MOLECULAR PHARMACOLOGY, 28:62–71

Stereoselective Metabolism of 7-Chlorobenz(*a*)anthracene by Rat Liver Microsomes

Absolute Configurations and Optical Purities of *trans*-Dihydrodiol Metabolites

PETER P. FU, LINDA S. VON TUNGELN, AND MING W. CHOU
National Center for Toxicological Research, Jefferson, Arkansas 72079

Received January 14, 1985; Accepted April 16, 1985

SUMMARY

Metabolism of 7-chlorobenz(*a*)anthracene (7-Cl-BA) by liver microsomes from untreated rats, and from rats pretreated with either 3-methylcholanthrene or phenobarbital was studied. The metabolites were isolated by HPLC and characterized by UV-visible, mass, and proton NMR spectral analyses and identified as 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiols, 3-hydroxy-7-Cl-BA, and 4-hydroxy-7-Cl-BA. Proton NMR spectral analysis indicated that 7-Cl-BA *trans*-3,4-dihydrodiol preferentially adopted a quasidiequatorial conformation while *trans*-5,6- and 8,9-dihydrodiols preferentially adopted a quasidiaxial conformation. Comparison of circular dichroism spectra with those of 7-bromobenz(*a*)anthracene *trans*-dihydrodiol metabolites of known absolute stereochemistry indicated that the major enantiomeric 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiols had *R,R* absolute configuration. Application of chiral stationary phase HPLC for direct resolution of the *trans*-dihydrodiols and their hydrogenated and dechlorinated derivatives enabled determination of the optical purity of each dihydrodiol metabolite obtained from the three microsomal systems. *In vitro* incubation of 7-Cl-BA under molecular oxygen-18 produced 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiols, each containing one oxygen-18 atom. Mass spectral analysis of the dehydration products of the oxygen-18-containing *trans*-dihydrodiol metabolites indicated that 7-Cl-BA 3*S*,4*R*-epoxide and 7-Cl-BA 8*R*,9*S*-epoxide were the predominant enantiomeric intermediates.

INTRODUCTION

Polycyclic aromatic hydrocarbons require metabolic activation to exert their biological activities, including mutagenicity and carcinogenicity. This metabolism occurs in a highly stereoselective manner (1–8), and it is known that structural features of the PAH substrates can affect both the regio- and stereoselectivity of this process (1–8). *In vitro* hepatic microsomal metabolism of PAHs[1] in general produces *trans*-dihydrodiols as major metabolites, and the *trans*-dihydrodiol enantiomers have been found to exhibit markedly different biological activities (1). Both methyl and fluoro substituents have long been employed to study the structure-activity relationships of PAHs. Recent metabolic studies using rat liver microsomes have shown that both methyl and fluoro substituents may alter the stereoselective preference of the metabolizing enzymes in the formation of the enantiomeric *trans*-dihydrodiols (2–10). The stereoselective metabolism of 7-F-BA (9) and 7-Br-BA (11) was found to produce predominantly the *R,R* enantiomer of the *trans*-dihydrodiols. Although the metabolism of chlorinated biphenyls has been reported (12), to our knowledge there are no reports on the stereoselective metabolism of a chlorinated PAH. Therefore, we were interested in studying the stereoselective metabolism of benz(*a*)anthracene with a chlorine substituent at the C-7 position. We report here stereoselective metabolism of 7-Cl-BA by rat liver microsomes. The results indicate that the three *trans*-dihydrodiol metabolites are formed in very high optical purities predominantly as the *R,R* enantiomers. Since a dihydrodiol metabolite is formed through two enzymatic steps, epoxidation of the substrate followed by hydration of the epoxide, mechanistic studies employing oxygen-18 were also conducted. The results indicate that both enzymatic reactions were highly stereoselective and that, due to steric and electronic effects

Part of this work was presented at the annual meeting of the American Association for Cancer Research, Toronto, Canada, May 22–25, 1984.

[1] The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; 7-F-BA, 7-chlorobenz(*a*)anthracene (other halogenated benz(a)anthracenes are similarly designated); 7-Cl-BA *trans*-3,4-dihydrodiol, *trans*-3,4-dihydroxy-3,4-dihydro-7-Cl-BA (other dihydrodiols are similarly designated); BA, benz(*a*)anthracene; 3-MC, 3-methylcholanthrene; THF, tetrahydrofuran; HPLC, high performance liquid chromatography; psig, pound/sq. inch.

0026-895X/85/010062-10$02.00/0

of the halogen substituent, both the *peritrans*-5,6- and 8,9-dihydrodiols were forced to adopt quasidiaxial conformations.

MATERIALS AND METHODS

Materials. 7-Cl-BA was synthesized by chlorination of BA with anhydrous cupric chloride in carbon tetrachloride by a modification of the procedure for the synthesis of 9-chloroanthracene (13). This compound was purified by column chromatography over Florisil eluted with 10% benzene in hexane followed by recrystallization from benzene-hexane as yellowish needles; mass spectrum (70 eV), M^+ m/z 262; proton NMR (500 MHz), 7.72 (m, 2, H-3, H-10), 7.78 (m, 2, H-2, H-9), 7.93 (d, 1, H-5), 8.01 (d, 1, H-4), 8.32 (d, 1, H-11), 8.36 (d, 1, H-6), 8.52 (d, 1, H-8), 9.02 (d, 1, H-1), and 9.50 ppm (s, 1, H-12); $J_{1,2} = 8.2$, $J_{5,6} = 9.4$, and $J_{10,11} = 8.6$ Hz. The *trans*-3,4-, 5,6-, and 8,9-dihydrodiols of 7-Br-BA, which were predominantly in the *R,R* absolute configuration (11), were prepared by incubation of 7-Br-BA with liver microsomes of rats pretreated with 3-MC as previously described (11). Racemic BA 8,9,10,11-tetrahydro-*trans*-8,9-diol was prepared by catalytic hydrogenation of BA *trans*-8,9-dihydrodiol (11). Adam's catalyst (PtO_2) was purchased from Ventron Chemical Co., and all solvents used were HPLC grade.

Metabolism studies and HPLC analysis of 7-Cl-BA metabolites. Liver microsomes from untreated immature male Sprague-Dawley rats (80–100-g body weight; control microsomes) and rats pretreated with 3-MC (MC-microsomes) and with phenobarbital (PB-microsomes) were prepared as previously described (14). Incubation mixtures contained 25 mmol of Tris-HCl, pH 7.5; 1.5 mmol of magnesium chloride; 0.5 mmol of NADP (Sigma, sodium salt); 1 mmol of glucose 6-phosphate (Sigma, monosodium salt); 50 units of glucose 6-phosphate dehydrogenase (Sigma, type II); 500 mg of microsomal protein, and 40 μmol of 7-Cl-BA (dissolved in 20 ml of acetone) in a total incubation volume of 500 ml. Incubations using either the control, MC-, or PB-microsomes were conducted with shaking for 60 min at 37° and the reaction was quenched by addition of 500 ml of acetone. The metabolites and residual substrate were extracted with ethyl acetate (1 liter). The organic solvent was evaporated under reduced pressure. The residue was suspended in acetone (2 × 10 ml) and the insoluble materials were removed by centrifugation. The acetone was evaporated and the residue was redissolved in THF/methanol (1:1, v/v) for reversed phase HPLC separation of the metabolites. Multiple incubations were conducted in order to isolate a sufficient amount of the dihydrodiols for structural identification and determination of the optical purity.

Reversed phase HPLC was performed with a Beckman system consisting of two model 100A pumps, a model 210 injector, a model 420 solvent programmer, and a Waters Associates (Milford, MA) model 440 absorbance (254 nm) detector. Metabolites were separated using a 20-min linear gradient of 50–100% methanol in water at a flow rate of 2 ml/min on a DuPont Zorbax ODS column (6.2 × 250 mm).

Catalytic hydrogenation and dechlorination of 7-Cl-BA trans-3,4- and 8,9-dihydrodiols. The 7-Cl-BA *trans*-3,4-dihydrodiol metabolite (0.7 mg) was dissolved in 1 ml of THF and then hydrogenated (15 psig) over Adam's catalyst (5 mg) in a Parr apparatus at ambient temperature for 2 hr. Acetone (2 ml) was then added and the mixture was vortexed. The insoluble catalyst was removed by centrifugation. The catalyst was washed with acetone (2 × 2 ml) and the combined acetone washes were evaporated under reduced pressure. The product was purified on a DuPont Zorbax SIL column (4.6 × 250 mm) eluted isocratically with 30% THF in hexane at 2 ml/min (retention time, 14 min). The product had a UV-visible spectrum similar to that of anthracene, a mass spectrum with a molecular ion at m/z 298, and therefore was identified as 7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol.

