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STEREOSELECTIVE FORMATION OF BENZ[a]ANTHRACENE (+)-(5S,6R)-OXIDE AND (+)-(8R,9S)-OXIDE BY A HIGHLY PURIFIED AND RECONSTITUTED SYSTEM CONTAINING CYTOCHROME P-450c

P. J. van Bladeren[1], R. N. Armstrong[2], D. Cobb[2], D. R. Thakker[1], D. E. Ryan[3], P. E. Thomas[3], N. D. Sharma[4], D. R. Boyd[4], W. Levin[3], and D. M. Jerina[1]

[1]Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205.
[2]Department of Chemistry, University of Maryland, College Park, MD 20034.
[3]Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110.
[4]Department of Chemistry, Queen's University of Belfast, Belfast BT9, 5AG, N. Ireland.

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The principal oxidative metabolites formed from benz[a]anthracene (BA) by the rat liver microsomal monooxygenase system are the 5,6- and 8,9-arene oxides. In order to determine the enantiomeric composition and absolute configuration of these metabolically formed arene oxides, an HPLC procedure has been developed to separate the six isomeric glutathione conjugates obtained synthetically from the individual enantiomeric arene oxides. Both (+)- and (-)-BA 5,6-oxide gave the two possible positional isomers, but only one positional isomer was formed in each case from (+)- and (-)-BA 8,9-oxide. When [^{14}C]-BA was incubated with a highly purified and reconstituted monooxygenase system containing cytochrome P-450c, and glutathione was allowed to react with the arene oxides formed, radioactive adducts were formed predominantly (>97%) from the (+)-(5S,6R) and (+)-(8R,9S) enantiomers. The present results are in accord with theoretical predictions of the steric requirements of the catalytic binding site of cytochrome P-450c.

INTRODUCTION

For several polycyclic aromatic hydrocarbon metabolites, expression of mutagenic and carcinogenic activity is known to be markedly dependent upon their enantiomeric purity and absolute configuration (1-4). Thus it is of considerable interest to determine the stereoselectivity of enzymes involved in the formation of such metabolites. In the livers of rats treated with 3-methylcholanthrene, >70% of the total cytochromes P-450 consist of cytochrome P-450c (5,6). This enzyme has been purified to apparent homogeneity (7) and has a much higher specific activity towards polycyclic aromatic hydrocarbons (PAH's) than any of the other purified forms of cytochrome P-450 (8).

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Recently a model has been proposed for the substrate binding site of cytochrome P-450c (9) from which predictions of the stereochemical outcome of the oxidation of PAH's can be made. In order to test the predictive properties of this model, the present communication establishes the enantiomeric purity and absolute configurations of the 5,6- and 8,9-oxides formed from benz[a]anthracene (BA) upon incubation with a highly purified and reconstituted system containing cytochrome P-450c. In the presence of epoxide hydrolase, BA is converted almost entirely (>98%) into dihydrodiols by the reconstituted system (10). Since the 5,6- and 8,9-dihydrodiols represent 49% and 44% of these dihydrodiols, respectively, the corresponding BA 5,6- and 8,9-oxides are preponderant metabolites of the hydrocarbon. Similar results have been obtained with liver microsomes from control and treated animals (10,11).

The enantiomeric composition and absolute configurations of the enzymatically formed BA 5,6- and 8,9-oxides has now been determined through chromatographic comparison of their glutathione conjugates with the set of conjugates obtained synthetically from the individual enantiomeric arene oxides. Previous studies have described the separation of isomeric glutathione conjugates formed from benzo[a]pyrene 4,5-oxide (12,13) and styrene oxide (14).

