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Comparative regioselective and stereoselective metabolism of 7-chlorobenz[*a*]anthracene and 7-bromobenz[*a*]anthracene by mouse and rat liver microsomes

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Quantitative metabolism of 7-chlorobenz[*a*]anthracene (7-Cl-BA) and 7-bromobenz[*a*]anthracene (7-Br-BA) by liver microsomes of uninduced mice and rats was studied. Both enzymatic systems metabolize 7-Cl-BA preferentially at the C-8 and C-9 aromatic double bond region, ~42 and ~56% respectively, of the total metabolites. 7-Cl-BA and 7-Br-BA were metabolized considerably at C-3 and C-4, C-5 and C-6, C-8 and C-9, and C-10 and C-11. While 7-Cl-BA *trans*-3,4-dihydrodiol was formed in a 7–8% yield of the total metabolites in both enzymatic systems, 7-Br-BA *trans*-3,4-dihdyrodiol was formed 16.0 and 9.9% respectively, from the mouse and rat liver microsomal metabolism. In mutagenicity assays with the *Salmonella typhimurium* tester strain TA100 in the presence of S9 activation enzymes, both of these *trans*-3,4-dihydrodiols exhibited higher mutagenicity than 7-Cl-BA and 7-Br-BA, while the other *trans*-dihydrodiol metabolites were either essentially inactive or weaker than the parent compounds. These results suggest that 7-Cl-BA *trans*-3,4-dihydrodiol and 7-Br-BA *trans*-3,4-dihydrodiol are the proximate metabolites of 7-Cl-BA and 7-Br-BA. Metabolism of 7-Cl-BA and 7-Br-BA by mouse liver microsomes was also in a stereoselective manner, preferentially giving *trans*-dihydrodiol metabolites an *R, R* stereochemistry.

Introduction

Polycyclic aromatic hydrocarbons (PAHs*) are the most common particulate environmental contaminant formed from incomplete combustion of organic matter, and many PAHs have been found to be carcinogenic in experimental animals (1). PAHs require metabolic activation in order to exert their biological activities, including mutagenicity and carcinogenicity (1–3). We have long been interested in determining how substituents affect the metabolism of PAHs, so that insight into mechanisms by which these compounds induce tumors can be obtained (4). In this regard, besides the commonly used methyl and fluoro substituents (5–14), we have also employed the chloro and bromo substituents to probe the geometric regions of PAHs that are involved in metabolic activation (14–18). The results together with the report by Shou and Yang (19) on the stereoselective metabolism of 1-bromopyrene indicate that metabolism of chloro- and bromo-PAHs is highly stereoselective. Recently, chlorinated and brominated PAHs have been detected in the environment, including municipal incinerator fly ash (20,21), urban air, snow and automobile exhaust (22). Some brominated and chlorinated PAHs have been shown to be potent mutagens to *Salmonella typhimurium* assay in the presence of mammalian activation enzymes (22–26), and 7-Br-BA is a weak tumor initiator (1). These reports have prompted us to study further the quantitative metabolism of the chlorinated and brominated PAHs and determine the metabolic activation pathways for mutation induction. We have previously reported the stereoselective metabolism of 7-bromobenz[*a*]anthracene (7-Br-BA) by rat liver microsomes, from which 7-Br-BA *trans*-3,4-, 5,6-, 8,9- and 10,11-dihydrodiols, and 4-hydroxy-7-Br-BA were identified; and the absolute configuration of the *trans*-dihydrodiols were determined to be predominantly *R,R* (16). We have also reported the metabolism of 7-chlorobenz[*a*]anthracene (7-Cl-BA) by rat liver microsomes, from which the 7-Cl-BA *trans*-3,4-, 5,6- and 8,9-dihydrodiols were identified and their optical purity was determined (17). In this paper, we report the quantitative stereo- and regioselective metabolism of 7-Cl-BA and 7-Br-BA by both mouse liver microsomes and rat liver microsomes. We have identified several more phenolic metabolites and studied the quantification for regioselectivity. Furthermore, the mutagenic activity of the metabolites as well as the parent substrates has been assayed so that the metabolic activation pathway toward mutation can be determined. By comparison with the results of benz[*a*]anthracene (BA), our results indicate that the chloro and bromo substituents can enhance the formation of the *trans*-3,4-dihydrodiol metabolite as well as increase the stereoselectivity of the metabolizing enzymes.

*Abbreviations: PAH, polycyclic aromatic hydrocarbon; 7-Br-BA, 7-bromobenz[*a*]anthracene; 7-Cl-BA, 7-chlorobenz[*a*]anthracene; BA, benz[*a*]anthracene; 7-Cl-BA *trans*-3,4-dihydrodiol, *trans*-3,4-dihydroxy-3,4-dihydro-7-Cl-BA (other dihydrodiols of 7-Cl-BA, 7-Br-BA and BA are similarly designated); NBS, *N*-bromosuccimide; 3-MC, 3-methylcholanthrene; THF, tetrahydrofuran; DMF, dimethylformamide; control microsomes, liver microsomes of uninduced mice or rats; 3-MC microsomes, liver microsomes of rats pretreated with 3-MC; B[*a*]P, benzo[*a*]pyrene; 7-MBA, 7-methylbenz[*a*]anthracene.

Materials and methods

7-Cl-BA and 7-Br-BA were prepared as previously described (16,17). Racemic BA 8,9,10,11-tetrahydro-*trans*-8,9-diol was prepared by catalytic hydrogenation of BA *trans*-8,9-dihydrodiol (16). BA, *N*-bromosuccimide (NBS), 3-methylcholanthrene (3-MC), triethylamine and tetrahydrofuran (THF) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Adam's catalyst (PtO_2) was obtained from Ventron Chemical Co., and all solvents used were HPLC grade.