7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol was dissolved in 1 ml of THF and 0.1 ml of triethylamine and then catalytically dechlorinated by hydrogenation (20 psig) over Adam's catalyst (10 mg) in a Parr apparatus at ambient temperature for 30 hr. After workup, the product was purified by injection onto a DuPont SIL column (4.6 × 250 mm) and eluted isocratically with 30% THF/hexane (retention time, 13 min). The product was identified as BA-1,2,3,4-tetrahydro-*trans*-3,4-diol based on comparison of its UV-visible and mass spectra with those of a synthetically prepared standard (15).

The 7-Cl-BA *trans*-8,9-dihydrodiol metabolite was catalytically hydrogenated to 7-Cl-BA 8,9,10,11-tetrahydro-*trans*-8,9-diol and then dechlorinated to BA 8,9,10,11-tetrahydro-*trans*-8,9-diol. 7-Cl-BA 8,9,10,11-tetrahydro-*trans*-8,9-diol was purified by injection onto a DuPont Zorbax SIL column (6.2 × 250 mm) and by eluting isocratically with 30% THF in hexane (retention time, 8 min). Its UV-visible spectrum was similar to that of phenanthrene and its mass spectrum exhibited a molecular ion at m/z 298. BA 8,9,10,11-tetrahydro-*trans*-8,9-diol was purified and identified as previously described (11).

Chiral HPLC direct resolution of 7-Cl-BA trans-dihydrodiol metabolites and their hydrogenated derivatives. Direct resolution of the enantiomers of the 7-Cl-BA *trans*-5,6-dihydrodiol metabolites and the partially hydrogenated derivatives was performed with a Pirkle I-A HPLC column (4.6 × 250 mm) (Regis Chemical Co., Morton Grove, IL) packed with (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine ionically bonded to spherical particles (5-μm diameter) of γ-aminopropylsilanized silica. HPLC was performed using a Waters Associates liquid chromatograph consisting of a model 6000A solvent delivery system and a model 440 absorbance detector (254 nm). Samples were eluted with a mixture of ethanol, acetonitrile, and hexane in various ratios at a flow rate of 2 ml/min. Each sample was initially purified by either reversed phase or normal phase HPLC before injection onto the Pirkle chiral stationary phase HPLC column. In each case, when direct resolution was achieved, the resolved *R,R* and *S,S* enantiomers were characterized by their UV-visible and mass spectra and/or by comparison of their HPLC retention times on the Pirkle column with those of the synthetically prepared standards. The percentage of the *R,R* and *S,S* enantiomers was determined by a Hewlett-Packard 3390A integrator.

Incubation of 7-Cl-BA under an oxygen-18 atmosphere. Incubation of 7-Cl-BA with MC-microsomes and cofactors was performed as described above except a closed system was used. The incubation mixture (250 ml) in a 1-liter round bottom flask was degassed with a water aspirator followed by flushing with argon. After repeating this procedure three times, oxygen-18 (99.8 atom %, Mound Facility, Monsanto Corp., Miamisburg, OH) was introduced into the flask with a syringe. The isotopic composition of the atmosphere was monitored and the oxygen-18/oxygen-16 ratio was determined during the course of the experiment with a Varian MAT-CH-5-DF mass spectrometer. The 7-Cl-BA *trans*-dihydrodiol metabolites were isolated by HPLC with the conditions described above. Using data obtained on a Finnigan model 4023 mass spectrometer, the isotopic abundance was calculated from the relative intensities of the molecular ions of the 7-Cl-BA metabolites containing oxygen-18 and oxygen-16.

Acid-catalyzed dehydration to phenols of oxygen-18-containing 7-Cl-BA trans-dihydrodiol metabolites. The oxygen-18-containing 7-Cl-BA *trans*-3,4-dihydrodiol (~50 μg) was dehydrated by stirring in a solution of 1 ml of 0.5 N HCl in water/THF (2:1, v/v) at 60° for 20 min. The resulting phenolic products were extracted with ethyl acetate and the solvent was removed under reduced pressure. The phenols were dissolved in methanol and purified on a DuPont Zorbax ODS HPLC column (6.2 × 250 mm) eluted isocratically with 77.5% methanol in water. The phenols of the oxygen-18-containing 7-Cl-BA *trans*-5,6- and 8,9-dihydrodiol metabolites were obtained in a similar manner.

Physicochemical properties of metabolites. Ultraviolet-visible absorption spectra of the metabolites in methanol were measured on a Beckman model 25 spectrophotometer. Mass spectra were recorded with a Finnigan model 4023 system. Proton NMR spectra were obtained with a Bruker WM 500 spectrometer. The dihydrodiols were dissolved in acetone-d_6 with a trace of D_2O for NMR spectral measurements. Chemical shifts are in parts per million relative to tetramethylsilane. CD spectra of dihydrodiols were determined with a quartz cell of 1-cm path length on a Jasco 500A spectropolarimeter. CD spectra are expressed as ellipticity for methanol solutions that read 1.0 *A* unit in a UV-visible spectrophotometer at the wavelength of maximum absorption in a quartz cell of 1-cm path length.

RESULTS

HPLC separation and spectral characterization of the 7-Cl-BA metabolites. The ethyl acetate-extractable me-

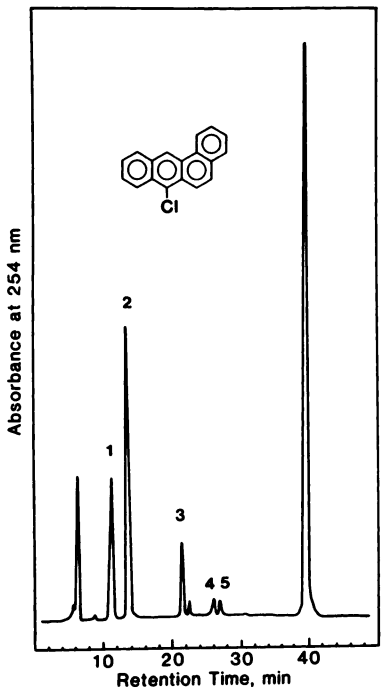

FIG. 1. *Reversed phase HPLC profile of the organic ethyl acetate-extractable metabolites obtained from incubation of 7-Cl-BA with liver microsomes of Sprague-Dawley rats pretreated with 3-methylcholanthrene*

For experimental conditions, see Materials and Methods.

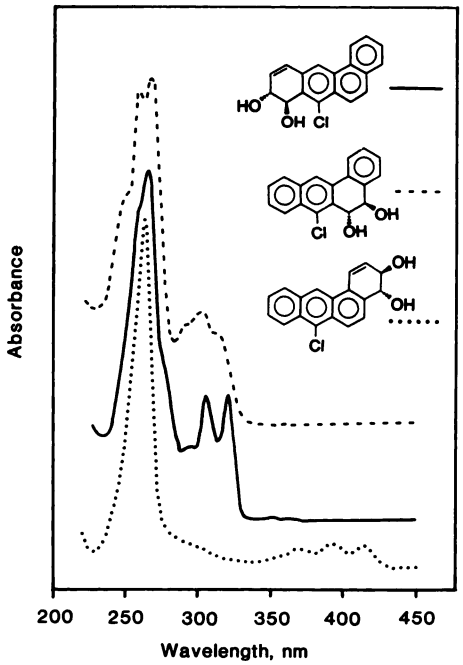

FIG. 2. *UV-visible spectra of metabolites identified as 7-Cl-BA trans-3,4-dihydrodiol (· · · · ·), 7-Cl-BA trans-5,6-dihydrodiol (– – –), and 7-Cl-BA trans-8,9-dihydrodiol (——)*

tabolites formed by incubation of 7-Cl-BA with MC-microsomes were separated by reversed phase HPLC (Fig. 1). Metabolites isolated from the chromatographic peaks 1–3 at 11.5, 13.5, and 21.5 min were identified as 7-Cl-BA *trans*-5,6-, 8,9-, and 3,4-dihydrodiols based on analysis of their UV-visible, mass, and NMR spectra.

