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STEREOSELECTIVE FORMATION OF BENZO(c)PHENANTHRENE (+)-(3S,4R) AND (+)-(5S,6R)-OXIDES BY CYTOCHROME P450c IN A HIGHLY PURIFIED AND RECONSTITUTED SYSTEM

P. J. van Bladeren[1], S. K. Balani[1], J. M. Sayer[1], D. R. Thakker[1], D. R. Boyd, D. E. Ryan[3], P. E. Thomas[3], W. Levin[3], and D. M. Jerina[1]

[1]Laboratory of Bioorganic Chemistry, NIDDK, The National Institutes of Health, Bethesda, MD 20892
[2]Department of Chemistry, The Queen's University of Belfast, Belfast BT9 5AG, N. Ireland
[3]Laboratory of Experimental Carcinogenesis and Metabolism, Roche Institute of Molecular Biology, Nutley, NJ 07110

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SUMMARY: The principal oxidative metabolites formed from benzo(c)phenanthrene (B(c)Ph) by the cytochromes P450 in liver microsomes from control and treated rats are the 3,4- and 5,6-arene oxides. A procedure is described which allows determination of the enantiomer composition and absolute configuration of these arene oxides based on HPLC separation of isomeric thiolate adducts formed with N-acetyl-L-cysteine in base. Incubation of [^{3}H]-B(c)Ph with highly purified cytochrome P450c in a reconstituted monooxygenase system followed by trapping of the metabolically formed arene oxides as above indicated that the 3,4-oxide was predominantly the (+)-(3S,4R)-enantiomer (90%) and that the 5,6-oxide consisted mainly of the (+)-(5S,6R)-enantiomer (76%). The results are discussed in terms of their implications about the catalytic binding site of cytochrome P450c.

The polycyclic aromatic hydrocarbons represent particularly useful substrates to probe the steric limitations of the catalytic binding sites of the cytochromes P450 because of their rigidity and size. This is especially true for cytochrome P450c, the major form (>70%) in the livers of 3-methylcholanthrene-treated rats (1), since it generally has a much higher turnover (10 to 200 fold) for these substrates when compared to other purified isozymes (2). The combined results of several previous studies (reviewed in 3) have led to the conclusion that the catalytic binding site of cytochrome P450c may be represented by a hydrophobic cleft or dishpan-like structure which is asymmetrically disposed relative to the heme iron where oxygen activation occurs (4).

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The present report describes the stereoselectivity of cytochrome P450c on metabolism of benzo(c)phenanthrene (B(c)Ph) to arene oxides at the 3,4- and 5,6-positions. Previous studies with liver microsomes from control and treated rats as well as a purified monooxygenase system reconstituted with cytochromes P450a, P450b or P450c had indicated that these two arene oxides constituted 3-17% and 77-97% of the total metabolites, respectively (5). Based on incubations in the presence of epoxide hydrolase, which converts arene oxides into trans-dihydrodiols, from 93 to 100% of the primary oxidative metabolites of B(c)Ph are represented by these two arene oxides.

EXPERIMENTAL

Materials. [^{3}H]-B(c)Ph (32 mCi/mmol) was prepared by hydrogenolysis of 5-bromobenzo(c)phenanthrene (5). Components of the reconstituted system including cytochrome P450c and epoxide hydrolase were obtained as described (2,6). Enantiomerically pure B(c)Ph 3,4-oxide (7) and B(c)Ph 5,6-oxide (8) were prepared and assigned absolute configurations in separate synthetic studies as were the corresponding trans-3,4-dihydrodiol (9) and 5,6-dihydrodiol (10). Dioxane was distilled from sodium and stored frozen.