Synthesis of [10,11-^{3}H]7-Br-BA

A solution of NBS (27 mg, 1.52 mmol), [10,11-^{3}H]BA (18.8 mg, 0.823 mmol, sp. act. 600 mCi/mmol) and dimethylformamide (DMF) (5 ml) was stirred overnight at ambient temperature under an argon atmosphere. The reaction was quenched by the addition of 50 ml of ice-cooled water. The precipitate was removed by suction filtration and washed with water. The residue was dissolved in acetone (10 ml) and the solvent was removed under reduced pressure. The crude product contained a trace of [10,11-^{3}H]BA and was purified by reversed-phase HPLC to purity of >99.9%.

Synthesis of [10,11-^{3}H]7-Cl-BA

[10,11-^{3}H]Benz[*a*]anthracene (22.43 mg, 0.0982 mmol, 590 mCi/mmol) in carbon tetrachloride (CCl_4), was added to $CuCl_2$ (0.195 mmol), necessary to prevent the formation of dichloro-BA. The reaction was purged with argon and heated to reflux for 22 h and allowed to stand overnight at room temperature. The $CuCl_2$ precipitate was removed by suction filtration. The filtrate was evaporated by passing a stream of argon over the mother liquid. The residue had a weight of 23.2 mg. Upon HPLC, the recovered BA and 7-Cl-BA were

found to be in a 2:1 ratio. The product was purified by HPLC using a Zorbax ODS column (9.4 × 250 mm) with 100% methanol at a flow rate of 4.0 ml/min. The retention times of BA and 7-Cl-BA were 7.8 and 12.2 min respectively.

Preparation of mouse and rat liver microsomes

The liver microsomes of male B6C3F1 mice (3 months old, 26–28 g body wt), obtained from the NCTR breeding colony, were prepared as previously described for the preparation of mouse liver microsomes (27). The liver microsomes of male Sprague–Dawley rats (100–120 g body wt), obtained from the NCTR breeding colony, were prepared similarly, with the modification that the livers were perfused with 0.15 M KCl before removal from the body. The protein content and the quantity of cytochrome P450 enzymes were assayed according to established procedures (28,29).

Metabolism of 7-Cl-BA and 7-Br-BA by mouse and rat liver microsomes

Metabolism with mouse liver microsomes. Metabolism of 7-Cl-BA and 7-Br-BA by mouse liver microsomes was conducted in a 300 ml volume. The incubation mixture contained 15 mmol of Tris–HCl, pH 7.5, 0.9 mmol of magnesium chloride, 0.3 mmol of NADP (Sigma, sodium salt), 0.6 mmol of glucose-6-phosphate (Sigma, monosodium salt), 30 units of glucose-6-phosphate dehydrogenase (Sigma, type II), 300 mg of microsomal protein and 24 μmol of 7-Cl-BA or 7-Br-BA (dissolved in 10 ml of acetone). Incubation was conducted with shaking for 1 h at 37°C, and the reaction was quenched by addition of 300 ml of acetone. The metabolites and residual substrate were extracted with ethyl acetate (600 ml). The organic fraction was evaporated under reduced pressure. The residue was suspended in acetone (2 × 5 ml) and the insoluble materials were removed by centrifugation. The acetone was removed and the residue was redissolved in methanol for use as standards in quantification (see below) and for reversed-phase HPLC separation of the metabolites.

Metabolism with rat liver microsomes. Metabolism of 7-Br-BA by liver microsomes of rats pretreated with 3-MC (3-MC microsomes) was similar to those described for the mouse liver microsomes, with the exception that a 500 ml incubation volume was used.

Quantification of the metabolites formed

Incubation of [10,11-^{3}H]7-Cl-BA (sp. act. 505.7 mCi/mmol) and [10,11-^{3}H]7-Br-BA (sp. act. 971.7 mCi/mmol) was conducted under conditions described above for the unlabeled 7-Cl-BA and 7-Br-BA, except that a 1 ml incubation volume containing 80 nmol/ml of substrate, 0.1 mg of microsomal protein of uninduced rats or mice, and a 10 min incubation period were used.

HPLC separation of the 7-Cl-BA and 7-Br-BA metabolites

Reversed-phase HPLC was performed with a Waters Associates system containing two 510 pumps, a 680 solvent programmer, a U6K injector and a 440 UV detector (254 nm). Metabolites and the recovered substrates were separated on a Du Pont Zorbax semi-preparative ODS column (9.4 × 250 mm). 7-Cl-BA and its metabolites were separated with a 20 min linear gradient 50–100% methanol in water at a flow rate of 2 ml/min. 7-Br-BA and its metabolites were separated with a 30 min linear gradient 60–100% methanol in water at a flow rate of 2 ml/min. A Hewlett-Packard 1040A detection system with the Data Processing Unit option was used to record the HPLC profiles as well as to obtain the UV–visible absorption spectra of the chromatographic peaks. To stabilize the *trans*-dihydrodiol metabolites, 1% of triethylamine was added to the elution solvents.

Catalytic hydrogenation and dehalogenation of the trans-3,4- and 8,9-dihydrodiol metabolites of 7-Br-BA and 7-Cl-BA

7-Br-BA *trans*-3,4-dihydrodiol (0.6 mg), obtained from metabolism of 7-Br-BA with 3-MC microsomes, was dissolved in 1 ml of THF and hydrogenated (15 p.s.i. on gauge) over Adam's catalyst (10 mg) in a Parr apparatus at ambient temperature for 3 h. Triethylamine (0.1 ml) was then added, and the reaction was allowed to continue for an additional 30 h. The reaction mixture was filtered through Celite and washed with acetone. The filtrate was collected and the solvent was evaporated under reduced pressure. The residue was purified by injection onto a Du Pont Zorbax SIL column (4.6 × 250 mm) and eluted isocratically with 30% THF in hexane (retention time, 13 min). The product was identified as BA 1,2,3,4-tetrahydro-*trans*-diol based upon comparison of its UV–visible absorption spectrum and HPLC retention time with those of a synthetically prepared standard (17,30).