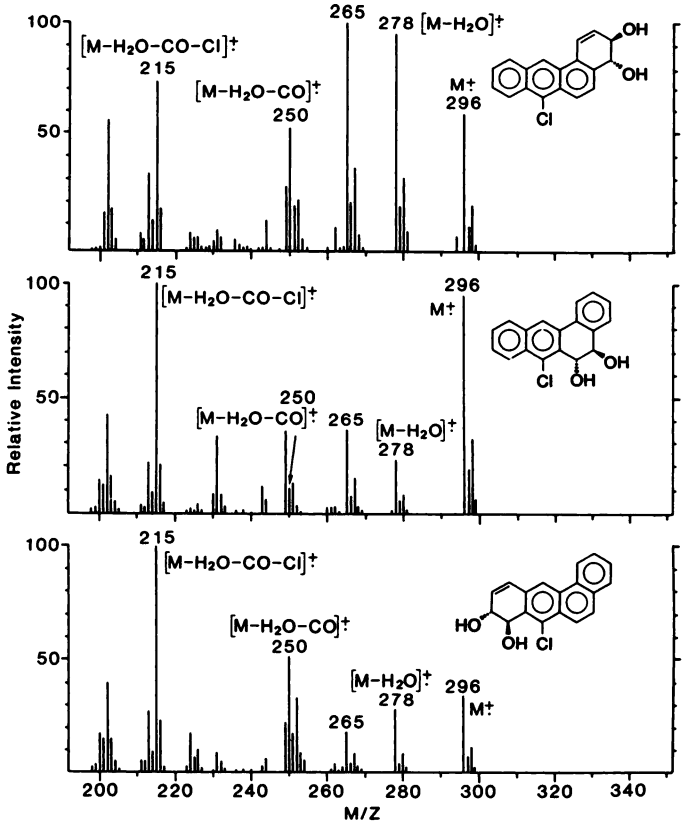

FIG. 3. *Mass spectra of metabolites identified as 7-Cl-BA trans-3,4-, 5,6-, and 8,9-dihydrodiols.*

The UV-visible absorption spectra of these dihydrodiols (Fig. 2) are similar to those of the respective BA *trans*-dihydrodiols. Mass spectral analysis (Fig. 3) indicated molecular ions at m/z 296 with characteristic fragment ions for dehydration at m/z 278 and the subsquent loss of CO (at m/z 250) and the chloro substituent (at m/z 215).

The structures of the *trans*-dihydrodiol metabolites were further confirmed by analysis of their high resolution proton NMR (500 MHz) spectral data. NMR spectra are shown in Fig. 4 and the assigned chemical shifts and coupling constants are given as follows.

7-Cl-BA trans-3,4-dihydrodiol. 4.58 (d, 1, H-3), 4.97 (d, 1, H-4), 6.34 (d, 1, H-2), 7.48 (d, 1, H-1), 7.60 (t, 1, H-9), 7.70 (t, 1, H-10), 8.05 (d, 1, H-5), 8.29 (d, 1, H-11), 8.46 (t, 2, H-6, H-8), and 8.96 ppm (d, 1, H-12); $J_{1,2}$ = 10.2, $J_{3,4}$ = 11.2, $J_{5,6}$ = 9.0, and $J_{10,11}$ = 7.7 Hz.

7-Cl-BA trans-5,6-dihydrodiol. 4.91 (d, 1, H-5), 5.60 (d, 1, H-6), 7.37 (t, 1H), 7.47 (m, 1H), 7.64 (m, 2H), 8.07 (d, 1H), 8.30 (d, 1, H-1), and 8.46 ppm (s, 1, H-12); $J_{1,2}$ = 8.3 and $J_{5,6}$ = 3.0 Hz.

7-Cl-BA trans-8,9-dihydrodiol. 4.42 (dd, 1, H-9), 5.37 (d, 1, H-8), 6.32 (dd, 1, H-10), 6.95 (d, 1, H-11), 7.72 (m, 2, H-2, H-3), 7.97 (d, 1, H-5), 8.03 (d, 1, H-4), 8.28 (d, 1, H-6), 8.65 (s, 1, H-12), and 8.86 ppm (d, 1, H-1); $J_{1,2}$ = 8.3, $J_{3,4}$ = 7.9, $J_{5,6}$ = 9.2, $J_{8,9}$ = 2.0, $J_{9,10}$ = 5.6, and $J_{10,11}$ = 9.5 Hz.

All NMR spectral patterns are consistent with the structural assignments. The NMR spectral patterns of the 7-Cl-BA *trans*-dihydrodiols are similar to those of

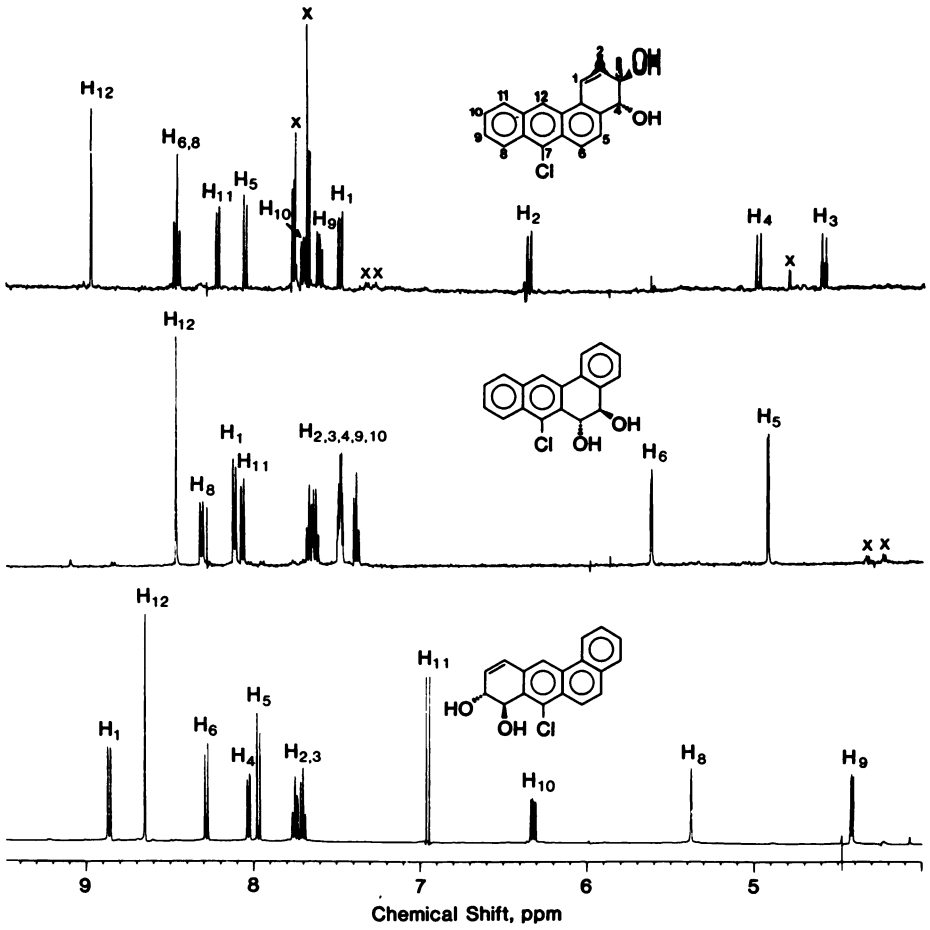

FIG. 4. *Proton NMR (500 MHz) spectra of metabolites identified as 7-Cl-BA trans-3,4-, 5,6-, and 8,9-dihydrodiols measured in acetone-d_6 with a trace of deuterated water*

Chemical shifts are in parts per million relative to tetramethylsilane. The chemical shifts and coupling constants of each compound are given in Results.

the analogous 7-F-BA *trans*-dihydrodiols and 7-Br-BA *trans*-dihydrodiols (11). The configuration and the conformation of a dihydrodiol may be determined from the NMR coupling constants between the carbinol protons and between the allylic carbinol and nonbenzylic olefinic protons (16). For 7-Cl-BA *trans*-3,4-dihydrodiol, the coupling constant between the carbinol protons is 11.2 Hz ($J_{3,4}$) and the coupling constant between the allylic and nonbenzylic olefinic protons is 2.3 Hz ($J_{2,3}$). These results indicate that 7-Cl-BA *trans*-3,4-dihydrodiol is in the *trans* configuration and preferentially adopts a quasidiequatorial conformation (16). In contrast, the coupling constants between the carbinol protons of 7-Cl-BA *trans*-5,6- and 8,9-dihydrodiols are small, at 3.0 ($J_{5,6}$) and 2.0 ($J_{8,9}$), respectively. These data indicate that both dihydrodiols are *trans* and that they preferentially adopt the quasidiaxial conformations (16).

