Effects of Inducers and Epoxide Hydrase on the Metabolism of Benzo[a]pyrene by Liver Microsomes and a Reconstituted System: Analysis by High Pressure Liquid Chromatography (carcinogen metabolism/microsomal cytochrome P-450)

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ABSTRACT The mobilities of 24 potential metabolites of benzo[a]pyrene were examined with high pressure liquid chromatography. Twelve phenols, five quinones, four dihydrodiols, and three oxides were studied. The chromatographic procedure employed allowed the separation and quantitation of benzo[a]pyrene metabolites into three major groups consisting of phenols, quinones, and dihydrodiols. Two of the benzo[a]pyrene oxides were unstable during chromatography, whereas the third oxide was more stable and chromatographed in the quinone fraction. Treatment of rats with phenobarbital or 3-methylcholanthrene enhanced the metabolism of benzo[a]pyrene by liver microsomes and altered the relative amounts of the various metabolites formed. In the absence of epoxide hydrase (EC 4.2.1.63), benzo[a]pyrene was metabolized primarily to phenols and quinones but was not appreciably metabolized to dihydrodiols by a solubilized, reconstituted cytochrome P-450 monooxygenase system. Addition of partially purified epoxide hydrase resulted in the formation of benzo[a]pyrene dihydrodiols with a concomitant decrease in the formation of phenolic metabolites, indicating that benzo[a]pyrene undergoes metabolism via arene oxides that are precursors for dihydrodiols and phenols.

The widespread occurrence of benzo[a]pyrene (BP) as an environmental contaminant (1) and the carcinogenic effects of BP in experimental animals (2) have stimulated interest in this compound for some 40 years. This interest is manifested by numerous metabolic studies of BP both in vivo (3–9) and in vitro (10–17), the testing of BP and its metabolites for their carcinogenic and mutagenic potency (18, 19), and binding studies of BP and its metabolites to nucleic acids and polynucleotides (15, 20–23). The growing evidence that arene oxides are ultimate carcinogens of polycyclic aromatic hydrocarbons (24, 25), and the established intermediacy of arene oxides in the metabolism of several of these hydrocarbons (26–29), make a critical examination of the metabolism of BP imperative. Recent studies of BP metabolism in vitro have led to the postulation of BP 4,5-oxide as an intermediate in rat liver microsomal systems (14) and in Syrian hamster liver microsomes (15), but none of the studies have taken fully into account the multiple pathways for metabolism of BP (Table 1). Due to the magnitude of the analytical problems, and the difficulties inherent in the synthesis of all potential BP metabolites, direct comparisons of possible metabolites with authentic reference materials have not been forthcoming. Product identifications have been based on limited comparisons of chromatographic, ultraviolet, and fluorescence data for metabolites with a relatively small number of standards which are sometimes of questionable origin.

Reported metabolic data (Table 1) suggest that BP 1,2-, 2,3-, 4,5-, 7,8-, and 9,10-oxides may be involved in biotransformations of BP. Arene oxides at ring junctures are considered less likely, based on profiles of metabolites from naphthalene (26) and substituted benzenes (30, 31). Also unlikely is the 8,9-oxide, since its stable tautomeric oxepin form (32) should have been detected previously. Lack of synthetic reference standards may account, in part, for the absence of suggested metabolism at the 11,12-K region. The present availability of synthetic reference compounds, which include twelve phenols, five quinones, three arene oxides, and four trans-dihydrodiols of BP, enables a more realistic interpretation of the metabolism of BP than had been possible in the past. The use of suitable reference compounds for product identification should lead to the identification of the bio-activated ultimate carcinogen(s) formed from BP. A recent report has described the use of high pressure liquid chromatography for separation of metabolites of BP (33). Independent studies in our laboratory have utilized similar methodology for the separation of BP metabolites. The present study describes the chromatographic mobility of 24 potential BP metabolites and describes the effects of induction by phenobarbital and 3-methylcholanthrene on the profile of BP metabolites formed by rat liver microsomes. In addition, the effects of epoxide hydrase [glycol hydro-lyase (epoxide-forming), EC 4.2.1.63] on the metabolism of BP by a partially purified mixed-function oxidase system (34) has been evaluated.

