, and 1.33 for microsomes from 3-methylcholanthrene-treated Sprague-Dawley rats. Components of the purified system reconstituted with cytochrome P-450*c* were obtained as described (5).

Incubation conditions. The standard incubation mixture contained 200 μmoles of potassium phosphate buffer (pH 7.4), 6 μmoles of $MgCl_2$, 2 μmoles of NADPH, 0.4–3.2 mg of microsomal protein, and 100 nmoles of racemic [^{3}H]-BA 1,2-dihydrodiol (dissolved in 70 μl of acetone) in a final volume of 2.0 ml. The reaction mixtures were incubated at 37° for 10 min with gentle shaking. Reactions were terminated by addition of 2 ml of acetone, and the metabolites and unreacted substrate were extracted with 4 ml of ethyl acetate. After centrifugation, the organic layer was carefully removed, dried over anhydrous sodium sulfate, and concentrated with a stream of dry nitrogen. Controls consisted of zero-time and boiled-protein incubation media and were treated in a similar manner. The concentrated extracts of reaction mixtures were stored at −80° until the time of analysis.

Incubations with the reconstituted system contained 200 μmoles of potassium phosphate buffer (pH 7.4), 6 μmoles of magnesium chloride, 1 μmole of NADPH, 1500–3000 units of microsomal NADPH cytochrome *c* reductase, 0.05 mg of dilauroylphosphotidylcholine, 0.1–0.4 nmole of cytochrome P-450*c*, 0.032 mg of purified epoxide hydrolase, and 100 nmoles of (+)- or (−)-^{3}H-BA 1,2-dihydrodiol (in 70 μl of acetone) in a final volume of 2.0 ml. Incubations were carried out for 10 min at 37°. Work-up and sample preparation were as above. Controls consisted of incubations without added reductase.

A large-scale incubation of unlabeled BA 1,2-dihydrodiol was carried out in order to isolate a quantity of metabolites sufficient for spectral characterization. In a final volume of 50 ml, the following components were incubated for 10 min at 37° with gentle shaking: 5 mmoles of potassium phosphate buffer (pH 7.4), 150 μmoles of $MgCl_2$, 50 μmoles of NADPH, 50 mg of microsomal protein from 3-methylcholanthrene-treated Sprague-Dawley rats, and 2.5 μmoles of dihydrodiol dissolved in 2.5 ml of acetone. The metabolites and unreacted substrate were extracted with 150 ml of a 2:1 mixture of ethyl acetate/acetone as described above.

HPLC analysis of metabolites. Concentrated extracts of incubation mixtures were dissolved in 100 μl of methanol, and 25-μl aliquots were injected on a DuPont Zorbax ODS column (0.64 × 25 cm) eluted with a linear gradient of 10–60% acetonitrile in water at a rate of gradient change of 1%/min and a flow rate of 1.2 ml/min. The eluant was monitored at 254 nm and collected in 0.36- to 0.60-ml fractions in order to determine the amount of radioactivity present. The acetonitrile/water gradient was used, since the *cis-1* tetraol and BA 1,2-dihydrodiol coelute in the methanol/water gradient used to separate the pairs of tetraols. Although *cis-1* and *cis-2* coelute in the acetonitrile system (Fig. 2), this posed no problem as described below.

In 10% dioxane/water containing 0.1 M sodium perchlorate at 25°, the BA 1,2-diol-3,4-epoxides-*1* and -*2* have half-lives of less than 1 min at pH 3 and greater than 4 hr at neutral to alkaline pH values. Thus, the diol epoxides would be expected to survive the conditions of incubation and work-up intact. For this reason, preliminary incubations included a mild acid treatment (cf. ref. 13) at the end of the incubation period to convert all diol epoxides to tetraols. However, acid-treated and untreated incubation mixtures gave the same ratios of tetraols when analyzed using the acetonitrile-water gradient. Furthermore, direct injection of the diol epoxides on the column resulted in complete conversion to tetraols in ratios similar to those found for acid-catalyzed hydrolysis; about 23% *cis-1* from diol epoxide-*1* and <2% *cis-2* from diol epoxide-*2*. Thus the acid treatment of the incubation mixtures could be avoided due to on-column hydrolysis of the diol epoxides. In addition, the tetraol *cis-2* was not considered as a potential metabolite since it