The metabolites contained in the chromatographic peaks 4 and 5 eluting at 26 and 27 min were identified as 3- and 4-hydroxy-7-Cl-BA, respectively. The identification was based upon comparison of their UV-visible and mass spectra and HPLC retention times with those of the standards obtained by acid-catalyzed dehydration of 7-Cl-BA *trans*-3,4-dihydrodiol.

Absolute configurations of the 7-Cl-BA trans-dihydrodiol metabolites. Each of the major enantiomers of the 7-Br-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiol metabolites, obtained from incubation of 7-Br-BA with MC-microsomes, has been previously determined to possess an *R*, *R* absolute configuration (11). These dihydrodiols also have similar conformations as the 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiols. Therefore, comparison of the CD spectra of the 7-Cl-BA *trans*-dihydrodiols with those of the 7-Br-BA *trans*-dihydrodiols could be used to determine the absolute configurations of the former. The CD spectra of 7-Br-BA 3*R*,4*R*-dihydrodiol and 7-Cl-BA *trans*-3,4-dihydrodiol, obtained from incubation of 7-Cl-BA with MC-microsomes, are shown in Fig. 5. Because these two CD spectra have similar Cotton effects, the major enantiomer of the 7-Cl-BA *trans*-3,4-dihydrodiol metabolite has an *R*,*R* absolute configuration. The CD spectra of 7-Br-BA 5*R*,6*R*-dihydrodiol and 7-Cl-BA *trans*-5,6-dihydrodiol, obtained from incubation of 7-Cl-BA with MC-microsomes, are shown in Fig. 6 and those of 7-Br-BA 8*R*,9*R*-dihydrodiol and 7-Cl-BA *trans*-8,9-dihydrodiol from MC-microsomes are shown in Fig. 7. Because of the similarity of the CD Cotton effects between the analogous dihydrodiols, the major enantiomers of the 7-Cl-BA *trans*-5,6- and 8,9-dihydrodiol metabolites also have *R*,*R* absolute configurations. The assignments

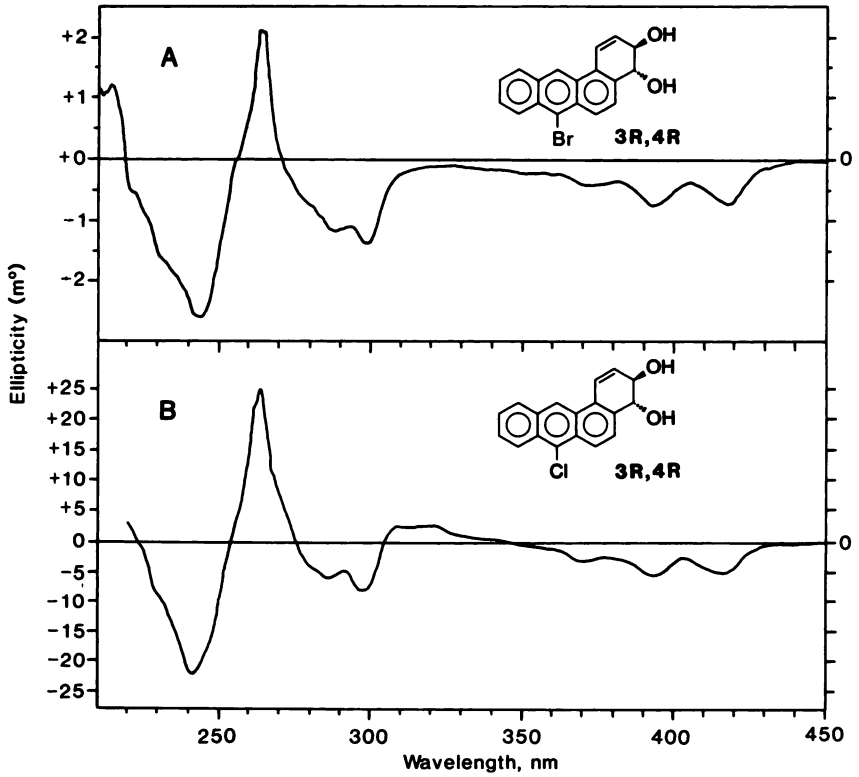

FIG. 5. *CD spectra of (A) 7-Br-BA 3R,4R-dihydrodiol and (B) 7-Cl-BA trans-3,4-dihydrodiol metabolite measured in methanol* See Materials and Methods for experimental details.

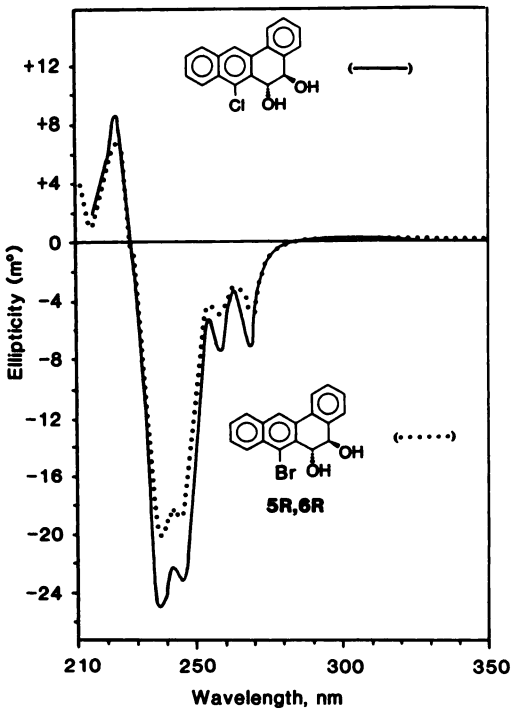

FIG. 6. *CD spectra of 7-Cl-BA trans-5,6-dihydrodiol (——) and 7-Br-BA 5R,6R-dihydrodiol (· · · · ·) measured in methanol.*

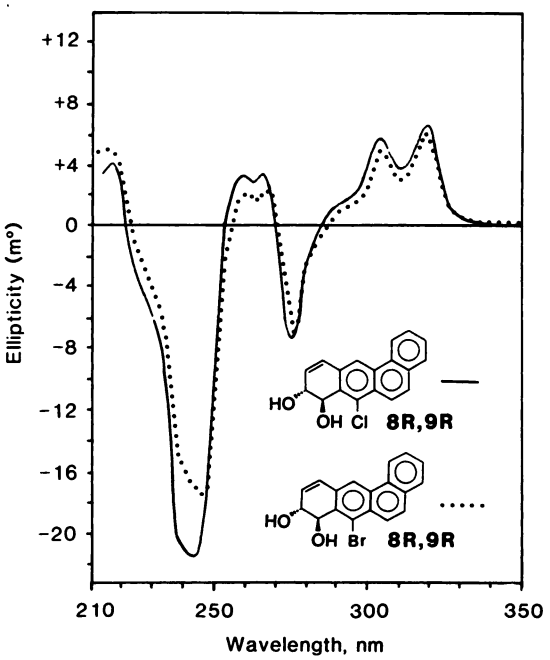

FIG. 7. *CD spectra of 7-Cl-BA trans-8,9-dihydrodiol (——) and 7-Br-BA 8R,9R-dihydrodiol (· · · · ·) measured in methanol.*

of the absolute configurations can be further confirmed by analysis of the optical (enantiomeric) purities of the 7-Cl-BA *trans*-dihydrodiol metabolites which are presented below.

Optical purity of the 7-Cl-BA trans-3,4-dihydrodiol metabolite. Attempts to resolve the enantiomers of the 7-Cl-BA *trans*-3,4-dihydrodiol, formed from incubation of 7-Cl-BA with MC-microsomes on a Pirkle chiral stationary phase HPLC column (17), were not successful. Therefore, 7-Cl-BA *trans*-3,4-dihydrodiol was catalyti-

