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STEREOSELECTIVE HYDROXYLATION AT THE ALIPHATIC CARBONS OF 7,8- AND 9,10-DIHYDROBENZO[A]PYRENES BY RAT LIVER MICROSOMES

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SUMMARY: Optically active 7-hydroxy-7,8-dihydrobenzo[a]pyrene and 8-hydroxy-7,8-dihydrobenzo[a]pyrene were identified as two of the major metabolites formed by incubation of 7,8-dihydrobenzo[a]pyrene with rat liver microsomes. Optically active 9-hydroxy-9,10-dihydrobenzo[a]pyrene and 10-hydroxy-9,10-dihydrobenzo[a]-pyrene were similarly identified as two of the minor metabolites of 9,10-dihydrobenzo[a]pyrene. The formation of these metabolites was abolished either by prior treatment of liver microsomes with carbon monoxide or the absence of NADPH, but was not inhibited by an epoxide hydrolase inhibitor. The results indicate that the aliphatic carbons of dihydro polycyclic aromatic hydrocarbons may undergo stereoselective hydroxylation reactions catalyzed by the cytochrome P-450 system of rat liver microsomes.

Polycyclic aromatic hydrocarbons (PAHs)[1] are formed in incomplete combustion of organic materials (1,2). During the pyrolytic process, molecular fragmentation yields free radicals and partially saturated aromatic ring structures, which then undergo ring closure and dehydrogenation (2). Therefore, partially saturated PAHs may be generated during pyrolytic reactions to pollute the environment (3). Of the two dihydro-PAHs that have been tested for carcinogenicity by subcutaneous injection into mice, 7,8-dihydrobenzo[a]pyrene (7,8-H_2BaP) was proved to be a powerful carcinogen whereas the isomeric 9,10-dihydrobenzo[a]-pyrene (9,10-H_2BaP) was virtually inactive (unpublished results cited in ref. 4). Metabolic formation of 7,8-dihydrodiol 9,10-epoxides, the known biologically reactive metabolites of BaP (5,6), from both 7,8-H_2BaP and 9,10-H_2BaP has been thought to be impossible (7). Evidence presented in this communication indicates that BaP is a metabolite formed in the metabolism of both 7,8-H_2BaP and

[1] Abbreviations: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[a]pyrene; H_2BaP, dihydro-BaP; H_4BaP, tetrahydro-BaP; HPLC, high performance liquid chromatography; CSP, chiral stationary phase; THF, tetrahydrofuran; G-6-P, glucose-6-phosphate; PCBs, polychlorinated biphenyls (Aroclor 1254); CD, circular dichroism.

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9,10-H_2BaP by rat liver microsomes. The results also indicate that the cytochrome P-450 system of these microsomes can catalyze stereoselective hydroxylation reactions at the aliphatic carbons of both 7,8-H_2BaP and 9,10-H_2BaP.

MATERIALS AND METHODS

Materials: 9,10-Dihydrobenzo[a]pyrene-7(8H)-one (7-keto-BaP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). 7,8-Dihydrobenzo[a]pyrene-10(9H)-one (10-keto-BaP) was synthesized as described (8). Reduction of 7-keto-BaP and 10-keto-BaP in dry THF/methanol (1:1, v/v) with $NaBH_4$ yields 7-hydroxy-7,8,9,10-H_4BaP and 10-hydroxy-7,8,9,10-H_4BaP, respectively. Dehydration of 7-hydroxy-7,8,9,10-H_4BaP and 10-hydroxy-7,8,9,10-H_4BaP with HCl in dry benzene yields 9,10-H_2BaP and 7,8-H_2BaP, respectively. 9,10-Dihydrobenzo[a]pyrene-8(7H)-one (8-keto-BaP) and 7,8-dihydrobenzo[a]pyrene-9(10H)-one (9-keto-BaP) were synthesized as described (9-11). Reduction of 8-keto-BaP and 9-keto-BaP with $NaBH_4$ yields 8-hydroxy-7,8,9,10-H_4BaP and 9-hydroxy-7,8,9,10-H_4BaP, respectively. $LiAlH_4$, 3,3,3-trichloropropylene oxide were purchased from Aldrich Chemical Co.. Platinum (IV) oxide was purchased from ALFA Products (Danvers, MA). Hydrogenation of dihydro-BaP to tetrahydro-BaP was carried out in THF in the presence of PtO_2 with hydrogen at 1 atm.