Similarly, the 7-Br-BA *trans*-8,9-dihydrodiol metabolite from 3-MC microsomes was catalytically hydrogenated and debrominated to BA 8,9,10,11-tetrahydro-*trans*-8,9-diol, which was purified and identified as previously described (17). The 7-Br-BA *trans*-3,4-dihydrodiol and 7-Br-BA *trans*-8,9-dihydrodiol obtained from mouse liver microsomal metabolism were similarly catalytically converted to the corresponding BA tetrahydro-diols.

7-Cl-BA *trans*-3,4-dihydrodiol and 7-Cl-BA *trans*-8,9-dihydrodiol obtained from mouse liver microsomal metabolism were similarly converted to BA 1,2,3,4-tetrahydro-*trans*-3,4-diol and BA 8,9,10,11-tetrahydro-*trans*-8,9-diol respectively, as previously described (17).

Chiral HPLC direct resolution of trans-dihydrodiol metabolites of 7-Br-BA and 7-Cl-BA and their hydrogenated derivatives

Direct resolution of the enantiomers of the 7-Br-BA *trans*-5,6-dihydrodiol, 7-Cl-BA *trans*-5,6-dihydrodiol, and the BA 1,2,3,4-tetrahydro-*trans*-3,4-diol and BA 8,9,10,11-tetrahydro-*trans*-8,9-diol 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. 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. In each case, when direct resolution was achieved, the resolved *R,R* and *S,S* enantiomers were characterized by comparison of their UV–visible absorption spectra and HPLC retention times with those previously published or those of the synthetically prepared standards (17). The percentage of the *R,R* and *S,S* enantiomers was determined by a Hewlett-Packard 3390A integrator.

Physicochemical properties of metabolites

UV–visible absorption spectra of metabolites in methanol were measured on a Beckman DU-65 spectrophotometer. The radioactivity of the metabolites collected from the HPLC separation was measured with a Searle Analytical Mark III liquid scintillation spectrometer. CD spectra of the dihydrodiols were determined in 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.

Mutagenicity assay

Reversion to prototrophy using *Salmonella typhimurium* histidine auxotrophic tester strain TA100, obtained from Dr Bruce Ames of the University of California, Berkeley, CA, was measured essentially as described previously (31). In preparation for daily experiments, the cells were grown at 37°C for 12 h in Oxoid Nutrient Broth No. 2 (KC Biological, Lenexa, KS) in an incubator shaker. S9 fractions were prepared from the livers of male Sprague–Dawley rats pretreated with polychlorinated biphenyls (PCB). All assays were performed in triplicate. Colonies were counted with a Bactronic colony counter (New Brunswick Scientific, Edison, NJ). All compounds were tested on at least two separate occasions with similar results. A treatment which resulted in at least twice the number of revertants per plate above that obtained in the solvent control was considered to produce a positive response.

Results

HPLC separation and identification of metabolites

Metabolism of 7-Cl-BA by mouse liver microsomes. The ethyl acetate-extractable metabolites formed by incubation of 7-Cl-BA with mouse control microsomes were separated by reversed-phase HPLC (Figure 1). With the standards available from our previous study on the stereoselective metabolism of these compounds, and the better separation capability by using a Zorbax ODS semi-preparative column (9.4 × 250 mm), several new metabolites were identified. Besides the previously identified 7-Cl-BA *trans*-3,4-dihydrodiol, 7-Cl-BA *trans*-5,6-dihydrodiol, 7-Cl-BA *trans*-8,9-dihydrodiol and 4-hydroxy-7-Cl-BA, the newly identified metabolites are 7-Cl-BA *trans*-10,11-dihydrodiol, 5-hydroxy-, 6-hydroxy-, 8-hydroxy- and 9-hydroxy-7-Cl-BA. The identification of the 5-, 6-, 8- and 9-hydroxy-7-Cl-BA was based on the comparison of their UV–visible absorption spectra (Figures 2 and 3) and HPLC retention times with those of the previously prepared standards (17). The 7-Cl-BA *trans*-10,11-dihydrodiol identification was based on the analysis of its UV–visible absorption and mass spectral data.

Metabolism of 7-Br-BA by mouse liver microsomes. The ethyl acetate-extractable metabolites formed from incubation of 7-Br-BA with mouse control microsomes were similarly separated by the same reversed-phase HPLC system (Figure 4). In addition to the previously identified metabolites, which include 7-Br-BA *trans*-3,4-, 5,6-, 8,9- and 10,11-dihydrodiols and 4-hydroxy-7-Br-BA, by comparison with the UV–visible absorption spectra of the hydroxy-7-Cl-BA analogs (Figures 2 and 3) and their mass spectral data, 5-, 8- and 9-hydroxy-7-Br-BA were identified.