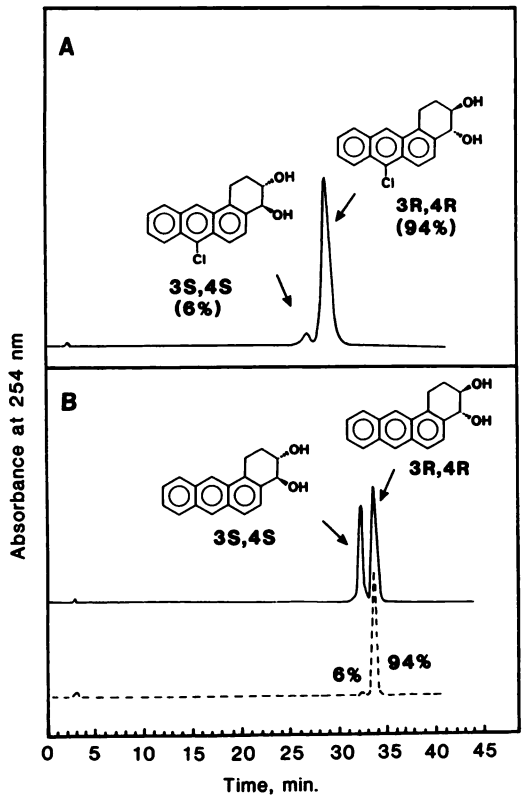

FIG. 8. *Chiral stationary phase HPLC direct resolution*
A, 7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol obtained from catalytic hydrogenation of the 7-Cl-BA *trans*-3,4-dihydrodiol metabolite formed from metabolism of 7-Cl-BA by liver microsomes of rats pretreated with 3-methylcholanthrene. B, synthetically prepared racemic BA 1,2,3,4-tetrahydro-*trans*-3,4-diol (——) and BA 1,2,3,4-tetrahydro-*trans*-3,4-diol (– – –) formed from further catalytic dechlorination of the 7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol described in A.

cally hydrogenated to give 7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol. Resolution was then achieved on the Pirkle column using a mixture of ethanol, acetonitrile, and hexane (2:1:40) at a flow rate of 2 ml/min (Fig. 8A). Analysis of the results indicated that it contained 94% 3*R*,4*R* enantiomer and 6% 3*S*,4*S* enantiomer. Thus, we concluded that the 7-Cl-BA *trans*-3,4-dihydrodiol metabolite from MC-microsomes also contained 94% 3*R*,4*R* enantiomer and 6% 3*S*,4*S* enantiomer. Therefore, the optical purity, which is the difference between the two enantiomers, of 7-Cl-BA *trans*-3,4-dihydrodiol from MC-microsomes, was found to be 88%.

In order to ensure that the results of absolute configuration and optical purity were reliable, the 7-Cl-BA 1,2,3,4-tetrahydro-*trans*-3,4-diol was catalytically dechlorinated to the corresponding BA 1,2,3,4-tetrahydro-*trans*-3,4-diol which was injected onto the Pirkle column. The synthetically prepared BA 1,2,3,4-tetrahydro-*trans*-3,4-diol was previously reported to be resolved by the Pirkle column with the 3*S*,4*S* enantiomer eluting prior to the 3*R*,4*R* enantiomer (17). The HPLC profiles of the BA 1,2,3,4-tetrahydro-*trans*-3,4-diols obtained from synthesis and from metabolism are shown in Fig. 8B. These results clearly confirmed that the major enantiomer of the 7-Cl-BA *trans*-3,4-dihydrodiol metabolite from MC-microsomes has a 3*R*,4*R* absolute configuration and that the optical purity of this dihydrodiol metabolite is 88%.

The optical purities of the 7-Cl-BA *trans*-3,4-dihydrodiols obtained from both control microsomes and from PB-microsomes were similarly determined and found to be 20% (Table 1).

Optical purity of the 7-Cl-BA trans-5,6-dihydrodiol metabolite. Direct resolution of this dihydrodiol was achieved with the Pirkle chiral stationary phase HPLC column (Fig. 9) by eluting with a mixture of ethanol, acetonitrile, and hexane (2:1:40) at 2 ml/min. The 7-Cl-BA *trans*-5,6-dihydrodiol metabolite from MC-microsomes was found to contain 94% 5*R*,6*R* enantiomer (retention time, 56 min) and 6% 5*S*,6*S* enantiomer (retention time, 63 min). Thus, the optical purity of 7-Cl-BA *trans*-5,6-dihydrodiol from MC-microsomes was 88% (Table 1). The optical purities of 7-Cl-BA *trans*-5,6-dihydrodiols from control microsomes and from PB-microsomes were similarly determined and were found to be 78 and 84%, respectively.

Optical purity of the 7-Cl-BA trans-8,9-dihydrodiol metabolite. 7-Cl-BA *trans*-8,9-dihydrodiol could not be directly resolved on the Pirkle column, and therefore it was catalytically hydrogenated to give the tetrahydro derivative, 7-Cl-BA 8,9,10,11-tetrahydro-*trans*-8,9-diol.

TABLE 1

Optical purity of 7-chlorobenz(a)anthracene trans-dihydrodiol metabolites obtained from metabolism of 7-chlorobenz(a)anthracene with rat liver microsomes

Dihydrodiol	Microsomes	Enantiomer		Optical purity
		R,R	*S,S*	
		(%)		(%)
3,4-Dihydrodiol	MC	94	6	88
	PB	60	40	20
	Control	60	40	20
5,6-Dihydrodiol	MC	94	6	88
	PB	89	11	78
	Control	92	8	84
8,9-Dihydrodiol	MC	99	1	98
	PB	95	5	90
	Control	94	6	88

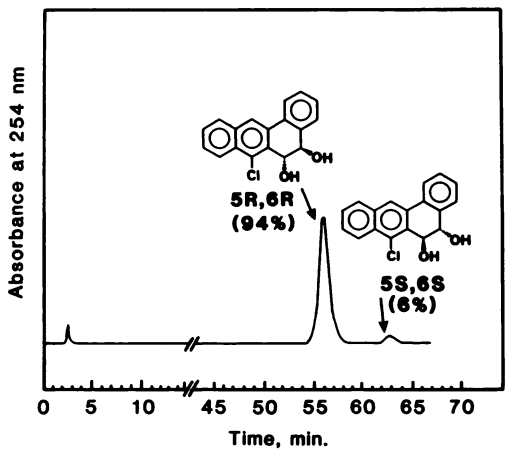

FIG. 9. *Chiral stationary phase HPLC direct resolution of the 7-Cl-BA trans-5,6-dihydrodiol metabolite obtained from incubation of 7-Cl-BA with liver microsomes of rats pretreated with 3-methylcholanthrene.*

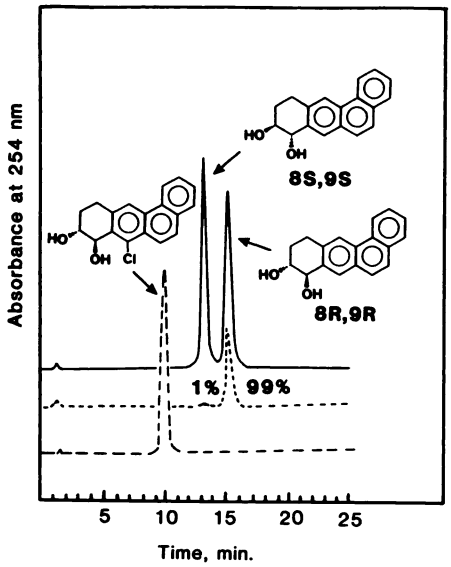

FIG. 10. *Chiral stationary phase HPLC direct resolution of the BA 8,9,10,11-tetrahydro-trans-8,9-diol formed from both catalytic hydrogenation of a synthetically prepared racemic BA trans-8,9-dihydrodiol (——) and from catalytic hydrogenation and dechlorination of the 7-Cl-BA trans-8,9-dihydrodiol metabolite obtained from incubation of 7-Cl-BA with liver microsomes of rats pretreated with 3-methylcholanthrene (– – –)*

The chromatogram of 7-Cl-BA 8,9,10,11-tetrahydro-*trans*-8,9-dihydrodiol (— —) is included for comparison.

Unfortunately, this derivative was also not resolved (Fig. 10). Therefore, it was catalytically dechlorinated to BA 8,9,10,11-tetrahydro-*trans*-8,9-diol which was resolved using a mixture of ethanol, acetonitrile, and hexane (2:1:12, Fig. 10). Since synthetically prepared BA 8,9,10,11-tetrahydro-*trans*-8,9-diol had been previously resolved on the Pirkle column (Fig. 10) (11), comparison of the HPLC profiles of the synthetic preparation with that from metabolism by MC-microsomes indicated that the enzymatically formed 7-Cl-BA *trans*-8,9-dihydrodiol contained 99% 8*R*,9*R* enantiomer and 1% 8*S*,9*S* enantiomer. Therefore, the optical purity of the dihydrodiol from MC-microsomes was 98% (Table 1). The 7-Cl-BA *trans*-8,9-dihydrodiols from control microsomes and from PB-microsomes were similarly determined to be 88 and 90%, respectively (Table 1).