Chromatography: Reversed-phase and chiral stationary phase HPLC were performed using a Waters Associates (Milford, MA) liquid chromatograph consisting of a Model 6000A solvent delivery system, a Model M45 solvent delivery system, a Model 660 solvent programmer, and a Model 440 absorbance (254 or 280 nm) detector. Samples were injected via a Valco model N60 loop injector (Valco, Houston, TX). Metabolites of 7,8-H_2BaP were separated with a DuPont Zorbax ODS column (25 cm x 4.6 mm ID). The column was eluted at ambient temperature with a 30-min linear gradient of 65% to 95% methanol in water (v/v) at a solvent flow rate of 1.2 ml/min. Metabolites of 9,10-H_2BaP were separated with a Waters Associates μBondapak C_{18} column (30 cm x 3.9 mm ID). The column was eluted with a 30-min linear gradient of 70% to 95% methanol in water (v/v) at a solvent flow rate of 1.0 ml/min. Enantiomers of 7-hydroxy- and 10-hydroxy-7,8,9,10-H_4BaP were resolved with an HPLC column (25 cm x 4.6 mm ID, Regis Chemical, Morton Grove, IL) packed with spherical particles (5 micrometer diameter) of γ-aminopropyl-silanized silica ionically bonded with (R)-N-(3,5-dinitrobenzoyl)phenylglycine (12). Samples were eluted isocratically with premixed solvents (v/v) of 5% (for 7-hydroxy-7,8,9,10-H_4BaP) or 2% (for 10-hydroxy-7,8,9,10-H_4BaP) of solvent A (ethanol:acetonitrile, 2:1, v/v) in hexane at a flow-rate of 2 ml/min (13,14).

Incubation of Dihydro-BaP with Rat Liver Microsomes: Male Sprague-Dawley rats weighing 80-100 g were treated i.p. with PCBs (50 mg dissolved in 0.5 ml of corn oil/kg body weight) once daily on each of four consecutive days. Liver microsomes were prepared as described (15). Metabolites were obtained by incubation of 7,8-H_2BaP or 9,10-H_2BaP (8 μmol in 4 ml acetone) under yellow light at 37°C for 30 min in 100-ml incubation mixtures (pH 7.5) each containing 5 mmol of Tris-HCl, 0.3 mmol of $MgCl_2$, 10 units of G-6-P dehydrogenase (type XII, Sigma), 10 mg of $NADP^+$, 65 mg of G-6-P, and 100 mg protein equivalent of rat liver microsomes. The dihydro-BaP and its metabolites were extracted with acetone (100 ml) and ethyl acetate (200 ml). The organic phase was dehydrated with anhydrous $MgSO_4$ and evaporated to dryness under reduced pressure. The residue was redissolved in 0.2 ml of methanol containing 2% (by vol.) of triethylamine for separation of metabolites by HPLC.

Spectral Analysis: Ultraviolet-visible absorption spectra of samples in methanol and in 0.1 N methanolic NaOH were determined using a Varian model 118C spectrophotometer. Mass spectral analysis was performed on a Finnigan model 4000 gas chromatograph-mass spectrometer-data system by electron impact with a solid probe at 70 eV and 250°C ionizer temperature. CD spectra of samples in methanol

were measured at room temperature using a Jasco model 500A spectropolarimeter equipped with a model DP-500 data processor. CD spectra are expressed by ellipticity (in millidegrees) as described (16).