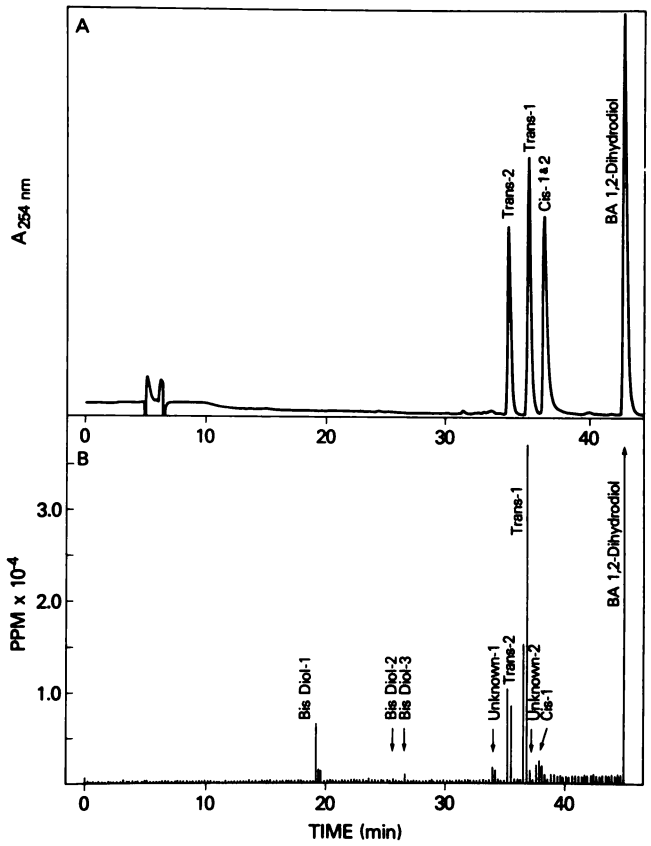

FIG. 2. *HPLC separation on a DuPont Zorbax ODS column (eluted with an acetonitrile/water gradient*

A, Separation of the synthetic standards: BA 1,2-dihydrodiol and the acid-catalyzed hydrolysis products of the BA 1,2-diol-3,4-epoxides-*1* and -*2* (tetraols *cis-1* and *cis-2* coelute with the acetonitrile gradient used). B, The profile of metabolites formed from racemic [^{3}H]-BA 1,2-dihydrodiol by liver microsomes from phenobarbital-treated mature, male rats of the Long-Evans strain after correction for radioactivity in a zero time incubation. Incubations and chromatographic conditions are described under Materials and Methods. The microsomal protein concentration was 1.6 mg/2 ml of incubation medium.

was not detected in more than trace amounts under the present conditions of analysis.

RESULTS

Rates of microsomal metabolism. The rate of metabolism (nanomoles of products per nanomole of cytochrome P-450 per minute) of racemic BA 1,2-dihydrodiol by rat liver microsomes from control and treated rats has been examined (Table 1). As had been observed previously for the related pseudo-diaxial chrysene 3,4-dihydrodiol (23), rates of metabolism were variably dependent upon the microsomal protein concentration used in the 10-min incubations. The rates given in Table 1 are for the lowest protein concentrations examined which gave good conversion of substrate. Doubling the protein concentration from 1.6 to 3.2 mg/2.0 ml of incubation medium caused a ~50% decrease in rate with liver microsomes from control and phenobarbital-treated rats, whereas doubling the protein concentration from 0.8 to 1.6 mg/2.0 ml of incubation medium with liver microsomes from 3-methylcholanthrene-treated rats had little effect on the rate. With microsomes from control or phenobarbital-treated Long-Evans rats, the dihydrodiol was metabolized at a rate of 0.5 nmole/nmole of cytochrome P-450 per minute, whereas treatment with 3-methylcholanthrene caused a 4-fold increase in rate. Microsomes from 3-methylcholanthrene-treated Sprague-Dawley rats gave an identical rate. Over-all rates of metabolism are actually ~25% higher than those given in Table 1, since the data do not take into account radioactivity above blank which remains in the aqueous phase after extraction (e.g., 2%, 3%, and 4%, respectively, for the three Long-Evans preparations). Zero time incubations retained less than 1% of the radioactivity in the aqueous phase after extraction.