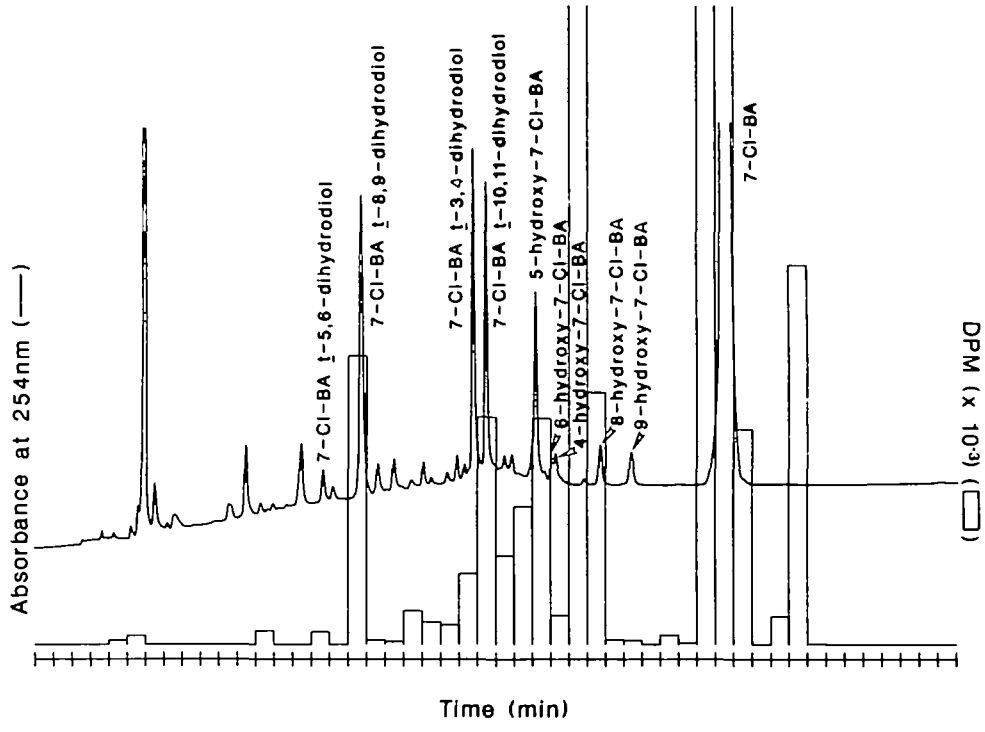

Fig. 1. Reversed-phase HPLC profile of the organic solvent-extractable metabolites obtained from incubation of 7-Cl-BA with mouse liver microsomes, and radioactivity profiles of metabolism of [^{3}H]7-Cl-BA by mouse liver microsomes For experimental conditions, see Materials and methods.

Absolute configuration of the trans-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA from mouse liver microsomal metabolism

The absolute configuration of 7-Br-BA *trans*-3,4-, 5,6-, 8,9- and 10,11-dihydrodiol metabolites obtained from incubation of 7-Br-BA with 3-MC microsomes and of 7-Cl-BA *trans*-3,4-, 5,6- and 8,9-dihydrodiols obtained from incubation of 7-Cl-BA with 3-MC microsomes has been previously reported by us (16,17). In all cases, the major enantiomers of each *trans*-dihydrodiol have an *R,R* stereochemistry and the CD spectra of each metabolite have been published (16,17). Therefore, direct comparison of the CD spectra obtained from mouse liver microsomal metabolism with those obtained from rat liver microsomal metabolism enabled the determination of the absolute configuration of the *trans*-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA from the mouse liver microsomal metabolism. In this way, we have determined that the major enantiomers of all the *trans*-3,4-, 5,6-, 8,9- and 10,11-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA have an *R,R* stereochemistry.

Optical purity of the trans-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA

7-Br-BA trans-dihydrodiol metabolites from 3-MC microsomes. The absolute configuration of the 7-Br-BA *trans*-3,4-, 5,6- and 8,9-dihydrodiol metabolites obtained from incubation of 7-Br-BA with 3-MC microsomes has been previously reported by us (16). In all cases, the major enantiomers of each *trans*-dihydrodiol have an *R,R* stereochemistry (16). Direct resolution of 7-Br-BA *trans*-5,6-dihydrodiol was achieved by employing the Pirkle chiral stationary phase HPLC column (Figure 5A) eluted with a mixture of ethanol, acetonitrile and hexane (2:1:40) at 2 ml/min. The results indicated that this *trans*-5,6-dihydrodiol contained 98% 5*R*,6*R* enantiomer and 2% 5*S*,6*S* enantiomer. Thus, the optical (enantiomeric) purity was found to be 96%.

However, similar to the results for direct resolution of the 7-Cl-BA *trans*-3,4- and 8,9-dihydrodiols (17), attempts to resolve

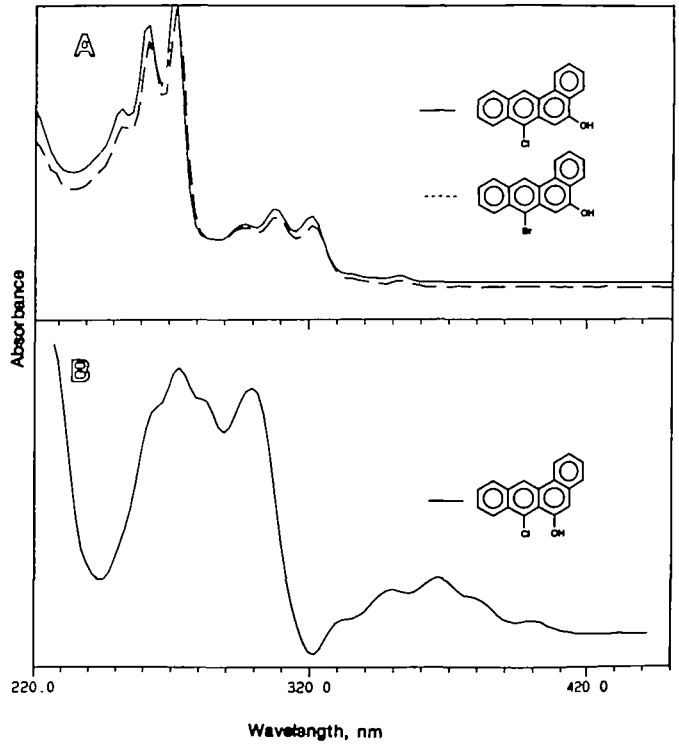

Fig. 2. UV–visible absorption spectra of metabolites identified as (**A**) 5-hydroxy-7-Cl-BA (——) and 5-hydroxy-7-Br-BA (---) and (**B**) 6-hydroxy-7-Cl-BA.