Incubation of 7-Cl-BA under an oxygen-18 atmosphere and dehydration of the resulting oxygen-18-containing trans-dihydrodiol metabolites. 7-Cl-BA was incubated with MC-microsomes under molecular oxygen-18 for 1 hr. The resulting *trans*-dihydrodiol metabolites were isolated by reversed phase HPLC and then analyzed for oxygen-18 incorporation by mass spectral analysis. Each of the 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiol metabolites was found to contain one atom of oxygen-18 with a molecular ion at m/z 298 (Figs. 11–13).

In order to determine which hydroxyl group in each of the *trans*-dihydrodiol metabolites was derived from molecular oxygen, the three dihydrodiol metabolites were converted to phenolic products by acid-catalyzed dehydration, and the phenols were separated by HPLC. 3-Hydroxy- and 4-hydroxy-7-Cl-BA, obtained from acid-catalyzed dehydration of 7-Cl-BA *trans*-3,4-dihydrodiol,

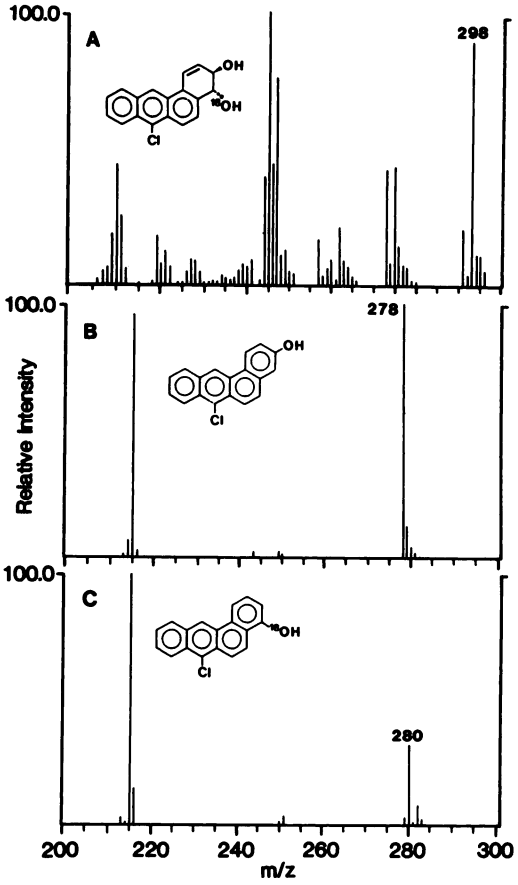

FIG. 11. *Mass spectra*

A, the oxygen-18-containing 7-Cl-BA *trans*-3,4-dihydrodiol metabolites obtained from incubation of 7-Cl-BA under an oxygen-18 atmosphere with liver microsomes of rats pretreated with 3-methylcholanthrene. B, the 3-hydroxy-7-Cl-BA. C, the 4-hydroxy-7-Cl-BA *trans*-3,4-dihydrodiol metabolite described in (A). See Materials and Methods for experimental details.

was isolated by reversed phase HPLC employing a DuPont Zorbax ODS column (6.2 × 250 mm) eluted isocratically with 77.5% methanol in water at a flow rate of 2 ml/min. The retention times for 3-hydroxy-7-Cl-BA and 4-hydroxy-7-Cl-BA were 37 and 42.5 min, respectively, and their UV-visible spectra were similar to those of 3- and 4-hydroxy-BA, respectively. Mass spectral analysis revealed that 3-hydroxy-7-Cl-BA did not contain oxygen-18 (molecular ion at m/z 278), while 4-hydroxy-7-Cl-BA contained one atom of oxygen-18 (molecular ion at m/z 280 (Fig. 11). These results indicate that the oxygen atom of the hydroxyl group attached at C-4 of the 7-Cl-BA *trans*-3,4-dihydrodiol metabolite was derived from molecular oxygen (Fig. 14A).

Similarly, 8- and 9-hydroxy-7-Cl-BA were obtained from acid-catalyzed dehydration of the oxygen-18-containing 7-Cl-BA *trans*-8,9-dihydrodiol. These phenolic compounds were first separated on a reversed phase Zorbax ODS column (9.4 × 250 mm) eluted with a 30-min linear gradient of 50–100% methanol in water at a flow rate of 2.8 ml/min (8- and 9-hydroxy-7-Cl-BA had retention times of 34.3 and 35.1 min, respectively). They were further purified by normal phase HPLC employing

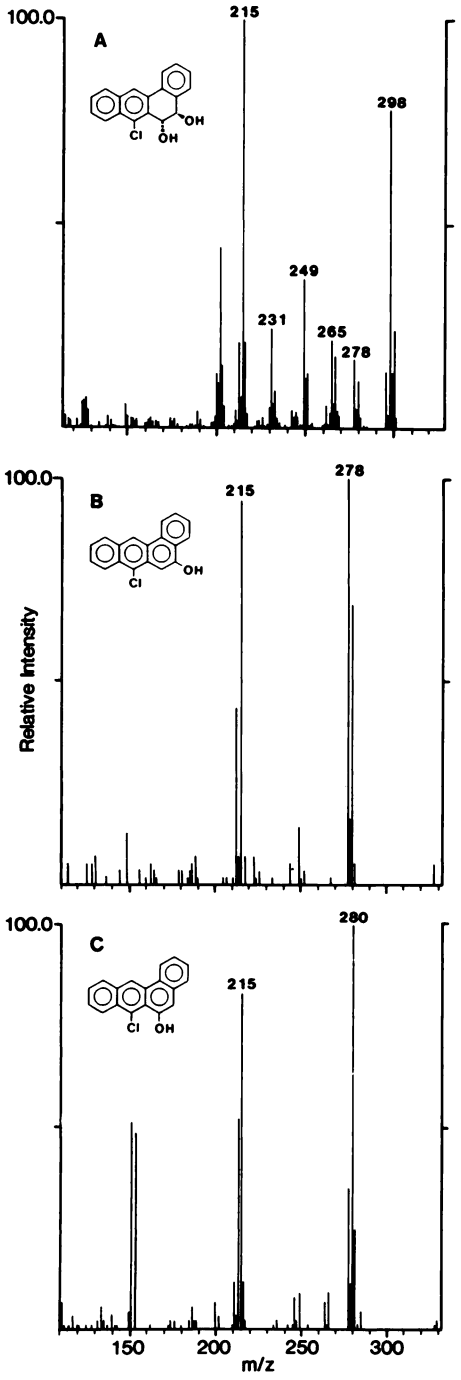

FIG. 12. *Mass spectra*

A, the oxygen-18-containing 7-Cl-BA *trans*-5,6-dihydrodiol metabolite obtained from incubation of 7-Cl-BA under an oxygen-18 atmosphere with liver microsomes of rats pretreated with 3-methylcholanthrene. B, the 5-hydroxy-7-Cl-BA. C, the 6-hydroxy-7-Cl-BA products obtained from acid-catalyzed dehydration of the oxygen-18-containing 7-Cl-BA *trans*-5,6-dihydrodiol metabolite described in A.

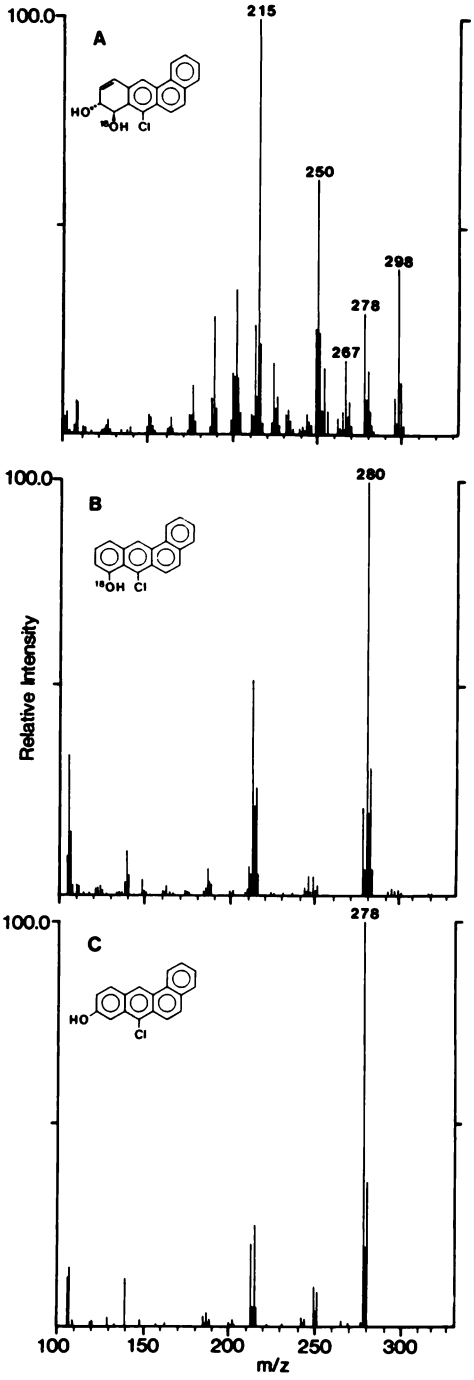

FIG. 13. *Mass spectra*

A, the oxygen-18-containing 7-Cl-BA *trans*-8,9-dihydrodiol metabolite obtained from incubation of 7-Cl-BA under an oxygen-18 atmosphere with liver microsomes of rats pretreated with 3-methylcholanthrene. B, the 8-hydroxy-7-Cl-BA. C, the 9-hydroxy-7-Cl-BA products obtained from acid-catalyzed dehydration of the oxygen-18-containing 7-Cl-BA *trans*-8,9-dihydrodiol metabolite described in A.