Metabolite identification. Major metabolites formed by the four microsomal preparations consisted of the diastereomeric pair of 1,2-diol-3,4-epoxides-*1* and -*2* (Fig. 3) as well as three bis-dihydrodiols (Fig. 4). The three bis-dihydrodiols are numbered on the basis of their increasing order of elution from the HPLC column. As noted under Materials and Methods, the diol epoxides hydrolyze to tetraols (Fig. 1) upon analysis by HPLC. Metabolically formed tetraols (large-scale incubation) were characterized by their chromatographic and spectral identity with the synthetic standards. Characterization of three of the metabolites as bis-dihydrodiols rests mainly on their mass spectra, which showed strong molecular ions at *m/e* 296 (M^+) accompanied by losses of one or two molecules of water. Since six diastereomers, each of which has a pair of enantiomers, are possible from the racemic substrate (Fig. 4), no attempt was made to assign a specific structure to the three bis-dihydrodiols. The bis-dihydrodiols-*2* and -*3* have practically identical ultraviolet spectra which are markedly different from that of bis-dihydrodiol-*1* (Fig. 5). The ultraviolet spectra of the metabolically formed tetraols and bis-dihydrodiols were unaffected by addition of alkali, as would be expected for nonphenolic metabolites. Two minor unknown

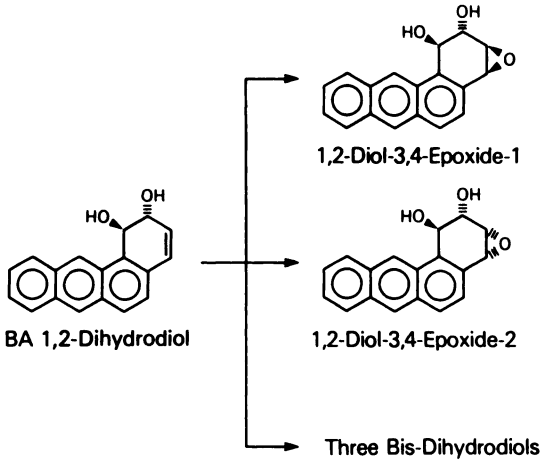

FIG. 3. *Metabolites formed from BA 1,2-dihydrodiol by rat liver microsomes*

Only the (−)-(1*R*,2*R*)-enantiomer is shown. See Fig. 4 for possible structures of the bis-dihydrodiols.

TABLE 1

Effect of inducing agents on the liver microsomal metabolism of racemic BA 1,2-dihydrodiol by mature Long-Evans and Sprague-Dawley rats

Experimental conditions are described under Materials and Methods. The substrate concentration was 100 nmoles/2.0 ml of incubation medium, and the protein concentrations were 1.6 mg/2.0 ml of incubation medium for control and phenobarbital-treated rats and 0.8 mg/2.0 ml of incubation medium for 3-methylcholanthrene-treated rats.