EXPERIMENTAL

Materials. The enantiomerically pure (+)-(5S,6R), (-)-(5R,6S), (+)-(8R,9S) and (-)-(8S,9R) arene oxides of BA were prepared as previously described (15,16) as were 5- and 6-hydroxyBA (17). Acetates of the phenols were prepared with pyridine-acetic anhydride. Rat liver cytochrome P-450c from 3-methylcholanthrene-treated rats was purified to homogeneity (7). Liver microsomal NADPH cytochrome c reductase was purified (18) to a specific activity of 39,800 units/mg protein assayed at 22°C (19). One unit of activity is equal to 1 nmol cytochrome c reduced per min. [^{14}C]-Benz[a]anthracene (25.5 mCi/mmol) and [^{3}H]-glutathione (glycine-2-^{3}H, 1.8 Ci/mmol) were obtained from New England Nuclear, Boston, MA. The glutathione was diluted to 1.2 mCi/mmol prior to use.

Synthesis of glutathione conjugates. A solution of 10 µmol of the requisite arene oxide in 0.5 ml of dioxane was added to 20 µmol of glutathione and 60 µmol of sodium hydroxide dissolved in 0.5 ml of water, conditions previously used in the synthesis of glutathione conjugates of benzo[a]pyrene 4,5-oxide (20). The mixture was agitated in a shaking water bath for 2 hrs at 45°C, extracted with 3 x 2 ml of ether, adjusted to pH 4-5 with acetic acid, and lyophilized to dryness. Both (+)- and (-)-BA 5,6-oxide formed equal amounts of the two possible trans adducts which result from attack of glutathione at either the 5- or 6-position of the arene oxide. The pair of positional isomers from each enantiomer of the 5,6-oxide were separated by semi-preparative HPLC on an Altex Ultrasphere ODS column (4.6 mm x 25 cm) eluted at 0.5 ml/min with 40% CH_3OH/60% 25 mM ammonium acetate (pH 4.0). The (+)- and (-)-BA 8,9-oxides each formed a single chromatographic peak after reaction with glutathione, presumably trans-9-glutathionyl-8-

hydroxy-8,9-dihydrobenz[a]anthracenes (21,22,23). UV spectra of the conjugates showed the required 5,6- or 8,9-dihydro BA chromophores.

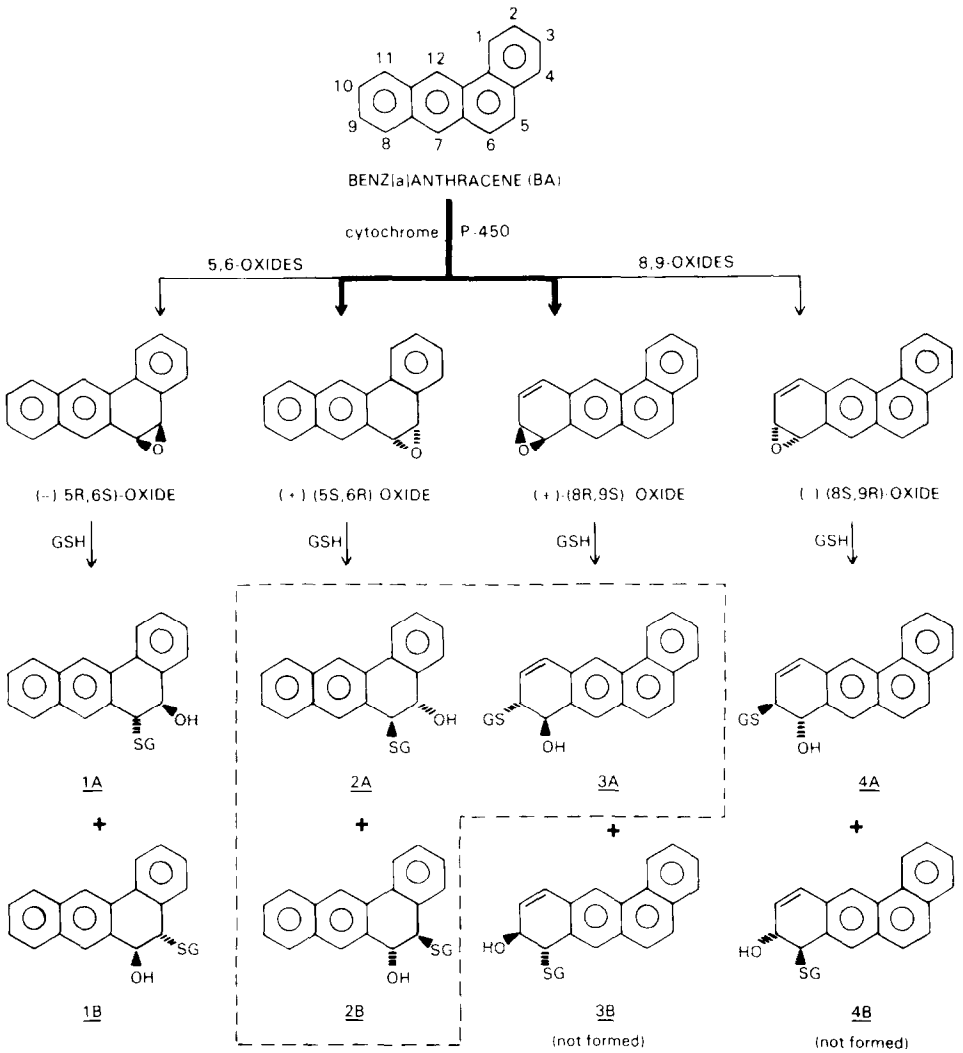
Structures of the BA 5,6-Oxide Adducts. The position of attachment of glutathione to the 5,6-arene oxides isomer was determined by acetylation of the resulting hydroxyl group, oxidation of the thioether to the sulfoxide, and cis-pyrolytic elimination of the sulfoxide under conditions identical to those previously described (12). The sulfoxide acetates were pyrolyzed at 280°C to form 5- and/or 6-acetoxyBA by injection onto an all glass chromatography system (Hewlett-Packard) equipped with a 2 mm x 1.5 m column of 3% OV-101 on 100/120 mesh gas chrom Q. After a 2 min isothermal period at 225°C, the column was programmed at 2°C/min to 230°C. Retention times for BA, 5-acetoxyBA, and 6-acetoxyBA were 4.5, 12.0 and 12.7 min, respectively.