MATERIALS AND METHODS

Incubations. Incubations with hepatic microsomes obtained from immature male rats (Long-Evans strain) contained 100
Equipped spectrometry. (HPLC) dioxane and intervals. Radioactivity from each flask and added after 37°. The samples and epoxide hydrase (0, 448 mg lipid, 0.5 μmol of NADPH, 3 μmol of magnesium chloride, 100 μmol of potassium phosphate buffer (pH 7.4), 95 nmol of [14C]BP (specific activity 10.99 nCi/mmol added in 0.05 ml of acetone) per 1.0 ml of final volume, and were agitated in diffuse light under air for 10 min at 37°. The cytochrome P-450 or P-448 content of the microsomes from control, phenobarbital-pretreated, and 3-methylcholanthrene-pretreated animals was 0.66, 1.76, and 1.01 nmol/mg of protein, respectively. Incubations with the solubilized and reconstituted systems contained 0.2 nmol of cytochrome P-448 (34), 120 units of NADPH-cytochrome c reductase, 0.1 mg of lipid, 0.5 μmol of NADPH, 3 μmol of magnesium chloride, 100 μmol of potassium phosphate buffer (pH 6.8), epoxide hydrase (0, 72, 216, or 726 units, as indicated) (35), and 95 nmol of [14C]BP as above per 1.0 ml of incubation. The samples were agitated in diffuse light under air for 5 min at 37°. The reactions were terminated by vortexing for 30 sec after the addition of 1 ml of acetone and 2 ml of ethyl acetate. More than 99% of the radioactivity was found in the organic phase after centrifugation. 

**Analysis.** Extracts from two identical incubations were pooled. Two-milliliter portions of the organic phase were taken from each flask and evaporated in a stream of nitrogen in the dark. After addition of 4–5 μmol of BP 4,5-, 7,8-, and 9,10-dihydriodiol, BP 3,6-quione, 3-HOBP, and 9-HOBP as reference compounds, the residue was dissolved in 5–10 μl of dioxane and injected onto the high pressure liquid chromatography (HPLC) column. Fractions were collected at 0.5- or 1.0-min intervals. Radioactivity was measured by scintillation spectrometry. 

**HPLC** was conducted on a DuPont 830 instrument equipped with a 254-nm photometer. Metabolites were separated from BP into diol, quinone, and phenol fractions on a silica gel column. Fractions were collected and assayed for radioactivity.

**TABLE 1.** Reported metabolites of benzo[a]pyrene (BP)*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Refs.</th>
<th>Metabolite</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-HOBP</td>
<td>3, 5, 10, 37</td>
<td>2,3-dihydrodiol</td>
<td>41, 6†</td>
</tr>
<tr>
<td>9-HOBP</td>
<td>3, 5, 61, 10</td>
<td>BP, 4,5-dihydriodiol</td>
<td>6†, 9, 16, 33</td>
</tr>
<tr>
<td>6-HOBP</td>
<td>11, 13, 15, 33</td>
<td>1,6-dihydroxybenzo[a]pyrene</td>
<td>6†</td>
</tr>
<tr>
<td>10-HOBP</td>
<td>2, 3, 10</td>
<td>3,6-dihydroxybenzo[a]pyrene</td>
<td>6†, 10</td>
</tr>
<tr>
<td>8-HOBP</td>
<td>2, 3, 10</td>
<td>6,12-dihydroxybenzo[a]pyrene</td>
<td>6†</td>
</tr>
<tr>
<td>11-HOBP</td>
<td>3, 5, 10, 37</td>
<td>6-hydroxymethyl BP 17</td>
<td></td>
</tr>
</tbody>
</table>

* These references are representative but not complete. Many of the structural assignments are tentative, and some, such as the 2,3-dihydriodiol (37), have been shown to be in error. 
† These metabolites are reported as water-soluble conjugates. The numbering system for BP used in this paper is:

μg of microsomal protein, 0.5 μmol of NADPH, 3 μmol of magnesium chloride, 100 μmol of potassium phosphate buffer (pH 7.4), 95 nmol of [14C]BP (specific activity 10.99 nCi/mmol added in 0.05 ml of acetone) per 1.0 ml of final volume, and were agitated in diffuse light under air for 10 min at 37°. The cytochrome P-450 or P-448 content of the microsomes from control, phenobarbital-pretreated, and 3-methylcholanthrene-pretreated animals was 0.66, 1.76, and 1.01 nmol/mg of protein, respectively. Incubations with the solubilized and reconstituted systems contained 0.2 nmol of cytochrome P-448 (34), 120 units of NADPH-cytochrome c reductase, 0.1 mg of lipid, 0.5 μmol of NADPH, 3 μmol of magnesium chloride, 100 μmol of potassium phosphate buffer (pH 6.8), epoxide hydrase (0, 72, 216, or 726 units, as indicated) (35), and 95 nmol of [14C]BP as above per 1.0 ml of incubation. The samples were agitated in diffuse light under air for 5 min at 37°. The reactions were terminated by vortexing for 30 sec after the addition of 1 ml of acetone and 2 ml of ethyl acetate. More than 99% of the radioactivity was found in the organic phase after centrifugation.