the enantiomers of the 7-Br-BA *trans*-3,4-dihydrodiol and 7-Br-BA *trans*-8,9-dihydrodiol by the same Pirkle chiral stationary phase HPLC column were not successful. Therefore, both 7-Br-BA *trans*-3,4- and 8,9-dihydrodiols were catalytically hydrogenated and debrominated to the BA 1,2,3,4-tetrahydro-

trans-3,4-diol and BA 8,9,10,11-tetrahydro-*trans*-8,9-diol respectively. Both of these hydrogenated BA derivatives can be directly resolved by the Pirkle column. A mixture of ethanol, acetonitrile and hexane with a ratio of 2:1:40 at a flow rate of 2 ml/min (Figure 5B) was used for the direct resolution of BA 1,2,3,4-tetrahydro-*trans*-3,4-diol, with the 3*S*,4*S* enantiomer (1%) eluting prior to the 3*R*,4*R* enantiomer (99%). The identity of the chromatographic peaks was confirmed by comparison with those of the synthetically prepared BA 1,2,3,4-tetrahydro-*trans*-3,4-diol. Thus, these results indicate that the 7-Br-BA *trans*-3,4-dihydrodiol metabolite obtained from metabolism of 7-Br-BA by 3-MC microsomes had an optical purity of 98%. For direct resolution of BA 8,9,10,11-tretrahydro-*trans*-8,9-diol, a mixture of ethanol, acetonitrile and hexane, with a ratio of 2:1:12 at a flow rate of 2 ml/min, was used. By comparison with the UV–visible absorption spectra and the HPLC retention times of the 8*R*,9*R* and 8*S*,9*S* enantiomers from direct resolution of BA 8,9,10,11-tetrahydro-*trans*-8,9-diol (Figure 5C), the 8*S*,9*S* and 8*R*,9*R* enantiomers from the 7-Br-BA *trans*-8,9-dihydrodiol metabolite were confirmed and were found to have a 1:99 ratio. Therefore, the optical purity of the 7-Br-BA *trans*-8,9-dihydrodiol was 98%. All the results are summarized in Table I.

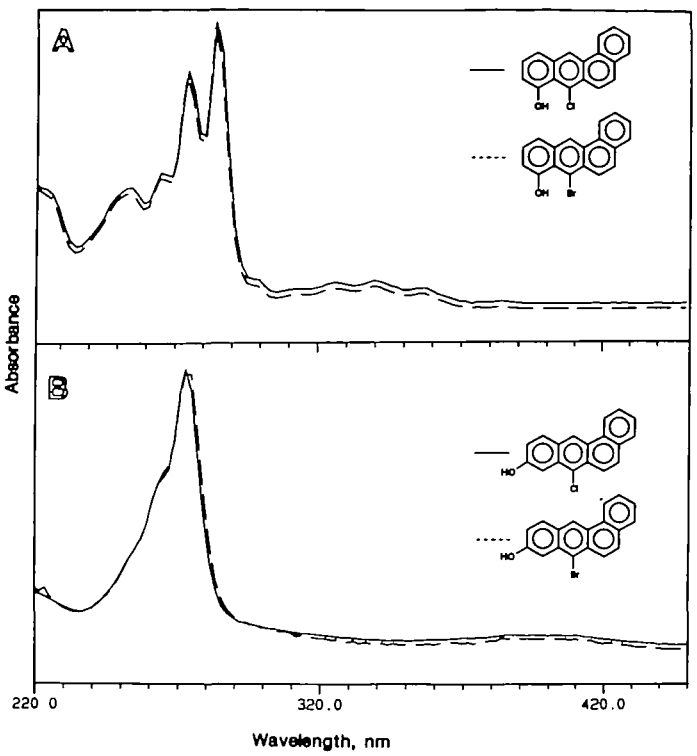

Fig. 3. UV–visible absorption spectra of metabolites identified as (**A**) 8-hydroxy-7-Cl-BA (——) and 8-hydroxy-7-Br-BA (---) and (**B**) 9-hydroxy-7-Cl-BA (——) and 9-hydroxy-7-Br-BA (---).

Trans-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA from mouse liver microsomal metabolism. Due to insufficient quantity of the metabolites, only the optical purity of the 7-Br-BA *trans*-3,4-dihydrodiol, 7-Cl-BA *trans*-3,4- and 8,9-dihydrodiol were similarly determined as those described above. As summarized in Table I, metabolism of 7-Br-BA by mouse liver microsomes yielded the 7-Br-BA *trans*-3,4-dihydrodiol with an enantiomeric ratio of 99% 3*R*,4*R* and 1% 3*S*,4*S*. For 7-Cl-BA *trans*-3,4-dihydrodiol, the ratio was found to be 88% 3*R*,4*R* enantiomer and 12% 3*S*,4*S* enantiomer. The 7-Cl-BA *trans*-8,9-dihydrodiol contained 70% 8*R*,9*R* enantiomer and 30% 8*S*,9*S* enantiomer.

Quantitation of metabolism of 7-Cl-BA and 7-Br-BA by mouse and rat control microsomes

[10,11-^{3}H]7-Cl-BA and [10,11-^{3}H]7-Br-BA were incubated with mouse and rat control microsomes for a period of 10 min. More

