A Comparison of Benzo(a)pyrene Metabolism by Liver and Lung Microsomal Enzymes from 3-Methylcholanthrene-treated Rhesus Monkeys and Rats

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INTRODUCTION

Microsomal AHH is responsible for the metabolic conversion of BP and other aromatic hydrocarbons to reactive metabolites that interact with intracellular macromolecules and thereby induce neoplasia. Although there are numerous reports of BP metabolism studies utilizing HPLC analysis with liver enzymes (2-4, 7, 10-13, 20), there are very few reports of studies utilizing lung enzyme preparations (6, 9).

Yet the lung is an organ directly exposed to varying concentrations of polycyclic aromatic hydrocarbons and is believed to be 1 of the primary target organs for hydrocarbon-induced carcinogenesis.

MATERIALS AND METHODS

Materials. [3H](generally labeled; specific activity, 1.5 Ci/m mole) was purchased from Research Products International, Elk Grove Village, Ill., and [7,10-14C]BP (specific activity, 25 mCi/m mole) was purchased from Amersham/Searle, Arlington Heights, Ill. BP metabolite standards were generous gifts from Dr. H. Gelboin and Dr. M. Litwack, National Cancer Institute, Bethesda, Md., and Dr. D. Jerina, National Institute of Arthritis, Metabolic, and Digestive Diseases, NIH. BP, 3-MC, glucose 6-phosphate, NADP, and glucose-6-phosphate dehydrogenase were purchased from Battette Columbus Laboratories, Columbus, Ohio 43201.
Sigma Chemical Co., St. Louis, Mo. Rhesus monkeys were obtained from Primate Imports, Port Washington, N. Y.

Both [3H]BP and [14C]BP were purified by thin-layer chromatography on aluminum oxide plates. The solvent system used was pentane-diethyl ether (19:1). Nonlabeled BP was purified by recrystallization in methanol. Purity of the radio-labeled BP was greater than 99% as determined by HPLC analysis.

**Animals and Treatment.** Mature male rhesus monkeys and 150- to 200-g male Sprague-Dawley rats were used in this study. Rats and rhesus receiving 3-MC were given a single i.p. injection (20 mg/kg) on 2 consecutive days and were sacrificed 24 hr after the last dose by decapitation. The nontreated and 3-MC-treated rats were divided into 3 groups with the livers and lungs of 5 animals pooled per group. The livers and lungs from 3 monkeys were assayed separately as the nontreated group, and 3 additional monkeys were evaluated individually after 3-MC treatment.

Immediately after decapitation, the livers and lungs were removed and rinsed in cold (0-4°C) 0.12 M Tris buffer (pH 7.4 at 37°C) containing 1.15% KCl. The livers and lungs were thoroughly minced with scissors, and the minced tissue was washed twice with cold buffer. All subsequent steps were performed in a refrigerated room at 0-4°C.

**Microsomal Enzyme Preparation.** Both tissues were homogenized in 3 volumes of buffer with a motor-driven, Potter-Elvenhem glass-Teflon homogenizer. Six complete passes of the Teflon pestle were used for liver, and 12 complete passes were used for lung. The homogenate was filtered through glass wool to remove pieces of connective tissue and then centrifuged at 9,000 x g for 20 min. The 9,000 x g supernatant was filtered through glass wool to remove pieces of connective tissue and then centrifuged for 45 min at 20,000,000 x g max. The resulting microsomal pellet was resuspended in 0.1 M Tris buffer, pH 7.4, at 37°C. The microsomal protein concentration was determined by the method of Lowry et al. (8), with bovine serum albumin as a standard.

**AHH Assay.** Unlabeled BP, [3H]BP, and [14C]BP were mixed in appropriate amounts in methanol to yield a final concentration of 2.5 mmoles/ml. The resulting specific activity was 78 mCi/m mole for [3H]BP and 12 mCi/m mole for [14C]BP. Substrate was prepared under red light illumination.