Microsomes	Individual metabolites as % of total metabolites							Total conversion[a,b]
	Bis-dihydrodiols			Tetraols			Total diol epoxides	
	1	*2*	*3*	*Trans-2*	*Trans-1*	*Cis-1*		
Long-Evans rats								
Control	13	7.4	7.6	14	47	7.1	68.1	8.1% (0.52)
Phenobarbital	8.9	1.8	2.3	21	61	3.8	85.8	12.1% (0.51)
3-Methylcholanthrene	32	11	7.5	24	12	1.9	37.9	16.5% (1.91)
Sprague-Dawley rats								
3-Methylcholanthrene	35	11	10	21	13	1.7	35.7	19.9% (1.87)

[a] Total metabolism denotes the percentage conversion of substrate (i.e., total radioactivity above blank) which emerges from the column before the substrate). Numbers in parentheses represent rates of metabolism, expressed in terms of nanomoles of substrate metabolized per nanomole of cytochrome P-450 per minute.

[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. These recoveries range from 81% to 96% and include two unknown peaks not tabulated (see Figs. 2 and 6). These unknowns range from <1% to as much as 7% in individual incubated samples.

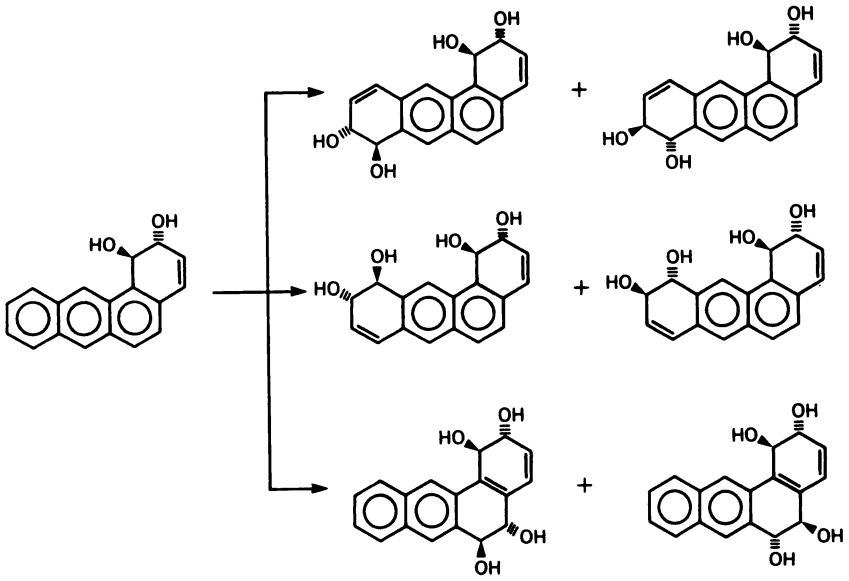

FIG. 4. *Possible structures of the bis-dihydrodiols formed from the (−)-(1R,2R)-dihydrodiol*
An enantiomeric set of equivalent diastereomers could be drawn for the (1*S*,2*S*)-dihydrodiol as well.

metabolites (cf. Figs. 2 and 6) were detected, but their amounts were too small to allow characterization. Notably, a 1,2-catechol or quinone would not be detected as a radioactive metabolite with the present substrate, labeled in the 1,2-positon.

Microsomal specificity. The profiles of metabolites formed by liver microsomes from control and phenobarbital-treated Long-Evans rats were practically identical. Diol epoxide-*1* predominated (54–65% of total metabolites) the profiles, followed by diol epoxide-*2* (14–21%), bis-dihydrodiol-*1* (9–13%) and bis-dihydrodiols-*2* and -*3*