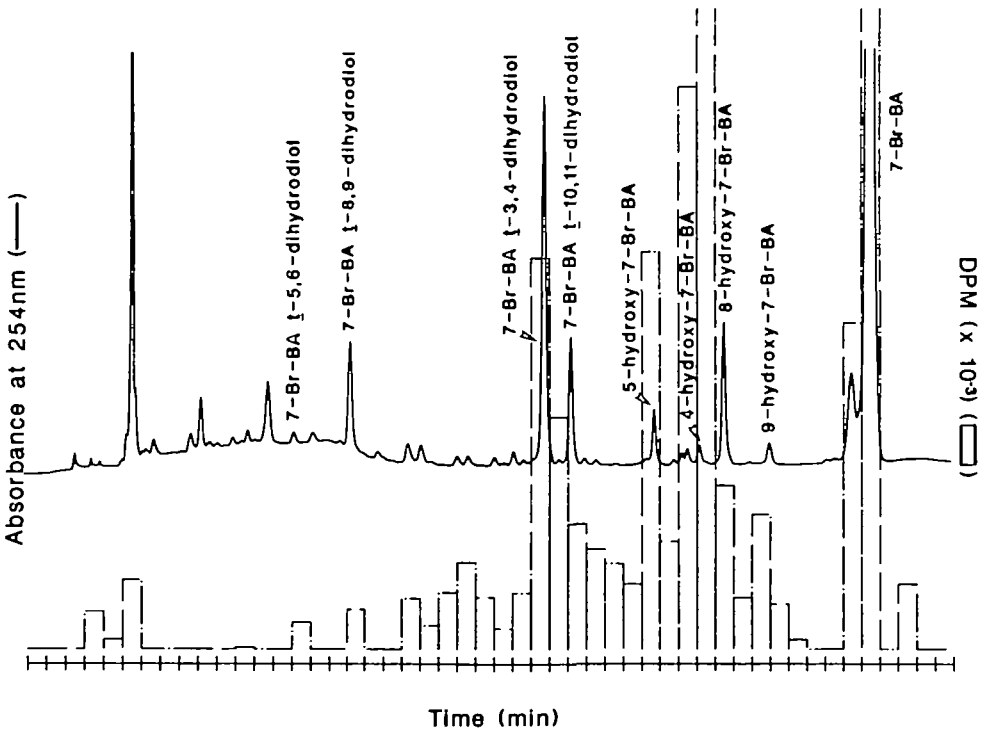

Fig. 4. Reversed-phase HPLC profile of the organic solvent-extractable metabolites obtained from incubation of 7-Br-BA with mouse liver microsomes, and radioactivity profiles of metabolism of 7-Br-BA by mouse liver microsomes.

than 99% of the total radioactivity was found in the ethyl acetate-extractable fractions. The radioactivity profile for metabolism of 7-Cl-BA and 7-Br-BA by mouse liver microsomes are shown in Figures 1 and 4 respectively. Under these incubation conditions, 7-Br-BA was metabolized faster than the 7-Cl-BA, and mouse control microsomes metabolize both compounds faster than the rat control microsomes. In both 7-Cl-BA and 7-Br-BA metabolism, considerable amounts of phenolic metabolites were formed. Both mouse and rat control microsomes preferentially metabolize 7-Cl-BA at the C-8 and C-9 region. 7-Br-BA *trans*-3,4- dihydrodiol was formed at a yield of 16.0 and 11.2%

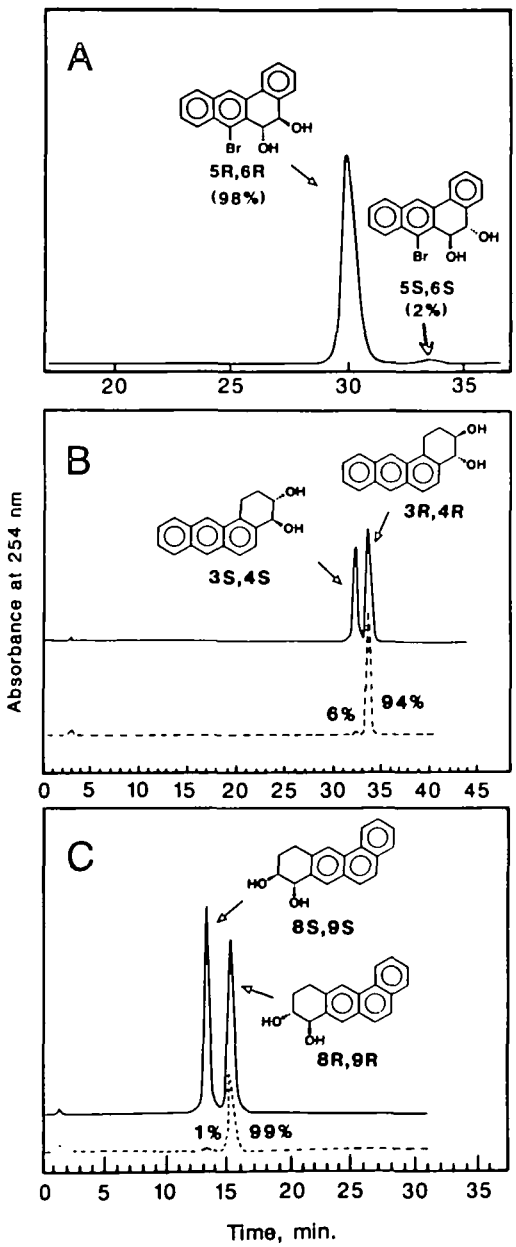

Fig. 5. Chiral stationary phase HPLC direct resolution of the 7-Br-BA *trans*-5,6-dihydrodiol metabolite obtained from incubation of 7-Br-BA with 3-MC microsomes (**A**), chiral stationary phase HPLC direct resolution of BA 1,2,3,4-tetrahydro-*trans*-3,4-diol standard (——) and from catalytic hydrogenation and debromination of the 7-Br-BA *trans*-3,4-dihydrodiol (—) obtained from incubation of 7-Br-BA with 3-MC microsomes (**B**), and chiral stationary phase HPLC direct resolution of BA 9,10,11-tetrahydro-*trans*-8,9-diol (—) obtained from catalytic hydrogenation and debromination of the 7-Br-BA *trans*-8,9-dihydrodiol obtained from incubation of 7-Br-BA with 3-MC microsomes and the synthetic standard (——) (**C**).

respectively from the metabolism with mouse and rat liver microsomes. Although the 7-Cl-BA *trans*-3,4-dihydrodiol formation was lower, at a 7.8 and 6.9% yield of the total metabolites from these two enzyme systems, the yields are still higher than that of the BA *trans*-3,4-dihydrodiol analog formed from the BA metabolism by rat control microsomes.