RESULTS AND DISCUSSION

The metabolites formed by incubation of 7,8-H_2BaP and of 9,10-H_2BaP with liver microsomes prepared from PCBs-treated male Sprague-Dawley rats were separated by reversed-phase HPLC and are indicated by numbers in Figs. 1 and 2, respectively. Each metabolite was characterized by uv absorption and mass spectral analyses, and in the case of mono-ol and diol metabolites, also by CD spectral analysis. The identified metabolites are indicated in the legend of each figure. BaP and some of its metabolites were detected as metabolites of

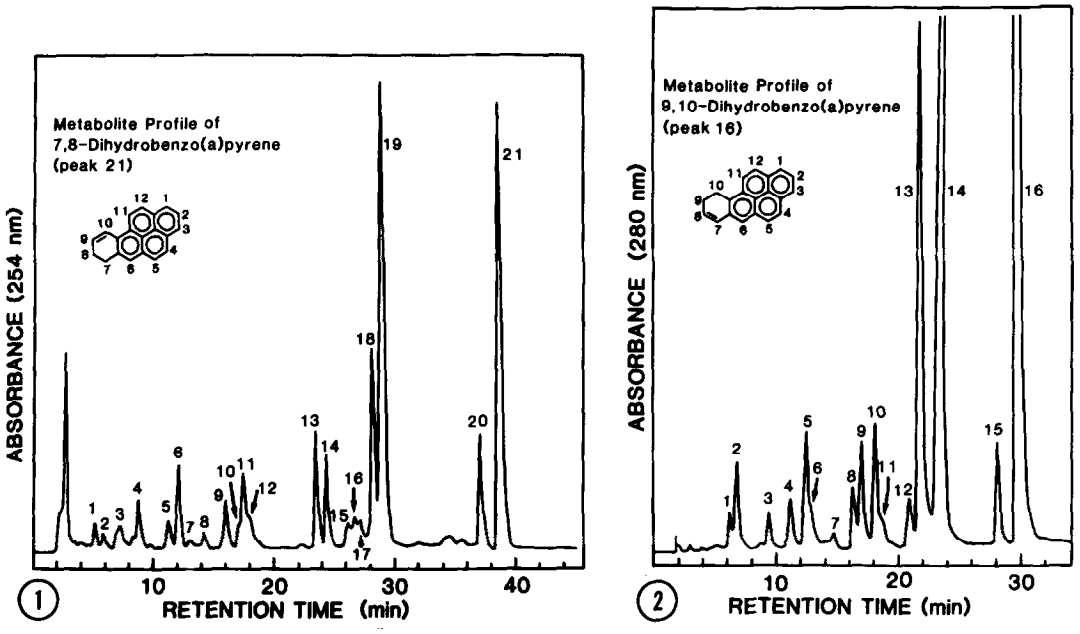

Fig. 1. Reversed-phase HPLC separation of the metabolites of 7,8-H_2BaP. Metabolites are numbered and the identified metabolites are: 4, BaP trans-9,10-dihydrodiol; 6, 7,8,9,10-H_4BaP trans-9,10-diol; 9, BaP trans-4,5-dihydrodiol; 10, BaP trans-7,8-dihydrodiol; 11, 7,8-H_2BaP 4,5-dihydrodiol; 12, 7,8,9,10-H_4BaP cis-9,10-diol; 13, 7-hydroxy-7,8-H_2BaP; 14, 8-hydroxy-7,8-H_2BaP; 15, BaP 1,6-quinone; 17, BaP 3,6-quinone; 18, phenols of BaP (major components are 1-hydroxy-BaP and 3-hydroxy-BaP); 19, phenols of 7,8-H_2BaP; 20, BaP. Peak 21 is the substrate 7,8-H_2BaP.

Fig. 2. Reversed-phase HPLC separation of the metabolites of 9,10-H_2BaP. Metabolites are numbered and the identified metabolites are: 5, 7,8,9,10-H_4BaP trans-7,8-diol; 6, 7,8,9,10-H_4BaP cis-7,8-diol; 9, 10-hydroxy-9,10-H_2BaP; 10, 9-hydroxy-9,10-H_2BaP; 11, BaP 3,6-quinone; 12, 9-hydroxy-BaP (major component); 13, 3-hydroxy-BaP (major component); 14, phenols of 9,10-H_2BaP (3-hydroxy-9,10-H_2BaP is the major component); 15, BaP. Peak 16 is the substrate 9,10-H_2BaP. The absorbance scale is expanded in order to show the presence of minor metabolite peaks.

both 7,8-H_2BaP and 9,10-H_2BaP. These findings are of special importance because they indicate that both 7,8-H_2BaP and 9,10-H_2BaP may be metabolically activated to 7,8-dihydrodiol 9,10-epoxides, the known carcinogenic metabolites of BaP (5,6). The bases for concluding that monohydroxylated products are formed at the aliphatic carbons of 7,8-H_2BaP and 9,10-H_2BaP are given below. The evidence for the identification of the other metabolites indicated in Figs. 1 and 2 will be presented in a later report.