AHH assays were carried out in a total volume of 4 ml in 0.1 M Tris buffer, pH 7.4, at 37°C. The final liver microsomal protein concentration was 0.4 mg/ml for nontreated animals and 0.2 mg/ml for 3-MC-treated animals. The final lung microsomal protein concentration was 1.0 mg/ml for both nontreated and 3-MC-treated animals. The [3H]BP and [14C]BP substrates were added to the incubation flask in methanol (40 µl/ml) to give a final concentration of 100 µmoles. The cofactor amounts per ml of incubation mixture were 1.3 µmoles NADP, 14.2 µmoles glucose 6-phosphate, 5.9 µmoles MgCl2, and 2 IU glucose-6-phosphate dehydrogenase. Incubations were carried out at 37°C in a darkened room with red light illumination. Liver microsomal assays were run for 10 min, and lung microsomal assays were run for 20 min. Product formation versus time of incubation was determined to be linear for all assay conditions utilized.

Assays were terminated by the addition of 1 ml cold acetone. The incubation mixture was extracted twice with 4 ml ethyl acetate. The two 4-ml extractions were combined and dried over anhydrous MgSO4, and taken to dryness under a stream of nitrogen gas. Dried extracts were stored at -20°C under nitrogen before analysis. Samples were resublimed in 0.1 ml methanol for HPLC analysis.

**HPLC Analysis.** Two DuPont Model 830 liquid chromatographs were used, both having gradient elution capability. Both chromatographs were equipped with Zorbax ODS columns (25-cm x 2.1-mm inside diameter). One chromatograph was connected to a DuPont Model 836 combination fluorescence/absorbance detector which monitors fluorescence and UV absorption simultaneously. The 2nd chromatograph utilized an absorbance detector.

The metabolites were separated by gradient elution. The initial solvent composition was 60% methanol, 39.9% water, and 0.1% phosphoric acid. The final solvent concentration was 85% methanol, 14.9% water, and 0.1% phosphoric acid. The inclusion of phosphoric acid aids in the resolution of BP metabolite peaks and has no effect upon the stability of BP metabolite standards while on the column (6, 7). A concave hyperbolic gradient system was used which fit the equation X = Y². The gradient rate was set at 1%/min going from 10 to 100%. The oven temperature was 34°C, and the column pressure was 2000 psi. The flow rate was approximately 0.8 ml/min.

Fractions of the effluent were collected every 24 sec in scintillation vials. Metabolites were detected either by their absorption in UV at 254 nm or by simultaneous fluorescence:UV absorption. Metabolite reference standards were chromatographed daily. 3H and 14C levels were determined as dpm with an Amersham/Searle Mark IV liquid scintillation spectrometer.

**RESULTS**

The in vitro BP metabolites were separated by HPLC and detected by UV absorption and fluorescence. Simultaneous detection by UV absorption and fluorescence qualitatively determined BP metabolite fractions because of the different fluorescence:UV absorption ratios observed for each BP metabolite standard (unpublished observations). Metabolite fractions were identified by cochromatography with authentic BP metabolite standards. However, the assumption that each fraction consists solely of a single BP metabolite cannot be made, even though they cochromatograph with BP metabolite standards. Water-soluble metabolites were not measured.

Chart 1 compares the metabolite profiles obtained with lung microsomes from nontreated and 3-MC-treated rhesus monkeys. The 9,10-diol and 7,8-diol fractions and the 3-OH-BP fraction show the greatest increase after 3-MC treatment. However, the 9,10-diol and 7,8-diol fractions and the 3-OH-BP fraction show the greatest increase after 3-MC treatment. Similarly, Chart 2 shows an unknown metabolite fraction, U', present in 3-MC-treated rat lung microsomal assays. As with the rhesus, BP metabolite formation in rat lung is greatly stimulated by 3-MC treatment. However, the dione fractions show the greatest increase after 3-MC treatment.
Charts 3 and 4 are liver metabolite profiles from 3-MC-treated rhesus and rats, respectively. The metabolite profiles are similar in both species, each having the same metabolite fractions. However, the predominant diol fraction in the rat is the 9,10-diol, whereas the 4,5-diol is the predominant one in the rhesus.