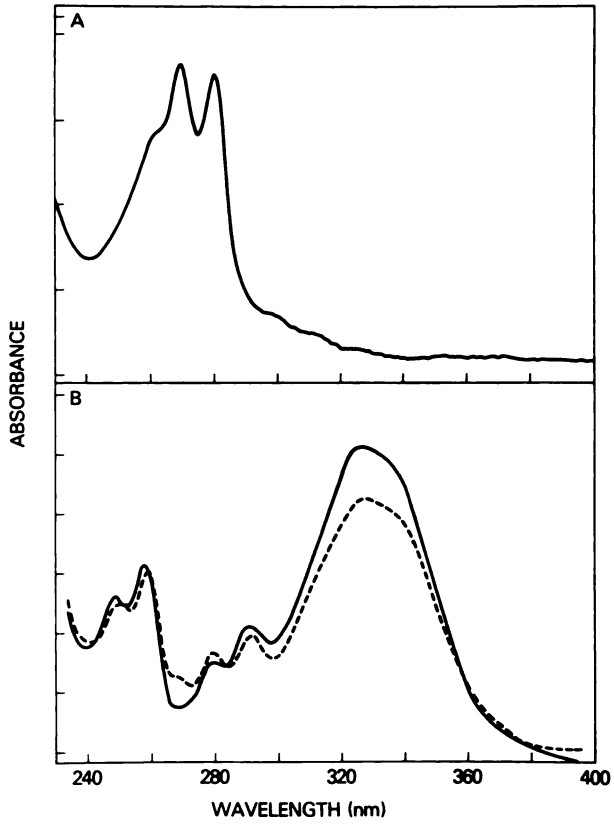

FIG. 5. *Ultraviolet spectra of the metabolically formed bis-dihydrodiol-1 (A) and bis-dihydrodiols-2 (——) and -3 (- - -) (B) in methanol/water (40:60).*

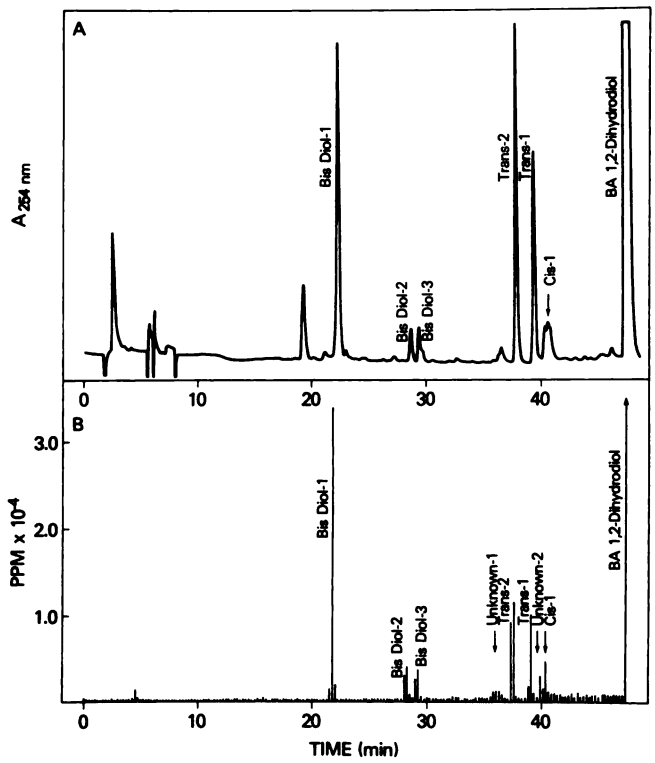

FIG. 6. *HPLC separation of the metabolites of racemic [^{3}H]-BA 1,2-dihydrodiol formed by liver microsomes (0.4 mg/2.0 ml) from 3-methylcholanthrene-treated, mature rats of the Sprague-Dawley strain*

A, The metabolic profile obtained by monitoring the column effluent at 254 nm. B, The metabolic profile obtained by quantitation of radioactivity in the effluent. Incubation and chromatographic conditions are described under Materials and Methods. The profile has been corrected for radioactivity in a zero-time incubation.

(2–8% each). Tetraols arising from diol epoxides accounted for ~70–85% of the total metabolites. Treatment of either Long-Evans or Sprague-Dawley rats with 3-methylcholanthrene resulted in practically identical profiles in which bis-dihydrodiol-*1* was the major metabolite (32–35% of total metabolites), followed by diol epoxide-*2* (21–24%). Tetraols arising from diol epoxides accounted for 36–38% of the total metabolites. Comparison of Fig. 2 (phenobarbital treatment) with Fig. 6 (3-methylcholanthrene treatment) illustrates this marked change in regioselectivity on treatment of the animals. Notably, diol epoxides constitute a higher percentage of total metabolites formed by liver microsomes from control of phenobarbital-treated rats as compared with liver microsomes from 3-methylcholanthrene-treated rats. Similar results were obtained for metabolism of the pseudo-diaxial chrysene (3R,4R)-dihydrodiol (23).