The compounds have been prepared by previously published procedures or unequivocal de novo synthesis. The materials are analytically pure and free of chromatographically or spectroscopically detectable impurities. Details of the procedures employed have been submitted for publication or are in press. The 7,8- and 9,10-dihydriodiol were obtained by the action of epoxide hydrase on the corresponding arene oxides (32).

† Retention times (in minutes) vary from run to run by ±2%. These arene oxides are unstable under the conditions employed and decompose to afford phenols. The 7,8- and 9,10-oxides isomerize predominantly to 7-HOBP and 9-HOBP, respectively.

Two coupled 1 m × 2.1 mm analytical DuPont ODS columns were used with a linear gradient of 35–85% methanol in water over 50 min, an inlet pressure of 2500 psig, and a flow rate of 0.7–1.1 ml/min.

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Two coupled 1 m × 2.1 mm analytical DuPont ODS columns were used with a linear gradient of 35 to 85% methanol in water at 2500 psig and a flow rate of 0.7–1.1 ml/min. The column was operated at room temperature to prevent possible destruction of arene oxides and labile phenols such as 1- and 6-HOBP. Retention times of the reference standards are shown in Table 2.

Results obtained from the microsomal and soluble systems were corrected for zero-time experiments analyzed as described above. The limit of detection of each metabolite fraction in this procedure was 0.01% of incubated BP.

**RESULTS AND DISCUSSION**

**Chromatographic Properties of BP Metabolites.** The HPLC procedure employed is highly efficient for separation and quantitation of phenols, quinones, and dihydriodiol produced from BP (Fig. 1). It is apparent, however, that rigorous identification of individual components within each group will require further study, as evidenced by the co-chromatography for many of the reference compounds presently available. The 9,10-dihydriodiol separates readily from the three other reference dihydriodiols, which overlap. Structural assignment to the metabolic dihydriodiol cannot be made with certainty until the unknown 1,2- and 2,3-dihydriodiol are available, or until further spectroscopic criteria can be applied. Three of the five available quinones (1,6-, 3,6-, and 4,5-) emerge from the column as a single badly tailing peak. The tail of this peak contains BP 6,12- and 11,12-quinones as well as BP 4,5-oxide. Mobility of the numerous other potential quinones is pres-
Fig. 1. Chromatographic profile of benzo[a]pyrene metabolites. (A) Composite high pressure liquid chromatography profile obtained with available reference compounds. (B) Chromatographic distribution of metabolites obtained from Exp. 6. The various ‘metabolite’ regions are indicated.

Effect of Inducers on Microsomal Metabolism of BP. The microsomal mixed-function oxidase system is responsible for the detoxification, as well as the activation, of polycyclic hydrocarbons. Microsomal metabolism results in the formation of arene oxides, which are highly reactive metabolites that covalently bind to macromolecules and which are more active than the parent hydrocarbon in causing malignant transformations of cultured cells. Treatment of animals with compounds which induce this enzyme system can result in either increased or decreased toxicity of the hydrocarbon. Quantitative differences between the activity of liver microsomes from control, phenobarbital-pretreated, and 3-methylcholanthrene-pretreated animals are seen in both the percentage of metabolism and in the profile of metabolites formed (Table 3). Since structures for the metabolites cannot be assigned with certainty, the quantitative data obtained from metabolic studies with [14C]BP are expressed only as diols 1, 2, and 3, quinones 1 and 2, and phenols 1 and 2 (Tables 3, 4; Fig. 1B). While quinone 2 is a metabolite fraction from control microsomes, it is not detectable as a separate peak with preparations from induced animals. Diol 1 predominates with control and 3-methylcholanthrene-induced microsomes, while diol 2 predominates with phenobarbital-induced microsomes. Phenol 1 is virtually absent with phenobarbital-induced microsomes. Direct comparison of the present results for microsomes from 3-methylcholanthrene-pretreated animals with a recent application of HPLC to the metabolism of 3-(33) is not possible, since higher protein concentrations, longer incubation times, and microsomes from a different strain of rat were employed.

