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# EVIDENCE FOR DISTINCT BINDING SITES IN THE CUMENE HYDROPEROXIDE-DEPENDENT METABOLISM OF BENZO[*a*]PYRENE CATALYZED BY CYTOCHROME P-450

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**Abstract**—A few constitutive cytochrome P-450 isozymes in male rat liver microsomes catalyzed the metabolism of benzo[*a*]pyrene (BP) in cumene hydroperoxide (CHP)-dependent reactions, which produced predominantly 3-hydroxyBP and BP quinones. This process varied with the concentration of CHP. At 0.05 mM CHP, 3-hydroxyBP was the major metabolite. An increase in CHP concentration reduced 3-hydroxyBP formation but increased the level of BP quinones. This change in metabolic profile was reversed by preincubation with pyrene. Pyrene selectively inhibited quinone formation and enhanced 3-hydroxyBP formation. Naphthalene, phenanthrene and benz[*a*]anthracene nonspecifically inhibited total metabolism. BP binding to microsomal protein correlated with quinone formation, suggesting a common precursor reactive intermediate. BP metabolism by female rat liver microsomes also depended on CHP concentration but was much less effective than that in the male. With females, quinones were the major metabolites at all CHP concentrations, and their formation was again modulated by pyrene. These data indicate that two distinct binding sites are responsible for the formation of 3-hydroxyBP and BP quinones.

Metabolism of benzo[*a*]pyrene (BP‡) by cytochrome P-450 produces three classes of products: BP phenols, BP dihydrodiols and BP quinones [1–5]. Cumene hydroperoxide (CHP) can replace NADPH and oxygen in supporting metabolism of BP, but in this case the metabolism is mediated by constitutive cytochrome P-450 isozymes [6]. The typical metabolic profile at low CHP concentrations (<0.15 mM) contains, in decreasing amounts, 3-hydroxyBP, BP quinones and BP 9,10-dihydrodiol. A higher CHP concentration (≥0.15 mM) enhances quinone formation but inhibits 3-hydroxylation of BP. This change in metabolic profile can be reversed by preincubation with BP or by increasing the concentration of BP [6]. These observations suggest that CHP interferes with the binding site involved in 3-hydroxylation. The present study further investigates the competitive effect of various polycyclic aromatic hydrocarbons (PAH) (Fig. 1) on BP metabolism under CHP-dependent conditions. The results of this study indicate that the cytochrome P-450 isozymes have different substrate binding sites which determine the positional specificity in the formation of BP metabolites. These findings may have implications in identifying cytochrome P-450 isozymes that display substrate specificity for BP.

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‡ Abbreviations: BP, benzo[*a*]pyrene; CHP, cumene hydroperoxide; DMSO, dimethyl sulfoxide; HPLC, high pressure liquid chromatography; and PAH, polycyclic aromatic hydrocarbons.

## MATERIALS AND METHODS

*Chemicals.* BP (Aldrich Chemical Co., Milwaukee, WI) was purified by silica gel chromatography and recrystallization from benzene-methanol. [$^{14}$C]BP (sp. act. 58.5 Ci/mole) was purchased from Amersham (Arlington Heights, IL) and diluted 200 times with unlabeled BP in dimethyl sulfoxide (DMSO) to give a 1.6 mM solution. The solution was checked periodically for purity by high pressure liquid chromatography (HPLC) and was stored frozen under argon. Ultrapure naphthalene, phenanthrene and pyrene were purchased from Baker Chemicals (Phillipsburg, NJ). [$^{14}$C]BP quinones were obtained from metabolic incubations and purified by HPLC. NADPH and CHP were purchased from the Sigma Chemical Co. (St. Louis, MO).