Stereoselectivity of cytochrome P-450c toward (+)- and (−)-benz[a]anthracene 1,2-dihydrodiol. Metabolism of the (+)-(1S,2S)- and (−)-(1R,2R)-dihydrodiols of BA was examined with a highly purified monooxygenase system reconstituted with cytochrome P-450*c* and epoxide hydrolase (Table 2). Good linearity with protein was observed, and the (−)-enantiomer was a 3-fold better substrate than the (+)-enantiomer. For the (−)-enantiomer, diol epoxide-2 was the major metabolite (46% of total metabolites). Bis-dihydrodiol-*1* was the next most important metabolite (36%), and diol epoxide-*1* could not be detected. For the (+)-enantiomer, bis-dihydrodiol-*3* predominated (55%), and about 4 times more diol epoxide-*1* was formed relative to diol epoxide-*2*. At best, only trace amounts of unknowns *-1* and *-2* were formed. Bis-dihydrodiols were absent from the profiles when epoxide hydrolase was omitted from the incubation medium, and several large peaks, presumably phenolic dihydrodiols, were present in the tetraol region. In addition, the purified system had a 30–50% lower rate of metabolism in the absence of epoxide hydrolase. This decrease in rate may be due to the presence of phenolic dihydrodiols, which may be inhibitors of cytochrome P-450 as was shown to be the case in the metabolism of chrysene 1,2-dihydrodiol (12). One of these phenolic dihydrodiols chromatographed in the region of unknown *-1* formed mainly in the microsomal preparations and may account for the lack of linearity there.

DISCUSSION

Metabolism of racemic BA 1,2-dihydrodiol, in which the hydroxyl groups prefer the pseudo-diaxial conformation, has been examined with liver microsomes from control and treated rats. Tetraols, which arise upon hydrolysis of the diastereomeric BA 1,2-diol-3,4-epoxides-*1* and *-2*, and bis-dihydrodiols account for 93–97% of the total metabolites which emerged from the HPLC column as identifiable peaks. With liver microsomes from control or phenobarbital-treated rats, diol epoxides account for ~70–85% of the total metabolites. In contrast, with liver microsomes from 3-methylcholanthrene-treated rats of either of two strains, diol epoxides represent only 36–38% of the total metabolites, and bis-dihydrodiols (51–56%) predominate in the profiles. The present results differ markedly from those of an earlier study with liver microsomes from 3-methylcholanthrene-treated Sprague-Dawley rats (24) in which the authors concluded that tetraols from diol epoxides were the predominant metabolites. With these microsomes, bis-dihydrodiol-*1* is actually the largest single metabolite (35% of total metabolites). The inability of the authors of the previous study (24) to recognize that bis-dihydrodiols were the predominant metabolites with these microsomes stems from the fact that quantitative conclusions about the percentage of individual metabolites were based solely on the height of HPLC peaks monitored at a specific wavelength. This led to inaccurate quantitation of individual metabolites.

The bis-dihydrodiols from racemic BA 1,2-dihydrodiol proved interesting. On the basis of their ultraviolet spectra (Fig. 5), they seem to involve only two of the three possible sites (the 5,6-, 8,9-, and 10,11-positions) on the starting dihydrodiol since the spectra of bis-dihydrodiols-*2* and *-3* are almost identical, and the spectrum of bis-dihydrodiol-*1* is markedly different. As was the case for the bis-dihydrodiols formed from BA 3,4-dihydrodiol (15), appropriate reference spectra are not available to aid in the identification of the site at which the second diol group has been introduced into the 1,2-dihydrodiol.