Mutagenicity of 7-Cl-BA, 7-Br-BA and their trans-dihydrodiol metabolites toward S.typhimurium

The mutagenicity of 7-Cl-BA, the *trans*-3,4-, 5,6- and 8,9-dihydrodiols of 7-Cl-BA, 7-Br-BA and the *trans*-3,4-. 5,6-, 8,9- and 10,11-dihydrodiols of 7-Br-BA was determined in the tester strain *S.typhimurium* TA100 in the presence of the S9 activation enzyme system. For comparison, the mutagenicity of benzo[*a*]pyrene (B[*a*]P) and 7-methylbenz[*a*]anthracene (7-MBA) was also determined. The mutagenicity results are shown in Table III. With the exception of B[*a*]P, all the compounds were assayed in four different doses: 5, 10, 15 and 20 nmol/plate. B[*a*]P exhibited the highest mutagenicity. For both the 7-Cl-BA and 7-Br-BA series, the *trans*-3,4-dihydrodiols exhibited higher mutagenic activity than the corresponding 7-Cl-BA and 7-Br-BA; the *trans*-8,9-dihydrodiols of 7-Cl-BA and 7-Br-BA and 7-Br-BA *trans*-10,11-dihydrodiol were weakly mutagenic; while the K-region 7-Cl-BA *trans*-5,6-dihydrodiol and 7-Br-BA *trans*-5,6-dihydrodiol were essentially inactive (Table III). A parallel mutagenicity was observed between the 7-Cl-BA and 7-Br-BA series. The mutagenic activity of 7-Cl-BA and its *trans*-dihydrodiols exhibited slightly higher mutagenic activity than their corresponding 7-Br-BA analogs.

Discussion

We previously described the metabolism of 7-Cl-BA and 7-Br-BA by rat liver microsomes, from which the absolute configuration of the *trans*-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA and the optical purity of the 7-Cl-BA *trans*-dihydrodiol metabolites were determined (16,17). 4-Hydroxy-7-Br-BA, 3-hydroxy-7-Cl-BA and 4-hydroxy-7-Cl-BA were also identified. In this paper, we describe the use of a semi-preparative ODS HPLC column and the identification of several previously unidentified phenolic metabolites of 7-Cl-BA and 7-Br-BA. With the newly identified metabolites, together with the availability of specifically tritium-labeled 7-Cl-BA and 7-Br-BA, direct comparison of quantitative and regioselective metabolism of 7-Cl-BA and 7-Br-BA by mouse and rat liver microsomes have been pursued. The results summarized in Table II indicate that the metabolizing enzymes contained in the mouse and rat liver microsomal fractions exhibit similar regioselectivity on the metabolism of 7-Cl-BA and 7-Br-BA. Both mouse and rat liver microsomal enzymes metabolize 7-Cl-BA preferentially at the C-8 and C-9 aromatic double bond region (~42 and 56% respectively, of the total metabolites formed), followed at the C-5 and C-6 K-region (19.6 and 14.7% respectively), then at the C-10 and C-11 region and the C-3 and C-4 region. Comparison of these results with those obtained from metabolism of BA under similar incubation conditions indicates that the chloro substituent at the C-7 position of BA does not significantly affect metabolism at the C-8 and C-9 region, but inhibits metabolism considerably at the C-5 and C-6 K-region (Table II). This effect exerted by the chloro substituent is similar to that observed for the metabolism of 7-F-BA by 3-MC microsomes (11).

It is worthwhile to note that in both microsomal systems the bromo substituent markedly affects the *trans*-3,4-dihydrodiol formation, giving 7-Br-BA *trans*-3,4-dihydrodiol a 16.0 and 9.9%

Table I. Optical purity of *trans*-dihydrodiol metabolites of 7-Cl-BA and 7-Br-BA obtained from metabolism of 7-Cl-BA and 7-Br-BA with rat and mouse liver microsomes[a]

Dihydrodiol	Microsomes	7-Cl-BA enantiomer (%)		7-Br-BA enantiomer (%)		BA[b] enantiomer (%)	
		R,R	*S,S*	*R,R*	*S,S*	*R,R*	*S,S*
3,4-Dihydrodiol	Control[c]	60[d] (88)[e]	40 (12)	ND[f]	ND		
	3-MC	94	6	99 (99)	1 (1)	90	10
	PB[c]	60	40	ND	ND		
5,6-Dihydrodiol	Control	92	8	ND	ND		
	3-MC	94	6	98	2	88	12
	PB	89	11	ND	ND		
8,9-Dihydrodiol	Control	94 (70)	6 (30)	ND	ND		
	3-MC	99	1	99	1	99	1
	PB	99	1	ND	ND		

[a]The data for percentage of the *R,R* and *S,S* enantiomers of the 7-Cl-BA *trans*-dihydrodiol metabolites obtained from incubation of 7-Cl-BA with rat liver microsomes are taken from our previously published results shown in ref. 17.
[b]Data were from Table II of ref. 11 for comparison.
[c]Control microsomes, liver microsomes of untreated animals; PB microsomes, liver microsomes of rats pretreated with phenobarbital.
[d]Data from metabolism by rat liver microsomes.
[e]Data from metabolism by mouse liver microsomes.
[f]ND, not determined.

Table II. Comparison of the metabolites formed from the metabolism of 7-Cl-BA, 7-Br-BA, 7-F-BA and BA by mouse and rat microsomes[a]

	pmol of product formed per nmol P450 per min (%metabolite formed)					
	7-Cl-BA		7-Br-BA		7-F-BA	BA
	Mouse microsomes	Rat microsomes	Mouse microsomes	Rat microsomes	Rat microsomes	Rat microsomes
3,4-Dihydrodiol	458 (7.8)	259 (6.7)	3653 (16.0)	1983 (19.9)	(9.8)	(1.6)
5,6-Dihydrodiol	171 (2.9)	115 (3.0)	698 (3.0)	285 (2.9)	(7.8)	(41.6)
8,9-Dihydrodiol	271 (4.6)	798 (20.8)	354 (1.5)	329 (3.3)	(30.8)	(48.9)
10,11-Dihydrodiol	1452 (24.8)	646 (16 8)	3400 (14.9)	1096 (11.0)	(9.8)	(3.8)
4-Hydroxy			4921 (21.5)	2072 (20.8)	(41.7)	
4-Hydroxy + 6-hydroxy	642 (11.0)	477 (12 4)				
5-Hydroxy	686 (11.7)	748 (19.5)	4768 (20.8)	2078 (20.9)		
8-Hydroxy	1959 (33.5)	707 (18.4)	2735 (12.0)	1109 (11.2)		
9-Hydroxy	214 (3.7)	93 (2.4)	2349 (10.3)	991 (10.0)		
Total metabolites identified	5853	3843	22878	9943		

[a]The percentages of radioactivity contained in each of the organic solvent extractable fractions are: metabolism of 7-Cl-BA by mouse control microsomes, 96.7%; metabolism of 7-Cl-BA by rat and control microsomes, 95.7%; metabolism of 7-Br-BA by mouse control microsomes, 97.5%; and metabolism of 7-Br-BA by rat control microsomes, 97.4%.