The metabolites contained in chromatographic peaks 13 and 14 of Fig. 1 are identified as 7-hydroxy-7,8-H_2BaP and 8-hydroxy-7,8-H_2BaP, respectively. The uv absorption spectra of these metabolites in methanol are similar to that of the parent compound, indicating that the carbons at the C_7 and C_8 positions are saturated. Mass spectral analyses indicate that they are monohydroxylated products of 7,8-H_2BaP yielding molecular ions at m/z 270 and characteristic fragment ions at m/z 252. The uv absorption properties are not changed when the metabolites are dissolved in alkaline solutions. These results indicate that the hydroxyl group in each metabolite is not phenolic and is at either the C_7 or the C_8 position of 7,8-H_2BaP. The exact location of the hydroxyl group in each of the two metabolites was established by conversion to the 7,8,9,10-tetrahydro derivatives by catalytic hydrogenation. The hydrogenation products of chromatographic peaks 13 and 14 of Fig. 1 were identical (with respect to uv absorption spectrum, mass spectrum and retention time on reversed-phase HPLC) to those of authentic 7-hydroxy-7,8,9,10-H_4BaP and 8-hydroxy-7,8,9,10-H_4BaP, respectively. Furthermore, when kept in methanol, both metabolites were readily dehydrated to BaP upon treatment with acid. Based on this evidence, the metabolites contained in chromatographic peaks 13 and 14 of Fig. 1 are established to be 7-hydroxy-7,8-H_2BaP and 8-hydroxy-7,8-H_2BaP, respectively.

CD spectral analyses (Fig. 3A) indicate that both monohydroxylated metabolites are optically active. Their optical purities could not be established from the CD spectra due to the lack of optically pure standards. Because the enantiomers of 7-hydroxy-7,8,9,10-H_4BaP have been resolved by a CSP-HPLC method (14,17), metabolite peak 13 was converted to 7-hydroxy-7,8,9,10-H_4BaP by cataly-