TABLE 2

Metabolism of (+)- and (−)-BA 1,2-dihydrodiol by a highly purified monooxygenase system reconstituted with cytochrome P-450c and epoxide hydrolase

Experimental conditions are described under Materials and Methods. The substrate concentration was 100 nmoles/2.0 ml of incubation medium. Each incubation sample contained epoxide hydrolase, 0.032 mg/2.0 ml.

Substrate enantiomer	Cytochrome P-450*c*/2 ml	Individual metabolites as % of total metabolites							Total conversion[a]
		Bis-dihydrodiols			Tetraols			Total diol epoxides	
		1	*2*	*3*	*Trans-2*	*Trans-1*	*Cis-1*		
	nmoles								
(−)-1*R*,2*R*	0.10	37	13	3	46	<0.5%	<0.5%	46	22.7% (22.7)
	0.20	35	16	2	46	<0.5%	<0.5%	46	38.5% (19.3)
(+)-1*S*,2*S*	0.20	23	5	56	3	12	~1%	16	14.7% (7.35)
	0.40	20	5	54	4	16	~1%	21	29.4% (7.35)

[a] Total conversion denotes the percentage conversion of substrate (i.e., total radioactivity above blank) which emerges from the column before the substrate. Recoveries were generally 80–90%. Rates of metabolism (numbers in parentheses) are expressed as nanomoles of substrate metabolized per nanomole of cytochrome P-450 per minute.

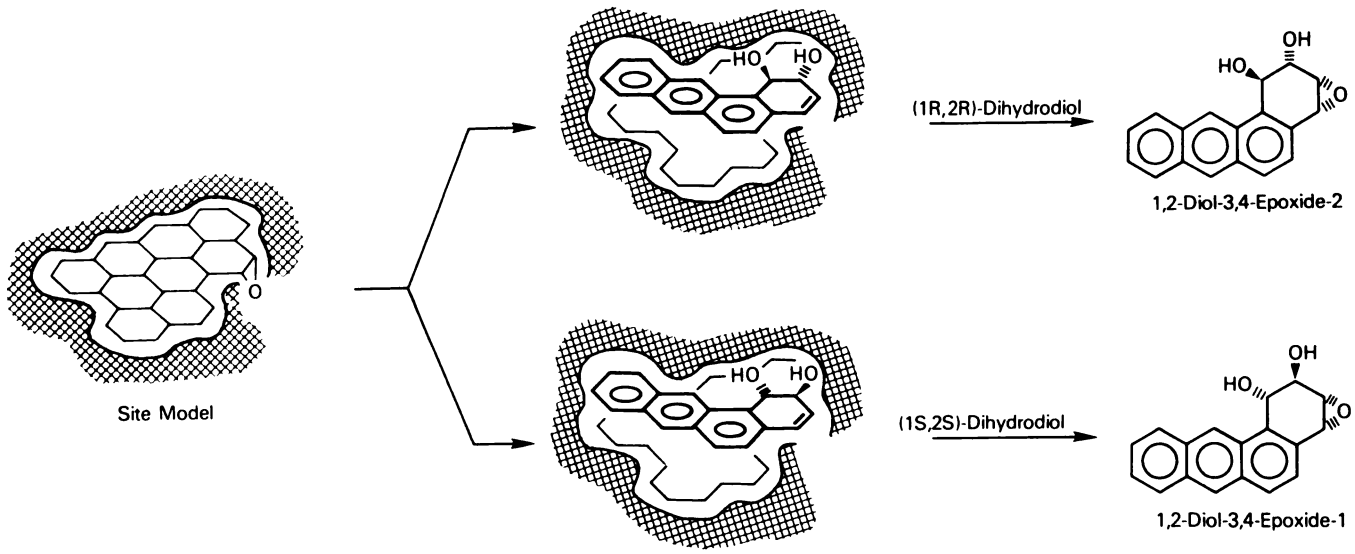

FIG. 7. *Predicted stereoselective epoxidation of BA 1,2-dihydrodiol by cytochrome P-450c (35)*
According to this model, the binding site of cytochrome P-450*c* ignores both the relative and absolute configurations of the diol group and selects for a single stereotopic face of the hydrocarbon. The diol epoxides shown are the predominant enantiomers formed. Note that carbon atoms 9, 10, 11 lie outside the original minimal boundary of the site, thus allowing additional site-mapping in this region.