a Zorbax Sil column (6.2 × 250 mm) eluted isocratically with THF/hexane (1:1, v/v) and had retention times of 10.8 and 7.8 min, respectively. They exhibited UV-visible spectra similar to the analogous hydroxy-BAs. Mass spectral analysis of these phenolic products indicated that, while 9-hydroxy-7-Cl-BA did not contain oxygen-18 (molecular ion at m/z 278), 8-hydroxy-7-Cl-BA contained one atom of oxygen-18 (molecular ion at m/z 280) (Fig. 13). These results indicate that the oxygen atom of the hydroxyl group attached at C-8 of the 7-Cl-BA *trans*-8,9-dihydrodiol metabolite was derived from molecular oxygen (Fig. 14B).

Similarly, 5- and 6-hydroxy-7-Cl-BA were obtained from acid-catalyzed dehydration of the oxygen-18-con-

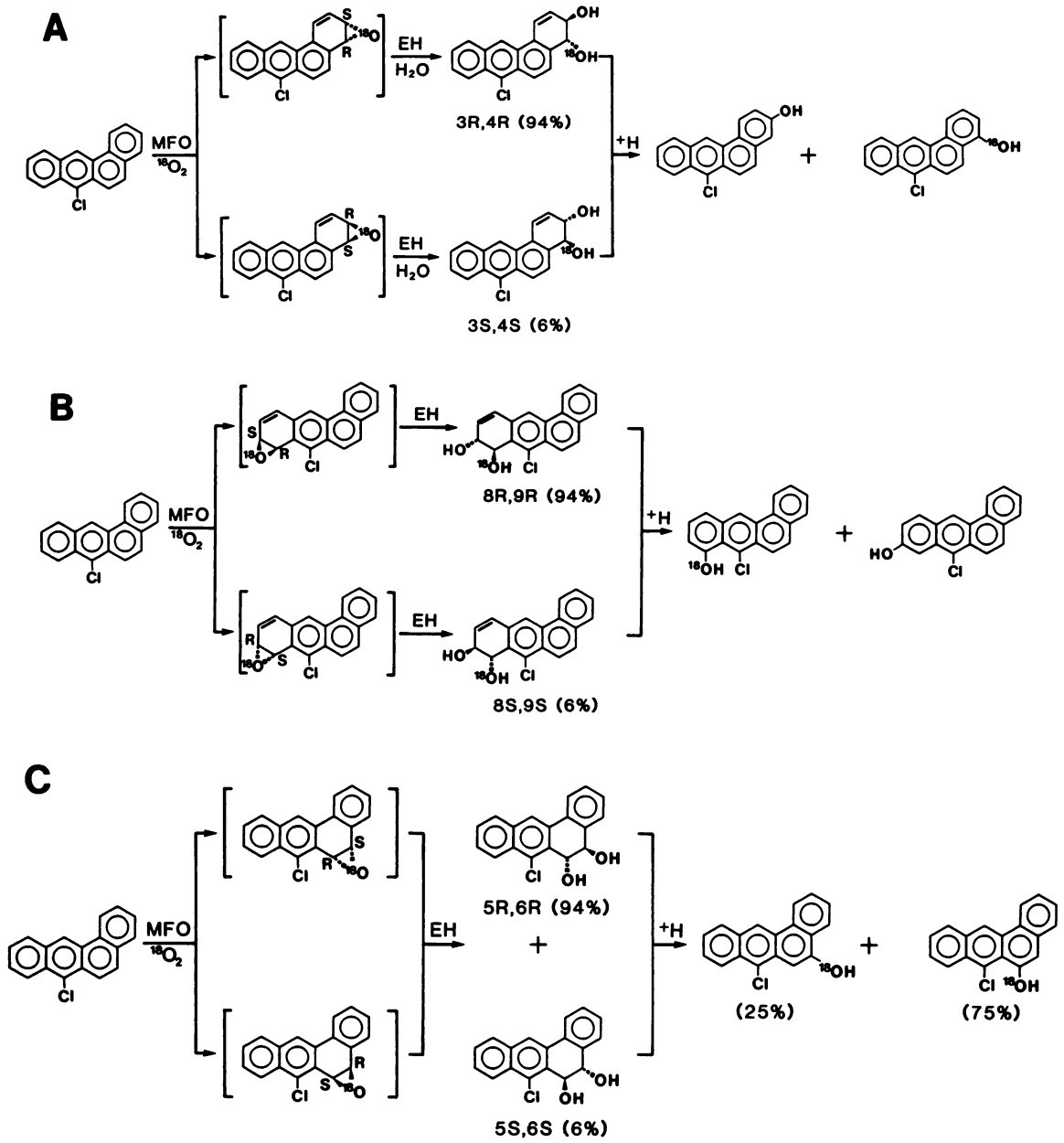

FIG. 14. *Stereoselective pathways of metabolism of 7-Cl-BA with liver microsomes of rats pretreated with 3-methylcholanthrene*

Formation of (A) 7-Cl-BA *trans*-3,4-dihydrodiol, (B) 7-Cl-BA *trans*-8,9-dihydrodiol, and (C) 7-Cl-BA *trans*-5,6-dihydrodiol studied by incubation under an oxygen-18 atmosphere, followed by acid-catalyzed dehydration of the resulting oxygen-18-containing *trans*-dihydrodiol metabolites. See Materials and Methods for experimental details. *MFO* and *EH* indicate steps catalyzed by the mixed function oxidases contained in the cytochrome P-450 enzyme systems and epoxide hydrolase, respectively.

taining 7-Cl-BA *trans*-5,6-dihydrodiol metabolite. They were separated by HPLC with conditions similar to those for separation of 3- and 4-hydroxy-7-Cl-BA. 5-Hydroxy-7-Cl-BA and 6-hydroxy-7-Cl-BA were eluted at 34.6 and 27.3 min, respectively. Mass spectral analysis of these phenols indicated that oxygen-18 was partially incorporated into both compounds, giving molecular ions at both *m/z* 278 and 280 (Fig. 12). Therefore, it cannot be concluded from which of the two hydroxyl groups that molecular oxygen was derived (Fig. 14C).

DISCUSSION

We have shown that rat liver microsomes metabolize 7-Cl-BA to *trans*-dihydrodiols in a highly stereoselective manner. Each of the 7-Cl-BA *trans*-3,4-, 5,6-, and 8,9-dihydrodiol metabolites formed from the three different microsomal preparations possessed predominantly *R,R* absolute stereochemistry. MC-microsomes exhibited highest stereoselectivity in the formation of the 7-Cl-BA *trans*-3,4- and 5,6-dihydrodiol (Table 1). For 7-Cl-BA *trans*-8,9-dihydrodiol formation, all three of the microsomal systems showed a very high stereoselectivity, with optical purities of 88–98%. Formation of a *trans*-dihydrodiol is through two enzymatic steps: first, epoxidation of the substrate which is catalyzed by cytochrome P-450, and second, hydrolysis of the epoxide to a *trans*-dihydrodiol which is catalyzed by epoxide hydrolase. The oxygen-

18 incorporation and acid-catalyzed dehydration experiments indicate that both cytochrome P-450 and epoxide hydrolase catalyze the transformations in a highly stereoselective manner for the formation of 7-Cl-BA *trans*-3,4- and 8,9-dihydrodiol metabolites (Fig. 14). Furthermore, these results indicate that 7-Cl-BA 3*S*,4*R*-epoxide and 7-Cl-BA 8*R*,9*S*-epoxide were the predominant enantiomeric intermediates. On the other hand, due to the incorporation of oxygen-18 into both the 5- and the 6-phenol, the exact stereoselectivity of both cytochrome P-450 and epoxide hydrolase toward the 7-Cl-BA *trans*-5,6-dihydrodiol formation remains unknown. However, the overall stereoselectivity of these two enzymatic steps for the 7-Cl-BA *trans*-5,6-dihydrodiol is very high, in the range of 78 to 88%, depending upon the enzyme sources (Table 1). The oxygen-18 incorporation and acid-catalyzed dehydration experiments also showed that epoxide hydrolase catalyzes the enzymatic hydration of the 7-Cl-BA 3,4- and 8,9-epoxide metabolites (Fig. 14) through a *trans* addition of a water molecule from the less hindered side of the chlorinated PAH epoxides. These results are consistent with the previous report that epoxide hydrolase catalyzes hydration by attacking at the least hindered epoxide carbon atom (18, 19). Thus, our results illustrate that the enzymatic systems which stereoselectively metabolize PAHs and their derivatives similarly metabolize 7-Cl-BA in a highly stereoselective manner. Since chlorinated pesticides and chlorinated drugs are commonly used, the results of our study suggest that metabolism of these compounds would also occur in a highly stereoselective manner.