Table III. Mutagenicity of 7-Cl-BA, 7-Br-BA, their *trans*-dihydrodiol metabolites, 7-MBA and B[*a*]P to *S.typhimurium* TA100 in the presence of liver S9 activation enzymes[a]

Compound	Revertants/plate			
	5 nmol	10 nmol	15 nmol	20 nmol
B[*a*]P	544 ± 23	963 ± 56	–	–
BA	145 ± 12	197 ± 18	280 ± 21	327 ± 25
7-Cl-BA	118 ± 14	128 ± 7	221 ± 13	301 ± 14
3,4-Dihydrodiol	226 ± 23	403 ± 24	535 ± 29	731 ± 29
5,6-Dihydrodiol	119 ± 8	125 ± 7	121 ± 9	116 ± 7
8,9-Dihydrodiol	127 ± 7	141 ± 14	191 ± 12	214 ± 21
7-Br-BA	109 ± 17	112 ± 9	197 ± 11	252 ± 21
3,4-Dihydrodiol	206 ± 19	380 ± 23	501 ± 12	629 ± 26
5,6-Dihydrodiol	119 ± 11	111 ± 21	114 ± 9	110 ± 9
8,9-Dihydrodiol	115 ± 12	117 ± 19	175 ± 12	193 ± 17
10,11-Dihydrodiol	117 ± 8	118 ± 12	186 ± 8	221 ± 24
7-MBA	173 ± 18	211 ± 22	292 ± 17	344 ± 34

[a]Spontaneous revision, 145 ± 11.

of the total metabolites from the mouse and rat liver microsomal metabolism respectively. This is higher than the formation of BA *trans*-3,4-dihydrodiol formed in only 1.6% from the metabolism of BA by rat control microsomes (Table II). The effect of the chloro substituent on the *trans*-3,4-dihydrodiol formation (up to 7.8 and 6.9%) is similar to that of the fluoro substituent observed for the metabolism of 7-F-BA by rat control microsomes (Table II). The mutagenicity assay indicates that among the *trans*-dihydrodiol metabolites, the *trans*-3,4-dihydrodiols of both 7-Cl-BA and 7-Br-BA exhibit higher mutagenic activity than the parent 7-Cl-BA and 7-Br-BA respectively, while all the other *trans*-dihydrodiols exhibit either no mutagenicity or a mutagenic response lower than the parent compounds (Table III). These results show that 7-Cl-BA *trans*-3,4-dihydrodiol and 7-Br-BA *trans*-3,4-dihydrodiol are the activated metabolites of 7-Cl-BA and 7-Br-BA respectively towards mutation. These results also support the concept that the *bay*-region dihydrodiolepoxides, which can be obtained from further metabolism of the *trans*-3,4-dihydrodiols of 7-Cl-BA and 7-Br-BA, are the probable ultimate metabolites of 7-Cl-BA and 7-Br-BA towards mutation. It was reported that while 7-Cl-BA exhibited similar tumorigenic activity as BA when injected s.c. in rats, 7-Br-BA showed slightly higher activity (1). These results are in concordance with the higher yield of the 7-Br-BA *trans*-3,4-dihydrodiol formation found by us in both the mouse and rat liver microsomal metabolism.

Because PAH K-region epoxides are more stable than their corresponding non-K-region isomers, and are good substrates of the epoxide hydrolase enzymes, the commonly detected K-region metabolites are *trans*-dihydrodiols and epoxides, and K-region phenols are seldom formed (1,32,33). Theoretical prediction and experimental results are all in agreement with the findings that the more stable the arene oxide is, the higher percentage of the *trans*-dihydrodiol formed through hydrolysis and the less of the corresponding phenols are obtained through non-enzymatic rearrangement (33–35). Therefore, the formation of 5- and 6-hydroxy-7-Cl-BA and 5-hydroxy-7-Br-BA as metabolites is unusual. Apparently, the chloro and bromo substituents *peri* to the K-region oxides exert both steric and/or electronic effects on the stability of the oxides. While isomerization of BA yields 5- and 6-hydroxy-BA in a nearly equal ratio (34,35), more 5-hydroxy-7-Cl-BA was formed than the 6-hydroxy-7-Cl-BA and only 5-hydroxy-7-Br-BA, but without the detection of 6-hydroxy-7-Br-BA, was obtained from the metabolism of 7-Br-BA by mouse and rat liver microsomes (Table II). These observations further support the steric and electronic effects on the K-region oxides by the chloro and bromo substituents at the *peri* position.

The results summarized in Table II indicate that the mouse and rat liver microsomes metabolize 7-Cl-BA and 7-Br-BA stereoselectively, and in all cases provide the *R,R* enantiomers in a much higher yield than the corresponding *S,S* enantiomers. Due to insufficient quantity of the metabolites formed, 7-Cl-BA *trans*-3,4- and 8,9-dihydrodiols and 7-Br-BA *trans*-3,4-dihydrodiol are the only metabolites whose optical purity was determined in the mouse liver microsomal metabolism. Comparison of these results with those from the rat control microsomes (17) indicates that the mouse liver microsomes and rat control microsomes exhibit similar stereoselectivity on the metabolism of 7-Cl-BA and 7-Br-BA. On the other hand, 7-Cl-BA and 7-Br-BA are metabolized by the 3-MC microsomes in a highly stereoselective manner (Table I). In all cases, the *trans*-dihydrodiols of 7-Cl-BA and 7-Br-BA show higher optical purity than those of the unsubstituted parent compound, BA. Further, the 7-Br-BA *trans*-dihydrodiols show either the same or higher optical purity than the 7-Cl-BA *trans*-dihydrodiols.

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