Incubation of [^{14}C]-BA. In a final volume of 2.0 ml, 0.025 μM cytochrome P-450c, 1500 units of microsomal NADPH-cytochrome c reductase, 0.06 mg/ml dilaurylphosphatidylcholine, 0.5 mM NADPH, 3 mM $MgCl_2$, and 50 μM [^{14}C]-benz[a]-anthracene in 0.1 M phosphate buffer (pH 7.25) were incubated for 10 min at 37°C. Incubations were terminated by addition of 0.15 ml of 100 mM glutathione (15 μmol), 1.5 ml of dioxane containing 1 μmol of (±) BA 5,6-oxide and 4 μmol of (±) BA 8,9-oxide, and 0.15 ml 2 M NaOH in that order. The resulting solution was agitated for 2 hrs at 45°C in a shaking water bath, extracted with ether, acidified, and lyophilized to dryness as above. In one instance, the initial incubation medium contained 8 mM glutathione, and only carrier arene oxides and base were added at the end of the incubation. In a third experiment, unlabeled BA and [^{3}H]-glutathione were used.

Analytical Separation of Glutathione Conjugates. The residue from the above lyophilization was dissolved in 250 μl of 100 mM Tris-phosphate (pH 2.5) and analyzed by HPLC on a Chromanetics 3μ Spherisorb ODS-2 column (4.6 mm x 10 cm) eluted at 1.5 ml/min with 8% acetonitrile/20% methanol/72% 100 mM Tris-phosphate (pH 2.5). Best results for this separation were obtained with new columns. Fractions of 0.9 ml were collected and assayed for radioactivity in Hydrofluor scintillation cocktail. Relative retention of 5,6-oxide adducts were the same as observed on the Altex column.