N-Acetyl-L-Cysteine Adducts. Like many other K-region arene oxides (11), B(c)Ph 5,6-oxide is quite stable at neutral to alkaline pH in aqueous media. At 37° C in 20% dioxane/water at pH 11, no detectable spectral change (347 nm) occurs within 15 min. In contrast, the arene oxide has a half-life of ∿100 sec when the above solution also contains 2.3 mM N-acetyl-L-cysteine. This pseudo-first order disappearance of the arene oxide is accompanied by a concomitant production of trans thiolate adducts whose separation will be described. B(c)Ph 3,4-oxide, like many other benzo-ring arene oxides (11), readily isomerizes to phenols throughout the acidic to basic pH range. When monitored at 302 nm, the arene oxide has a half-life of 300 sec at 37° C in 20% dioxane/water, pH 11. Neither the half-life nor the spectral change are influenced by the presence of 2.3 mM N-acetyl-L-cysteine. Thus, the 5,6-oxide can be selectively and quantitatively trapped by the thiol in the presence of the 3,4-oxide under these conditions. A 35-fold increase in the concentration of N-acetyl-L-cysteine (80 mM, pH 10.7) results in a decrease in the half-life of the 3,4-oxide to ∿100 sec, indicating that two-thirds of this arene oxide can be trapped as thiolate adducts under these latter conditions. In the metabolite trapping studies to be described, 150 mM N-acetyl-L-cysteine was used; this should trap ∿80% of the 3,4-oxide present, based on the observed rate at the lower thiolate concentration.

Trapping the 3,4-oxide formed in the incubation medium presents a significant problem since the oxide is isomerizing to phenols ($t_{1/2}$ ∿45 sec) simultaneously with its formation. Kinetic analysis (Fig. 1) indicates that the arene oxide concentration should plateau at 0.25 μM after ∿2.5 min with 0.1 nmol/ml of cytochrome P450c. Longer incubation times or increased protein concentrations could be counterproductive if phenols of B(c)Ph inhibit the oxidation, as was suggested by the 30% decrease in rate of metabolism when the reconstituted system was used in the absence of added epoxide hydrolase (5) which blocks phenol formation by efficient conversion of arene oxides to dihydrodiols.

Chromatography and Structures of Adducts. In the presence of thiolate anions, arene oxides form positional pairs of trans adducts (11). If the

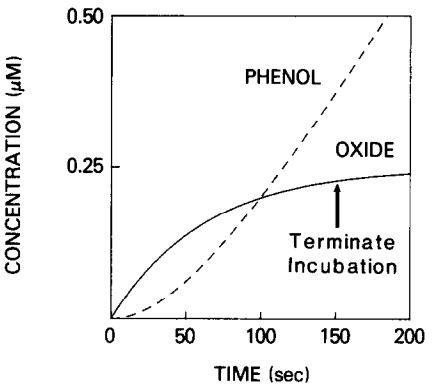


Fig. 1. Calculated time course of B(c)Ph 3,4-oxide and phenol formation in 0.1 M phosphate buffer, pH 7.4, at 37° C, in the presence of 0.1 nmol/ml of cytochrome P450c. The curves are based on the integrated rate equations [oxide] = $(c/k)(1-e^{-kt})$ and [phenol] = $ct - (c/k)(1-e^{-kt})$, where c is the rate of metabolic conversion of B(c)Ph to its 3,4-oxide and is taken as 0.004 μM/sec, as observed with the reconstituted enzyme in a previous study (5); k is the pseudo first order rate constant for isomerization of the oxide, and was determined to be 0.016 sec^{-1} under these conditions of buffer and temperature.

thiol is optically active, a given racemic arene oxide can form four separable diastereomeric adducts. Structures of the relevant adducts from the B(c)Ph 3,4- and 5,6-oxides are shown in Fig. 2.

The four adducts (structures assigned in ref. 8) from the enantiomeric B(c)Ph 5,6-oxides (Fig. 2; (+)-A and (+)-B from the (+)-(5S,6R)-oxide, (-)-A and (-)-B from the (-)-(5R,6S)-oxide) were separated chromatographically on an Applied Sciences Adsorbosphere ODS column (9.4 x 100 mm) eluted at 5 ml/min with 46% methanol in 0.05 M Tris-acetate buffer at pH 7.0 (8). For the present study, the adducts were also chromatographed as their methyl esters (excess diazomethane in methanol-ether) on the same column eluted with 1% tetrahydrofuran and 46% methanol in water at 5 ml/min: (-)-A 19.0 min, (+)-A 21.0 min, (+)-B 22.8 min and (-)-B 23.8 min as the esters. Adducts from the (+)-(5S,6R)-oxide elute as the outside pair for the free acids and as the inside pair for the esters.