CPK space-filling models suggest that a chloro substituent can exert a steric effect on a *peri*-hydroxyl group. Additionally, since a chloro atom has a high electronegativity, it can also exert an electronic effect on the peri-hydroxyl group. Therefore, as with a fluoro or a bromo substituent, the chloro substituent forces the 7-Cl-BA *trans*-5,6- and 8,9-dihydrodiols to adopt quasidiaxial conformations. Conformational analysis of a series of substituted *trans*-dihydrodiols indicates that all the substituents, methyl (7, 20), hydroxymethyl (20), fluoro (9, 10), chloro (results reported in this paper), and bromo (11, 21), can force the *peri-trans*-dihydrodiols to adopt the diaxial conformations. The exception is the nitro substituent of 7-nitrobenz(a)anthracene (22), which cause the *peri-trans*-dihydrodiols to adopt a mixture of quasi-diequatorial and diaxial conformations. Conformational analysis of substituted *trans*-dihydrodiols is an important aspect in the study of the structure-activity relationships of PAHs, since the mutagenic/carcinogenic activity of some PAH *trans*-dihydrodiol metabolites is affected by their conformation (1).

ACKNOWLEDGMENTS

We thank D. W. Miller for the NMR spectral measurements, and J. P. Freeman and L. E. Unruh for the mass spectral measurements.

REFERENCES

1. Conney, A. H. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes memorial lecture. *Cancer Res.* **42**:4875–4917 (1982).
2. Yang, S. K. The absolute stereochemistry of the major *trans*-dihydrodiol enantiomers formed from 11-methylbenz(a)anthracene by rat liver microsomes. *Drug Metab. Dispos.* **10**:205–211 (1982).
3. Yang, S. K., M. W. Chou, P. P. Fu, P. P. Wislocki, and A. Y. H. Lu. 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. *Proc. Natl. Acad. Sci. USA* **79**:6802–6808 (1982).
4. Fu, P. P., M. W. Chou, and S. K. Yang. *In vitro* metabolism of 12-methylbenz(a)anthracene: effect of the methyl group on the stereochemistry of a 5,6-dihydrodiol metabolite. *Biochem. Biophys. Res. Commun.* **106**:940–946 (1982).
5. Chiu, P.-L., H. B. Weems, T. K. Wong, P. P. Fu, and S. K. Yang. Stereoselective metabolism of benzo(a)pyrene and 7-methylbenz(a)pyrene by liver microsomes from Sprague-Dawley rats pretreated with polychlorinated biphenyls. *Chem.-Biol. Interact.* **44**:155–168 (1983).
6. Chou, M. W., P.-L. Chiu, P. P. Fu, and S. K. Yang. The effect of enzyme induction on the stereoselective metabolism of optically pure (−)1*R*,2*R* and (+)1*S*,2*S*-dihydroxy-1,2-dihydrobenz(a)anthracene to vicinal 1,2-dihydrodiol 3,4-epoxides. *Carcinogenesis* **4**:629–638 (1983).
7. Yang, S. K., and P. P. Fu. Stereoselective metabolism of 7-methylbenz(a)anthracene: absolute configurations of five dihydrodiol metabolites and the effect of dihydrodiol conformation on circular dichroism spectra. *Chem.-Biol. Interact.* **49**:71–88 (1984).
8. Yang, S. K., M. W. Chou, F. E. Evans, and P. P. Fu. Metabolism of 8-hydroxymethylbenz(a)anthracene by rat liver microsomes: stereochemistry of dihydrodiol metabolites and the effect of enzyme induction. *Drug Metab. Dispos.* **12**:403–413 (1984).
9. Chiu, P.-L., P. P. Fu, and S. K. Yang. 7-Fluorobenz(a)anthracene metabolism: stereoselectivity of rat liver microsomal enzymes and mutagenicity of metabolites. *Cancer Res.* **44**:562–570 (1984).
10. Thakker, D. R., H. Yagi, J. M. Sayer, U. Kapur, W. Levin, R. L. Chang, A. W. Wood, A. H. Conney and D. M. Jerina. Effects of a 6-fluoro substituent on the metabolism of benzo(a)pyrene 7,8-dihydrodiol to bay-region diol epoxides by rat liver enzymes. *J. Biol. Chem.* **259**:11249–11256 (1984).
11. Fu, P. P., and S. K. Yang. Stereoselective metabolism of 7-bromobenz(a)anthracene by rat liver microsomes: absolute configurations of *trans*-dihydrodiol metabolites. *Carcinogenesis* **4**:979–984 (1983).
12. Sundstrom, G., O. Hutzinger, and S. Safe. The metabolism of chlorobiphenyls—a review. *Chemosphere* **5**:267–298 (1976).
13. Nonhebel, D. C. 9-Chloroanthracene. *Org. Syn. Coll. V.* 206–208 (1973).
14. Chou, M. W., and S. K. Yang. Combined reversed-phase and normal-phase high performance liquid chromatography in the purification and identification of 7,12-dimethylbenz(a)anthracene metabolites. *J. Chromatogr.* **185**:635–654 (1979).
15. Yang, S. K., H. B. Weems, M. Mushtaq, and P. P. Fu. Direct resolution of mono- and diol enantiomers of unsubstituted and methyl-substituted benz(a)anthracene and benzo(a)pyrene by high-performance liquid chromatography with a chiral stationary phase. *J. Chromatogr.* **316**:569–584 (1984).
16. Zacharias, D. E., J. P. Glusker, P. P. Fu, and R. G. Harvey. Molecular structures of the dihydrodiols and diol epoxides of carcinogenic polycyclic aromatic hydrocarbons: X-ray crystallographic and NMR analysis. *J. Am. Chem. Soc.* **101**:4043–4051 (1979).
17. Weems, H. B., and S. K. Yang. Resolution of optical isomers by chiral high performance liquid chromatography: separation of dihydrodiols and tetrahydrodiols of benzo(a)pyrene and benz(a)anthracene. *Anal. Biochem.* **125**:156–161 (1982).
18. Hanzlik, R. P., M. Edelman, W. J. Michaely, and G. Scott. Enzymatic hydration of [^{18}O] epoxides, role of nucleophilic mechanisms. *J. Am. Chem. Soc.* **98**:1952–1955 (1976).
19. Lu, A. Y. H., and G. T. Miwa. Molecular properties and biological functions of microsomal epoxide hydrase. *Annu. Rev. Pharmacol. Toxicol.* **20**:513–531 (1980).
20. Yang, S. K., M. W. Chou, and P. P. Fu. Metabolic and structural requirements for the carcinogenic potencies of unsubstituted and methyl-substituted polycyclic aromatic hydrocarbons, in *Carcinogenesis: Fundamental Mechanisms and Environmental Effects* (B. Pullman, P. O. P. Ts'o, and H. V. Gelboin, eds.). D. Reidel Publishing Co., Dordrecht, 143–156 (1980).
21. Fu, P. P., and S. K. Yang. Stereoselective metabolism of 6-bromobenzo(a)pyrene by rat liver microsomes: absolute configuration of *trans*-dihydrodiol metabolites. *Biochem. Biophys. Res. Commun.* **109**:927–934 (1982).
22. Fu, P. P., and S. K. Yang. Stereoselective metabolism of 7-nitrobenz(a)anthracene by rat liver microsomes: absolute configurations of *trans*-dihydrodiol metabolites. *Carcinogenesis* **4**:979–984 (1983).

Send reprint requests to: Peter P. Fu, National Center for Toxicological Research, Jefferson, AR 72079.