RESULTS AND DISCUSSION

Under alkaline conditions, only trans adducts are expected (12,13,21,22,23) between arene oxides and the thiol group of the chiral tripeptide glutathione via nucleophilic displacement reactions. The set of eight possible adducts derived from the (+)- and (-)-enantiomers of BA 5,6- and 8,9-oxides are shown in Scheme I. Since none of these eight isomers are enantiomeric with each other, they should all be separable under appropriate chromatographic conditions. Each of the 5,6-oxides formed equal amounts of conjugate derived from attack at the 5- and 6-positions as expected (24), and all four of these adducts could be separated by HPLC (Fig. 1A). The positional attachment of glutathione in these adducts was deduced through their individual chemical degradation by a previously described technique (12) to either 5- or 6-acetoxyBA. Structures 1A and 2A (Scheme I) are based on the observation that the pyrolysis experiments resulted in the formation of >98% 5-acetoxyBA and <2% 6-acetoxyBA. Similarly, 1B gave >98% 6-acetoxyBA and <2% 5-acetoxyBA while 2B



Scheme I. Enantiomeric specificity in the metabolism of BA to its 5,6- and 8,9-oxides by cytochrome P-450c (heavy arrows). From the set of eight possible glutathione (GSH) conjugates, only those three in the dashed box could be identified in the sequential enzymatic oxidation and trapping experiment. Positional attachment of GSH in 3A and 4A is presumed from previous studies (21,22,23). Evidence for the formation of 3B or 4B was not obtained. Absolute configurations are as indicated. It should be noted that certain pairs of isomers, such as 3A and 4A, are diastereomers and not enantiomers since glutathione is optically active.

gave 90% 6-acetoxyBA and 10% 5-acetoxyBA. The (+)- and (-)-8,9-oxides each formed separable single adducts, presumably *via* attack at the more reactive 9-position. This result was anticipated from the prior observation that glutathione prefers to attack the 2-position of naphthalene (21) and anthracene 1,2-oxide (22).

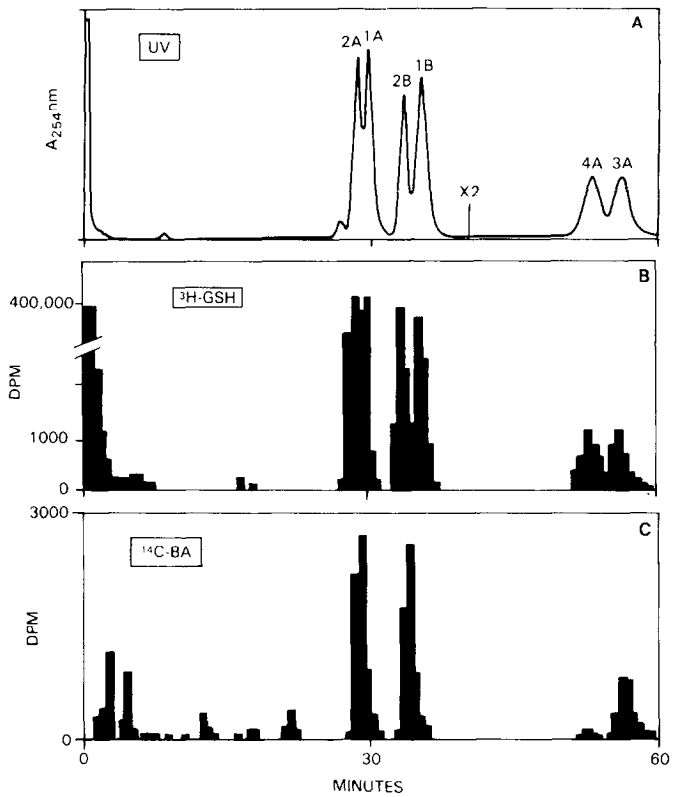


Fig. 1: HPLC traces on the Chromanetics column of: (A) the six glutathione conjugates synthesized from (±)-BA 5,6-oxide and (±)-BA 8,9-oxide; (B) the same conjugates prepared from [^{3}H]-glutathione; (C) the glutathione conjugates synthesized from enzymatically prepared [^{14}C]-BA 5,6- and 8,9-oxide. Adducts 2A and 2B are derived from (+)-BA 5,6-oxide, 1A and 1B from (-)-BA 5,6 oxide, 3A from (+)-BA 8,9-oxide and 4A from (-)-BA 8,9-oxide. For absolute configurations of the conjugates see Scheme I.