Specific enzyme activities (pmoles/mg/min) were calculated for each BP metabolite fraction. Table 1 lists the specific activities for the rhesus and rat lung metabolite pathways. Total lung activity was increased 7-fold in the rhesus and 42-fold in the rat by 3-MC treatment. The ratio of 3-MC to control level activities are the greatest for the 9,10-diol, 7,8-diol, and 3-OH-BP fractions in the rhesus, whereas the ratios for the 3 dione fractions and 9-OH-BP are largest in the rat.

The specific liver activities are listed in Table 2. The rhesus and rat liver activities are not as responsive to 3-MC treatment as the corresponding lung activities. Total rhesus liver activity increases approximately 2 times compared to the 3-fold induction observed in the rat. The 3-MC: non-treated control activity ratios in the rhesus are similar for 3 diol fractions and the 1,6-dione and 3,6-dione fractions. The activity ratios in the rat are largest for the 9,10-diol and 7,8-diol fractions.

The specific activity of each metabolite was converted to the percentage of total metabolism. Table 3 presents the metabolite percentages for all of the different species, tissues, and treatment groups. Treatment with 3-MC causes shifts in the metabolite percentages for all species and tissue groups. The relative amount of the diol metabolites changes the most with 3-MC treatment. For example, in the livers from 3-MC-treated rats, the 9,10-diol represents 14% of total BP metabolites compared to 4.8% in the control. The percentage of the 7,8-diol formed by rat livers increased from 5.1% in the control to 11.5% in the 3-MC-treated group. Similar changes were also observed for the 9,10-diol and 7,8-diol fractions obtained from control and 3-MC-treated rhesus lung. The metabolite percentage for the 4,5-diol fraction decreased from 8.1% in the control rhesus lung to 2.9% in the 3-MC-treated group. Unlike the rhesus lung, relative percentages of diol metabolites formed by rat lung diminish as a result of 3-MC treatment. The magnitude of the shift in the diol region percentages, however, is not as
Table 1  

Metabolism of BP by rat and rhesus lung microsomal enzymes  
Specific activities (pmoles/mg/min) were determined from 20-min assay incubations. Three rhesus monkeys were in each treatment group, whereas each of the 3 treatment groups of rats consisted of 5 animals pooled per group. Average values are reported for each treatment group. Assay and extraction conditions were fully described under "Materials and Methods."  

<table>
<thead>
<tr>
<th>Metabolite fraction</th>
<th>Rhesus</th>
<th>Rat</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control activity</td>
<td>3-MC activity</td>
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<tr>
<td>Pre-9,10</td>
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<td>4,5-Diol</td>
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<td>7,8-Diol</td>
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<tr>
<td>1,6-Dione</td>
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<td>6,12-Dione</td>
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<tr>
<td>9-OH-BP</td>
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<tr>
<td>Total</td>
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<td>1212</td>
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</tbody>
</table>

Table 2  

Metabolism of BP by rat and rhesus liver microsomal enzymes  
Specific activities (pmoles/mg/min) were determined from 10-min assay incubations. The hepatic microsomal protein concentration from nontreated animals was 0.4 mg/ml and 0.2 mg/ml for 3-MC-treated animals. Average values are reported for each metabolite fraction. Assay and extraction conditions are fully described under "Materials and Methods."  

<table>
<thead>
<tr>
<th>Metabolite fraction</th>
<th>Rhesus</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control activity</td>
<td>3-MC activity</td>
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<tr>
<td>Pre-9,10</td>
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<td>20.2</td>
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<td>9,10-Diol</td>
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</table>

great in the rhesus liver and rat lung as it is in rhesus lung and rat liver.  

The percentage of the 3-OH-BP fraction changed in the rhesus liver and lung. Treatment with 3-MC resulted in an increase in the percentage of 3-OH-BP in the lung and a decrease in the percentage in the liver. The percentage of 3-OH-BP remains relatively unchanged in rat liver and lung, as do the percentage values for 9-OH-BP.  