Four adducts are also obtained from racemic B(c)Ph 3,4-oxide. The major adduct from each enantiomer (Fig. 2, (+)-C and (-)-C) is presumed to arise by

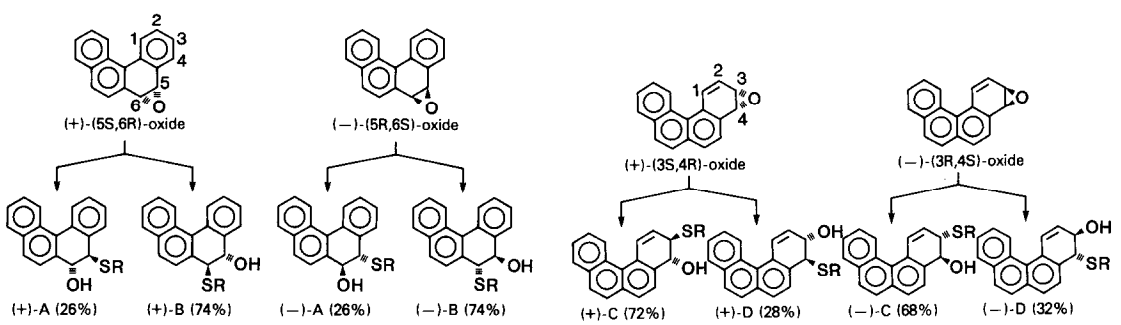


Fig. 2. Structures of the enantiomeric B(c)Ph 3,4- and 5,6-oxides and their derived thiol adducts. Major (B or C) and minor (A or D) trans thiol adducts are formed from each arene oxide in the presence of N-acetyl-L-cysteine and base. The (+)- and (-)- symbols associated with each adduct designate the specific rotation of the starting arene oxide. Racemic arene oxides gave equal amounts of (+)- and (-)- adducts (±2%) on the basis of absorbance at their λ_{max} upon HPLC.

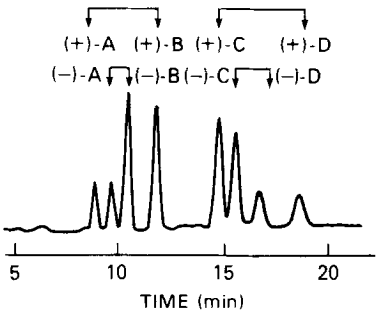


Fig. 3. Chromatographic separation of the eight N-acetyl-L-cysteine adducts derived from (+)- and (-)- B(c)Ph 3,4- and 5,6-oxides on a 100-mm Perkin-Elmer HS-3 C18 column eluted with 15% methanol and 15% acetonitrile in 50 mM Tris-acetate buffer (pH 7.0) at a flow rate of 1 ml/min. For structures of the adducts, see Fig. 2.

attack at the more reactive allylic 3-position of the 3,4-oxide as has been the case for several other benzo-ring arene oxides (6,11,12). Similarly, the minor adducts (Fig. 2, (+)-D and (-)-D) result from attack at the benzylic 4-position of the 3,4-oxide. Since reversal of these assignments would not affect the conclusions of the present study, further structural characterization was not undertaken. A solvent which gave an acceptable separation (Fig. 3) of the 3,4-adducts, and which also separated the 5,6-adducts, was identified through the use of the Perkin-Elmer Solvent Optimization System.

Trapping of B(c)Ph Oxides. For determination of the enantiomer composition of the B(c)Ph 5,6-oxide formed by cytochrome P450c, the reconstituted system contained 0.05 μM cytochrome P450c, 1200 units of NADPH-cytochrome c reductase, 0.02 mg of dilauroylphosphatidyl choline, 1 mM NADPH and 80 μM [^{3}H]-B(c)Ph (added in 50 μl of acetone) in a final volume of 1 ml of 0.1 M phosphate buffer (pH 7.4). Incubations were for 10 min at 37° C and were terminated by addition of 0.25 ml of dioxane containing 50 nmol of racemic B(c)Ph 5,6-oxide, 40 μl of 1 M sodium hydroxide, and 15 μl of 0.2 M N-acetyl-L-cysteine. The solutions were maintained at 37° C for 15 min, extracted twice with 2 ml of ether, and lyophilized to dryness. The pooled residue of 10 incubations was dissolved in 0.5 ml of methanol containing 5 drops of acetic acid. Direct HPLC of this solution provided the enantiomer composition of the adducts as free acids. For determination as esters, the solution was treated with excess diazomethane in ether before analysis.