Our ability to develop chromatographic conditions for the separation of the six glutathione conjugates which form from the (+)- and (-)-enantiomers of the BA 5,6- and 8,9-oxides (Fig. 1A) has allowed investigation of the stereochemistry of arene oxide formation by cytochrome P-450c. In an initial experiment, unlabeled BA was incubated in the reconstituted system, a large excess of racemic 5,6- and 8,9-oxides was added, and adducts were allowed to form with [^{3}H]-glutathione (Fig. 1B). Comparison of total radioactivity due to 5,6- versus 8,9-oxide conjugates indicated that, as expected, trapping of the 5,6-oxides was ∿20-fold more efficient because of the greater tendency of the 8,9-oxides to isomerize to phenols (25). In the presence of a 15-fold excess of glutathione, 74% of the 5,6-oxide was trapped as conjugates.

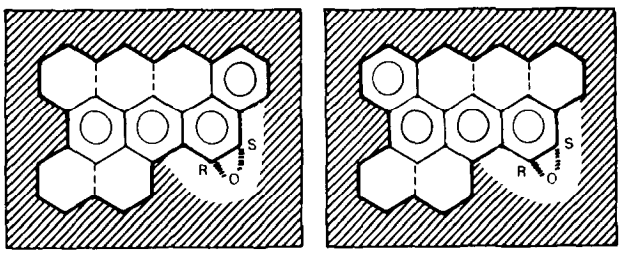


Fig. 2: The model for the substrate binding site of cytochrome P-450c as it has recently been proposed (9). BA is drawn bound to it in the way necessary to obtain the (+) 5,6- and (+) 8,9-oxides.

Incubations of [^{14}C]-BA in the reconstituted system were conducted such that ~20% of the substrate was metabolized (10). After workup and lyophilization, ~7% of the incubated substrate had been trapped as water soluble metabolites. The presence of 8 mM glutathione in the initial incubation only slightly increased the amount of conjugates trapped, presumably because little thiolate anion was present at the pH used. Chromatographic separation of the conjugates formed from [^{14}C]-BA (Fig. 1C) indicated that three of the six possible adducts greatly predominated. Peaks 2A and 2B derive from (+)-BA (5S,6R)-oxide. Comparison of radioactivity in these peaks with peaks 1A and 1B indicated that <2% of the (-)-enantiomer had formed. Similarily, comparison of peaks 3A and 4A indicated that >97% of the 8,9-oxide formed was the (+)-(8R,9S)-enantiomer. The small amount of radioactivity near peak 4A emerged from the column slightly before the standard and thus does not consist entirely of conjugate 4A (Fig. 1C).

The present results indicate exceptionally high stereoselectivity in the metabolism of BA to its 5,6- and 8,9-oxides by the reconstituted system containing cytochrome P-450c. Opposite stereoheterotopic faces of the hydrocarbon must be epoxidized in order to form the observed enantiomers. This is exactly the result predicted by a recent theoretical model of the steric requirements of the catalytic site of cytochrome P-450c (9). Predicted configurations and orientations of the product arene oxides within the proposed binding site are shown in Fig. 2. Formation of the opposite enantiomers would require one or more rings of the hydrocarbon to be outside the boundary of the proposed site.

The present results also allow conclusions regarding the mechanism of action of epoxide hydrolase. Since liver microsomes from 3-methylcholanthrene-treated rats form almost exclusively the (-)-(8R,9R)-enantiomer of the 8,9-dihydrodiol (26), epoxide hydrolase must selectively add water with inversion of configuration at the 9-position of the (+)-(8R,9S)-oxide. In this same system (26), 80% of the 5,6-dihydrodiol formed has the (+)-(5R,6R) configuration and 20% the (-)-(5S,6S) configuration. Since this dihydrodiol is formed from enantiomerically pure (+)-(5S,6R)-oxide as established in the present study, epoxide hydrolase has a fourfold preference for attack with inversion at the (5S)-carbon relative to the (6R)-carbon in this arene oxide. This same preference has been found for the hydration of (+)-BA 5,6-oxide by purified epoxide hydrolase (15).

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