The effect of 3-MC treatment on the percentage of the dione fractions is complex. The percentages of 1,6-dione metabolites decreased with 3-MC treatment in the rhesus lung and rat liver, whereas it increased in the rhesus liver and rat lung. The 3,6-dione fraction followed the same pattern as the 1,6-dione, although the changes were not as pronounced with the 3,6-dione fraction. Although the percentage of the 6,12-dione fraction remained essentially constant for control and 3-MC-treated rhesus lung and rat lung, 3-MC treatment resulted in diminished percentage of the 6,12-dione from rhesus and rat liver.  

**DISCUSSION**  

BP metabolite profiles have been compared by HPLC techniques for different animal species and tissues (1, 6, 7, 9, 13, 20). Differences in the BP metabolite profiles were noticed between human skin epithelial cultures and rat liver microsomes (1). Selkirk et al (13) determined similar metabolite patterns for human liver microsomes and human lymphocyte cell cultures. Initial studies by Leber et al. (7) revealed species differences in BP metabolite profiles obtained from hepatic microsomes. The present study shows similar metabolite profiles from rhesus and rat lung and from rhesus and rat liver. However, treatment with 3-MC
results in differing responses with regard to individual BP metabolite fractions.

Prior studies have examined the effect of 3-MC treatment on BP metabolite patterns in rats and mice. With the Sprague-Dawley rat, Yang et al. (20) showed that the formation of the 9,10-diol and 7,8-diol fractions by hepatic enzyme preparation was increased the most after 3-MC treatment, which is in agreement with the findings in the present study. Similarly, the greatest change in metabolite percentages was the increase in the 9,10-diol and 7,8-diol fractions. Working with the Long-Evans rat, Holder et al. (3) observed shifts in hepatic BP metabolite patterns upon 3-MC treatment, but the degree of change was not as great as was reported for the Sprague-Dawley strain. Species differences between rat and mouse hepatic BP metabolism after 3-MC treatment were apparent in the metabolite percentage values for the 9,10-diol and 7,8-diol fractions (4). These differences between the rat and mouse are similar to those reported between rhesus and rats in the present study.

Recent work by Seifried et al. (9) compared constitutive and 3-MC-induced lung metabolism of BP in 3 strains of laboratory mice. Although the levels of activity differed among strains, the comparative metabolite percentages in each strain were very similar and were not altered by 3-MC treatment in 2 of the mouse strains. This is in contrast to the changes in metabolite percentages observed in the present study with rats and rhesus monkeys. In the DBA/2J mouse, the metabolite percentages for the 9,10-diol and 7,8-diol fractions reportedly increased 2-fold as a result of 3-MC treatment. This mouse strain reacted similarly to the rhesus lung activity with 3-MC treatment.

The information presented in the present study and in similar studies with HPLC to determine BP metabolite profiles shows that exposure to polynuclear aromatic hydrocarbons results in increased AHH activity and, in some instances, significant changes in the ratios of the oxygenated hydrocarbon metabolites formed. The magnitude of change and the metabolites involved depend upon the tissue being examined and the animal species used. Species and tissue differences such as these may also occur in the formation of secondary BP metabolites.

Interest in the secondary metabolites of BP has increased with the evidence showing both isomers of 7,8-dihydroxybenzo(a)pyrene-9,10-oxide to be the most mutagenic of all BP metabolites (5, 15, 17). Recent evidence indicates that a high degree of stereospecificity is involved in both the formation of the 7,8-diol and the subsequent formation of the diol epoxide (14, 18, 19). Epoxide hydrolase activity has been shown to be essential in the formation of the highly mutagenic diol epoxide (16). Therefore, future studies examining the effects of animal species, treatment, and different tissues on BP metabolism should include determination of the formation of secondary metabolites, epoxide hydrolase activity, and enzyme conjugation of metabolites. Mutagenic studies with microsomal enzyme preparations from different species and different tissues for metabolic activation need additional attention.

REFERENCES


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