For determination of the enantiomer composition of the formed B(c)Ph 3,4-oxide, incubations were as above except that cytochrome P450c was 0.15 μM and 2500 units of reductase were used. After incubation for 2.5 min at 37° C, reaction was terminated by addition of 0.6 ml of dioxane containing 10 nmol each of racemic B(c)Ph 3,4- and 5,6-oxides as well as 1.5 ml of a solution prepared by mixing 1 part of 1 M N-acetyl-L-cysteine and 2.1 parts of 1 M sodium hydroxide. The solution was agitated for 15 min at 37° C, adjusted to pH 7.0 with 50% phosphoric acid, and extracted twice with 2 ml of ether. One third of the aqueous phase from ten tubes was applied to a C_{18} Sep Pak (Waters Associates) followed by 3 ml of water. Adducts were eluted with 4 ml of 90% acetonitrile in water which was concentrated to 1 ml prior to HPLC.

Enantiomer Composition of Dihydrodiols. Incubation of [^{3}H]-B(c)Ph with liver microsomes from control, phenobarbitol-treated and 3-methylcholanthrene-treated rats as well as with the purified system reconstituted with cytochrome P450c and epoxide hydrolase were essentially as described (5). For the present study, the dihydrodiols were isolated by HPLC on a Du Pont Zorbax SIL column (9.4 x 250 mm) eluted with 2.5% methanol and 15% ethyl acetate in cyclohexane at a flow rate of 5.7 ml/min; k' = 0.0, 1.7, 3.5 and 6.3 for B(c)Ph and the 5,6-, 3,4- and 1,2-dihydrodiols, respectively. For enantiomer

composition, radioactive dihydrodiols were diluted with racemic carrier, converted to diastereomeric mixtures of bis-esters with the acid chloride of (-)-menthyloxyacetic acid, and separated on Du Pont Zorbax SIL columns eluted with 5% ether in cyclohexane as described (9,10).

RESULTS AND DISCUSSION

The present study evaluates the stereoselectivity of homogeneous cytochrome P450c on formation of enantiomeric 3,4- and 5,6-oxides of B(c)Ph. Based on previous results in the presence of epoxide hydrolase, these arene oxides constitute 5% and 95%, respectively, of the primary oxidative metabolites of the hydrocarbon. Metabolically formed arene oxides were trapped as stable thiolate adducts with N-acetyl-L-cysteine. A total of eight nonenantiomeric adducts (Fig. 2) were formed from the two racemic arene oxides, and HPLC conditions were developed for their separation (see Methods and Fig. 3). At a low concentration of N-acetyl-L-cysteine ($\sim$2 mM) in base, only adducts from the 5,6-oxide were formed whereas a much higher concentration (150 mM) trapped the very labile 3,4-oxide as well. Analysis of adducts from metabolically formed 5,6-oxides, trapped at the low N-acetyl-L-cysteine concentration, either as their free acids or as their methyl esters indicated formation of the (+)-(5S,6R) to (-)-(5R,6S)-oxides in a ratio of 76:24. At the higher N-acetyl-L-cysteine concentration, total adducts from the 3,4-oxide compared to the 5,6-oxide were formed in the ratio of 4:96 which is identical to the ratio of 3,4- and 5,6-dihydrodiols when epoxide hydrolase was present. In the second trapping experiment, the (+)-(5S,6R)-oxide enantiomer was favored 77:23 as above, and the (+)-(3S,4R)-oxide was favored over the (-)-(3R,4S)-oxide in a ratio of 90:10.

Formation of these arene oxides is shown graphically in terms of our steric model for the minimum catalytic binding site of cytochrome P450c in Fig. 4. If steric factors alone were the only consideration, the present results suggest that two areas at the top of the model (hatched rings outside of the minimal boundary) are acceptable albeit not preferable binding regions. An earlier study on the metabolism of benz(a)anthracene 1,2-dihydrodiol to 1,2-diol 3,4-epoxides had indicated that yet a third region at the