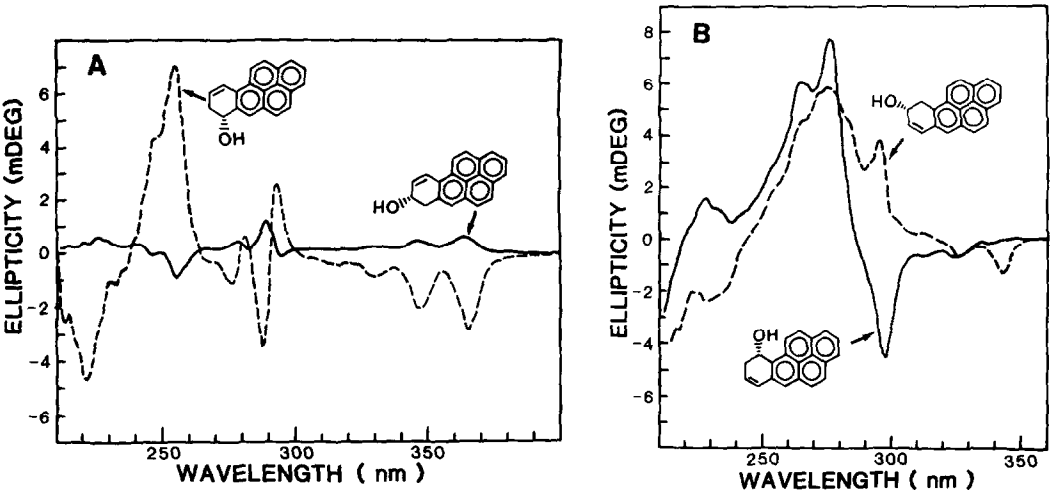

Fig. 3. (A) CD spectra of the metabolites identified as 7-hydroxy-7,8-H_2BaP (-----) and 8-hydroxy-7,8-H_2BaP (———), respectively.
(B) CD spectra of the metabolites identified as 9-hydroxy-9,10-H_2BaP (-----) and 10-hydroxy-9,10-H_2BaP (———), respectively.
The relative configuration of the hydroxyl group is shown. See text for discussion on the assignment of absolute configuration.

tic hydrogenation. This 7-hydroxy-7,8,9,10-H_4BaP was found to have an R/S enantiomer ratio of 93:7. The enantiomers of 8-hydroxy-7,8,9,10-H_4BaP were not resolved by CSP-HPLC, thus the optical purity of metabolite peak 14 cannot yet be determined. The enantiomers of the tetrahydro alcohols more strongly retained by the (R)-CSP employed were tentatively designated as the S enantiomer (17), based on the chiral recognition mechanisms proposed by Pirkle et al. (18).

The metabolites contained in chromatographic peaks 9 and 10 of Fig. 2 were similarly identified as 10-hydroxy-9,10-H_2BaP and 9-hydroxy-9,10-H_2BaP, respectively. The uv absorption spectra in methanol solutions were similar to that of 9,10-H_2BaP, indicating that the carbons at the C_9 and the C_{10} positions are saturated. Mass spectral analyses (molecular ions at m/z 270 and characteristic fragment ions at m/z 252) indicated that each metabolite contains a hydroxyl group. These two metabolites were gradually converted to BaP when they were stored in methanol at 4°C. They were also readily converted to BaP when treated with acid. Their uv absorption spectra in alkaline solution are similar to those in methanol solution, indicating that the hydroxyl group is at either the C_9 or the C_{10} position rather than at any of the aromatic ring positions.

The exact location of the hydroxyl group was established by the structure of the product formed by catalytic hydrogenation. The hydrogenation products of metabolite peaks 9 and 10 (Fig. 2) were identical to authentic 10-hydroxy-7,8,9,10-H_4BaP and 9-hydroxy-7,8,9,10-H_4BaP, respectively. These two isomeric tetrahydro alcohols were separable on both the CSP column (17) and the reversed-phase C_{18} column. Metabolites contained in peaks 9 and 10 of Fig. 2 are thus identified as 10-hydroxy-9,10-H_2BaP and 9-hydroxy-9,10-H_2BaP, respectively. These metabolites were found also to be optically active by their CD spectra (Fig. 3B). The optical purity of 10-hydroxy-9,10-H_2BaP (peak 9 of Fig. 2) was determined after conversion to 10-hydroxy-7,8,9,10-H_4BaP by catalytic hydrogenation. This tetrahydro product was found to have an R/S enantiomer ratio of 1:9 by the CSP-HPLC method previously described (14,17). The optical purity of 9-hydroxy-9,10-H_2BaP (peak 10 of Fig. 2) could not be determined because the enantiomers of 9-hydroxy-7,8,9,10-H_4BaP were not resolved by CSP-HPLC (13,17).

The formation of monohydroxylated metabolites at the aliphatic carbons of 7,8-H_2BaP and 9,10-H_2BaP was abolished if the liver microsomes were treated with carbon monoxide before the substrate was added into the incubation mixture or when NADPH was deleted from the incubation mixture. The enzymatic formation of these alcoholic metabolites was not inhibited by the presence of the microsomal epoxide hydrolase inhibitor 3,3,3-trichloropropylene oxide. These results indicate that the stereoselective hydroxylation reactions at the aliphatic carbons are catalyzed by cytochrome P-450 isozymes present in rat liver microsomes. Identification of BaP and some of its metabolites as products formed in the metabolism of either 7,8-H_2BaP or 9,10-H_2BaP suggests that, in addition to their unique pathways of metabolism, the dihydro-BaPs may be enzymatically activated and detoxified via the known metabolic pathways of BaP.

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