Presumably, bis-dihydrodiols-*2* and -*3* are diastereomers involving the same site on the parent dihydrodiol. Their relative amounts are ~1:1 with all of the microsomal preparations. Since the 5,6- and 8,9-positions of BA are major sites of cytochrome P-450-catalyzed oxidation (33, 34), these may also be the sites of bis-dihydrodiol formation from the 1,2-dihydrodiol. Because there are actually 12 possible isomeric bis-dihydrodiols (Fig. 4), no attempt was made to assign definitive structures.

Studies of the metabolism of the (−)-(1*R*,2*R*)- and (+)-(1*S*,2*S*)-dihydrodiols with a purified monooxygenase system reconstituted with cytochrome P-450*c* and epoxide hydrolase provided a further understanding of the microsomal results. Cytochrome P-450*c* accounts for >70% of the total cytochrome P-450 in the livers of 3-methylcholanthrene-treated rats. With the reconstituted system, the (−)-enantiomer is metabolized about 3 times faster than the (+)-enantiomer. For the bis-dihydrodiols-*2* and -*3*, the (−)-isomer forms mainly bis-dihydrodiol-*2* and the (+)-isomer mainly bis-dihydrodiol-*3*, such that a 1:1 ratio with liver microsomes from 3-methylcholanthrene-treated rats would be expected. A similar high degree of stereoselectivity for the formation of bis-dihydrodiols dependent on the substrate enantiomer has also been observed for metabolism of (+)- and (−)-BA 3,4-dihydrodiol (15).

Our recently described steric model for the catalytic binding site of cytochrome P-450*c* (35) has significant bearing on the present study. The model predicts that there should be high stereoselectivity in the epoxidation of the (+)- and (−)-enantiomers of BA 1,2-dihydrodiol. Specifically, the (−)-(1*R*,2*R*)-dihydrodiol (shown in Fig. 3) should be selectively epoxidized from the bottom face of the hydrocarbon to form a single enantiomer of diol epoxide-*2* and very little diol epoxide-*1*, and the (+)-(1*S*,2*S*)-enantiomer should form a single enantiomer of diol epoxide-*1* and very little diol epoxide-*2* (illustrated in Fig. 7). This is the result observed in the present study with the purified system reconstituted with cytochrome P-450*c*. As is evident from Table 1, the ratio of tetraols derived from diol epoxides-*1* versus epoxides-*2* is between 3:1 and 4:1 with liver microsomes from control and phenobarbital-treated rats, whereas this ratio is between 1:1.5 and 1:2 for liver microsomes from 3-methylcholanthrene-treated rats. This is a consequence of the different specificities of the multiple cytochromes P-450 in these preparations (36, 37).

In summary, the present report clarifies the metabolism of racemic BA 1,2-dihydrodiol with the demonstration that bis-dihydrodiols are actually the predominant metabolites formed by liver microsomes from 3-methylcholanthrene-treated rats, as was the case for the metabolism of BA 3,4-dihydrodiol (15). As predicted (35), cytochrome P-450*c* has high stereoselectivity in the metabolism of the enantiomeric 1,2-dihydrodiols to diol epoxides. The role of pseudo-diaxial versus pseudo-diequatorial hydroxyl groups in the regiospecificity of cytochrome P-450-catalyzed oxidations must remain an open question. One possible approach to this problem would be a comparative study of the metabolism of B[*a*]P 7,8-dihydrodiol and 6-fluoro B [*a*]P 7,8-dihydrodiol, since the introduction of the peri-fluorine substituent (2) causes this latter dihydrodiol to prefer the pseudo-diaxial conformation (9).

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