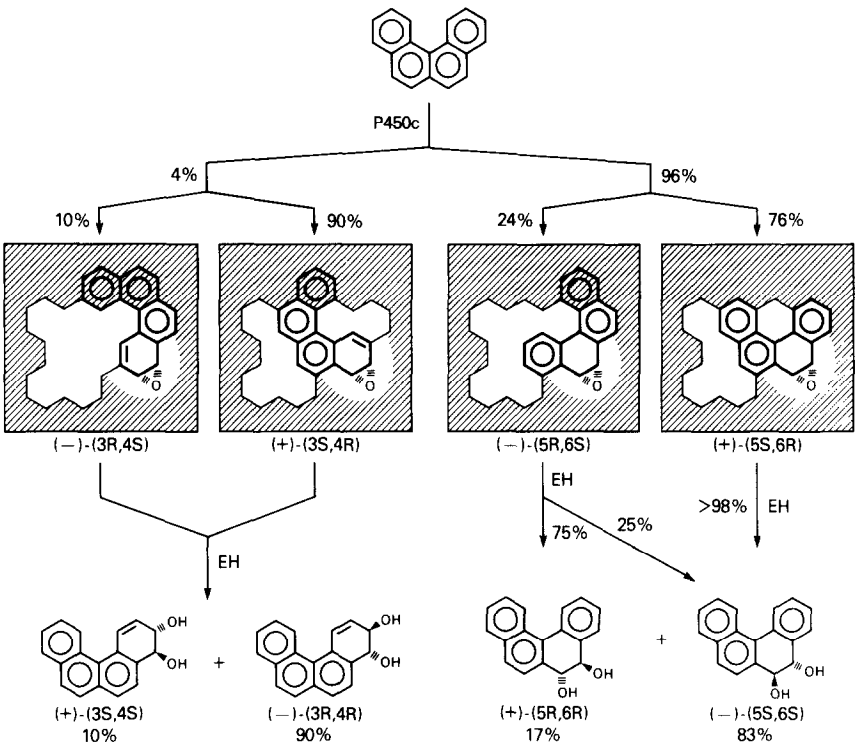
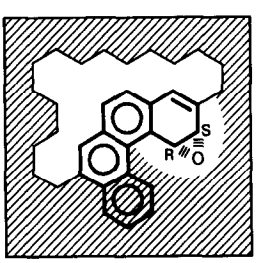


Fig. 4. Enantiomer compositions of the dihydrodiols formed from B(c)Ph by cytochrome P450c and epoxide hydrolase (EH). The unshaded area represents our previously proposed minimum boundary for the catalytic binding site of cytochrome P450c.

left top of the model was also acceptable for binding (13). In the same vein, cytochrome P450c and epoxide hydrolase fail to form any (<0.03% of total metabolites) 1,2-dihydrodiol from benzo(c)phenanthrene, and an earlier study (14) had shown that only trace amounts of 3,4-diol 1,2-epoxides were formed from B(c)Ph 3,4-dihydrodiol. Thus, the region shown below (hatched ring) seems particularly poor for binding.



Data in Table 1 provide enantiomer compositions for the 3,4- and 5,6-dihydrodiols as formed by liver microsomes from control and treated rats as well as by the purified system reconstituted with cytochrome P450c and

TABLE 1
Enantiomer Composition of Dihydrodiols Formed From Benzo(c)Phenanthrene

Protein	3,4-Dihydrodiol[a]		5,6-Dihydrodiol[b]	
	(-)-(3R,4R)	(+)-(3S,4S)	(+)-(5S,6S)	(-)-(5R,6R)
Control	89%	11%	58%	42%
PB	80%	20%	56%	44%
MC	94%	6%	85%	15%
P450c + EH	90%	10%	83%	17%

[a]Microsomal data from ref. 5.
[b]Earlier data (5) on the microsomally formed 5,6-dihydrodiol indicated it was racemic. We believe those results to be in error, and corrected enantiomer compositions for the three types of microsomal preparations are reported here.

epoxide hydrolase. As would be expected, data for the reconstituted system are practically identical to those for microsomes from 3-methylcholanthrene-treated rats. Based on the regioselectivity of epoxide hydrolase toward the enantiomers of B(c)Ph 5,6-oxide (shown in Fig. 4) and the amount of each enantiomeric oxide formed, the calculated ratio of (5S,6S) to (5R,6R)-dihydrodiol is 82:18, which is in excellent agreement with the ratio of these enantiomeric 5,6-dihydrodiols formed by the reconstituted system. A similar calculation has not been done for the 3,4-dihydrodiol because the optically active 3,4-oxides racemize above room temperature (7). This racemization (via the oxepin valence bond tautomer) does not affect the present trapping results since its half-life is >>150 sec at 37° C in phosphate buffer (pH 7.4), conditions under which no racemization was observed in the present study. It is, however, clear from initial studies that the (+)-(3S,4R)-oxide forms $\geq$85% of (-)-(3R,4R)-dihydrodiol with microsomal epoxide hydrolase, the same enantiomer which predominates from the parent hydrocarbon.

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