*Preparation of rat liver microsomes.* Male and female MRC-Wistar rats (8- to 10-weeks-old, Eppley Colony) were fasted overnight before being killed by cervical dislocation, and microsomes were prepared at 0–4°. Livers were perfused with 10 ml of 0.9% NaCl, 0.1 mM EDTA, pH 7.5, via the hepatic portal vein, minced and homogenized in 30–40 ml of 0.25 M sucrose, 0.1 mM EDTA, pH 7.5. Homogenates were centrifuged at 9,000 *g* for 10 min, and microsomes were obtained by ultracentrifugation of the supernatant fraction at 100,000 *g* for 60 min. The microsomal pellets were resuspended in the same sucrose solution at 1 ml/g liver and stored at −80°. Cytochrome P-450 content was measured by the method of Omura and Sato [7], and protein concentration was determined according to Lowry *et al.* [8]. Cytochrome P-450 contents in male and female rat liver microsomes were found to be 0.656 ± 0.020 and 0.666 ± 0.052 nmoles/mg protein respectively.

*Metabolism of* [$^{14}$C]*BP by rat liver microsomes.*

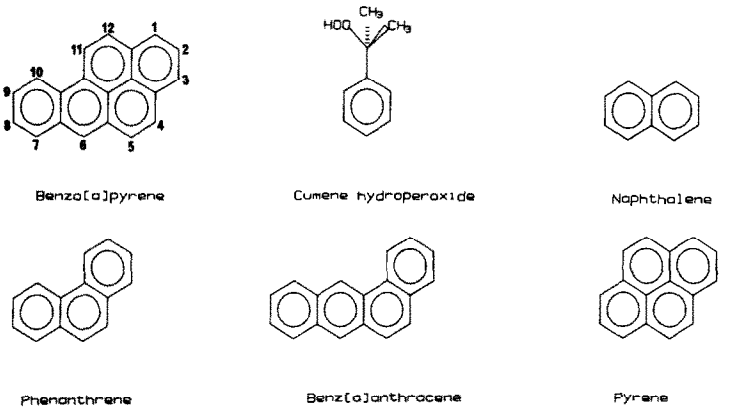


Fig. 1. Structures of compounds used in the metabolic studies.

Assays of [$^{14}$C]BP metabolism were performed in a volume of 1 ml with 1 mg microsomal protein, in 50 mM Tris–HCl, 150 mM KCl, 5 mM $MgCl_2$, pH 7.5. The mixtures were preincubated for 3 min at 37°. The reactions were initiated by adding 80 nmoles [$^{14}$C]BP in DMSO, immediately followed by the appropriate amount of CHP. Inhibitors (0.080 mM final concentration) were dissolved in DMSO and preincubated with the microsomes, where indicated. The final concentration of DMSO was 5%. Blanks were samples without CHP or microsomal protein. After incubation at 37° for 10 min, the reactions were terminated by adding 1 ml acetone to the samples. BP metabolites were extracted three times with 1.5 ml ethyl acetate and stored at −10° in the dark before analysis by HPLC. To measure binding of BP to protein, the protein in the aqueous phase was precipitated with 12 ml acetone, redissolved in 3 ml of 1% sodium dodecyl sulfate, and extracted with 3 ml ethyl acetate. Aliquots (0.5 ml) of the aqueous solution were counted for radioactivity. For binding of quinone to microsomal protein, 20 nmoles [$^{14}$C]BP quinones was substituted for [$^{14}$C]BP in incubation mixtures.

*Analysis of* [$^{14}$C]*BP metabolites by HPLC.* Samples of ethyl acetate extracts were processed immediately before HPLC analysis by evaporating to dryness under reduced light with a stream of oxygen-free argon and were reconstituted in 100 μl DMSO–methanol (1:1). The solutions were then injected onto an Altex Ultraphere 5 μm ODS reverse phase column attached to a Spectra Physics SP8700 solvent delivery system. The column was eluted with 60% methanol in water for 10 min, followed by a linear gradient to 100% methanol in 60 min at a flow rate of 1 ml/min at room temperature. The eluent was analyzed in series for absorbance at 254 nm, fluorescence and radioactivity. Radioactivity was monitored with a continuous flow system using a RAMONA detector (IN/US, Fairfield, NJ) with a 2.5 ml liquid cell. Liquid scintillation fluid had a flow rate of 2.5 ml/min. Radioactivity data were processed with an automatic data integration system. Recovery of radioactivity was greater than 95%. Three or more determinations, except as indicated, were made for each data point.