Differences in the overall catalytic activity of the microsomal preparations by the present assay were comparable to results observed previously with an assay that measures only fluorescent phenolic metabolites (36). Microsomes from 3-methylcholanthrene-pretreated animals were about 4.5 times more active per nmol of hemoprotein than microsomes from control animals, and microsomes from phenobarbital-pretreated animals were slightly less active per nmol of hemoprotein than the control.

Effect of Epoxide Hydrolase on the Metabolism of BP by a Partially Purified Cytochrome P-448-Containing System. Hepatic epoxide hydrolase is an important enzyme for the metabolism of aromatic compounds, since it converts the intermediate arene oxides formed by the mixed-function oxidase system to the corresponding dihydrodiols, which are nontoxic. Thus, the separation, purification, and reconstitution of the mixed-function oxidase system, and the removal of epoxide hydrolase activity from this system (34, 35), permit a study of the central role of the oxidase and hydrase in controlling the levels of cytotoxic and carcinogenic metabolites of benzo[a]pyrene. Further, in the present system, the epoxide hydrase activity results in the formation of the toxic metabolites only if the epoxide hydrolase is present and functional. The results presented in Table 3 indicate that this is the case for hepatic microsomes from control as well as from carcinogen-pretreated animals.

Table 3. Rat liver microsomal metabolism of benzo[a]pyrene

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% Metabolized</th>
<th>Diol</th>
<th>Quinone</th>
<th>Phenol</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1. Control</td>
<td>0.6</td>
<td>13.8*</td>
<td>9.2</td>
<td>9.7</td>
<td>18.8</td>
</tr>
<tr>
<td>2. 3-methylcholanthrene-pretreated</td>
<td>4.8</td>
<td>19.5*</td>
<td>8.7</td>
<td>11.8</td>
<td>23.4</td>
</tr>
<tr>
<td>3. Phenobarbital-pretreated</td>
<td>1.5</td>
<td>8.3*</td>
<td>11.8</td>
<td>4.6</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>0.06†</td>
<td>0.09</td>
<td>0.03</td>
<td>0.03</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Incubation mixtures contained 100 μg of microsomal protein, 0.5 μmol of NADPH, 3 μmol of MgCl₂, 100 μmol of potassium phosphate buffer (pH 7.4), 95 nmol of [14C]BP per 1.0 ml of final volume.

* The upper row of numbers for each experiment is the percent of each metabolite expressed as proportion of total radioactivity eluting before BP.
† The lower row of numbers of the specific activity of preparation, nmol of product formed/nmol P-450 or P-448 per min.
The activity of the reconstituted system (Exp. 4), measured radiochemically by phenol 2 production only, is about equivalent to the activity as measured by fluorimetry in terms of 3-HOBP (34). The comparison calculated from phenol 2 alone fails to take into account fluorescent 2- and 9-HOBP, which appear in the phenol 1 fraction. When both phenol peaks are considered, total phenol production is higher than when measured by fluorimetry. Although the fluorimetric assay for metabolism of BP has provided an adequate assay for the production of fluorescent phenols, it is inadequate when attempting to critically correlate metabolism with cytotoxicity and carcinogenicity of BP, since it fails to differentiate between the various fluorescent phenols formed or to take into account the nonphenolic metabolites (80% in Exp. 7).

BP metabolism results have sometimes been misinterpreted due to incomplete characterization of metabolites (11–13). Despite an early warning by Berenblum and Schoental (37), numerous mistakes in describing the metabolism of BP have been made through over-reliance on spectroscopic techniques such as fluorimetry, ultraviolet spectra, and even mass spectra, which provide limited structural information in the absence of adequate reference standards. Application of the techniques described in this paper, followed by additional chromatographic and spectroscopic methods involving the widest possible array of standards, should provide comprehensive and definitive answers to metabolic questions associated with BP.

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22. Meunier, M. & Chauveau, J. (1973) "Binding of benzo(a)- pyrene metabolite(s) to main and satellite calf thymus DNA's in vitro," FBBLS Lett. 31, 327-331.