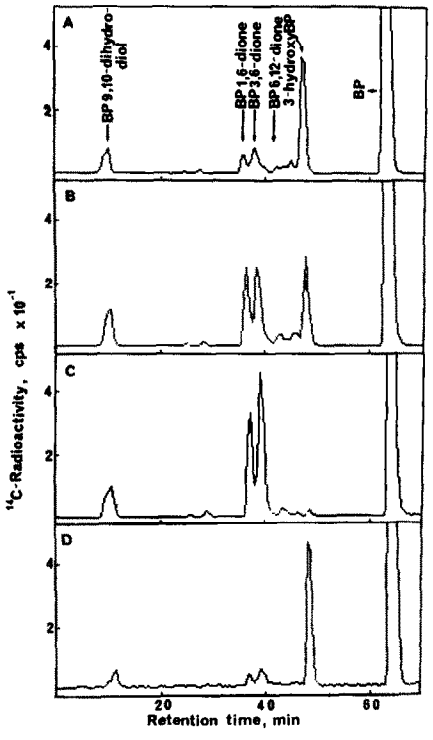


Fig. 2. Effect of CHP concentration and pyrene on HPLC profile of BP metabolites formed by male rat liver microsomes. (A) 0.05 mM CHP; (B) 0.10 mM CHP; (C) 0.15 mM CHP; and (D) 0.15 mM CHP plus 0.080 mM pyrene.

## RESULTS

The effects of CHP concentration on the HPLC profile of BP metabolites are shown in Fig. 2. At 0.05 mM CHP (Fig. 2A) 3-hydroxyBP was the major metabolite (50%). Quinones were also formed in substantial quantities (27%) and consisted of BP 1,6-, 3,6- and 6,12-dione. BP 9,10.dihydrodiol was also present (15%). Increased CHP concentration, 0.10 mM, decreased the amount of 3-hydroxyBP formed and increased the amount of quinones (Fig. 2B). At high CHP concentration (≥0.15 mM) quin-

Table 1. Effect of inhibitors on the cumene hydroperoxide-dependent [$^{14}$C]benzo[*a*]pyrene metabolism by rat liver microsomes

| | [$^{14}$C]BP metabolites (nmoles) | | | | | |
|---|---|---|---|---|---|---|
| | 0.05 mM CHP | | | 2 mM CHP | | |
| Inhibitor* | Ethyl acetate extractable metabolites | Quinones | 3-HydroxyBP | Ethyl acetate extractable metabolites | Quinones | 3-HydroxyBP |
| Solvent (control) | 8.8 (100)† | 1.8 (100) | 5.2 (100) | 11.6 (100) | 8.0 (100) | 0.4 |
| Benzo[*a*]pyrene | 6.8 (77) | 1.1 (60) | 3.8 (73) | 7.6 (66) | 5.9 (74) | 0.4 |
| Naphthalene | 5.6 (64) | 1.3 (69) | 3.4 (64) | 11.3 (97) | 8.0 (100) | 0.5 |
| Phenanthrene | 4.4 (50) | 1.1 (60) | 2.3 (44) | 8.2 (70) | 6.7 (84) | 0.2 |
| Benz[*a*]anthracene | 4.1 (47) | 0.7 (37) | 2.4 (47) | 6.8 (59) | 5.9 (73) | 0.1 |
| Pyrene | 6.1 (69) | 0.6 (30) | 4.6 (88) | 6.2 (53) | 1.1 (13) | 4.0 |

* Concentration of inhibitors was 0.080 mM.
† Numbers in parentheses indicate the percentage of metabolites with respect to control.

ones became the predominant metabolites (76–86%), whereas the formation of 3-hydroxyBP was reduced to trace amounts (Fig. 2C). To investigate the role of CHP in inhibiting 3-hydroxyBP formation, metabolism was conducted with 0.05 mM CHP and 0.05 or 0.10 mM cumene or cumenol (data not shown). Under these conditions neither compound affected BP metabolism, suggesting that the inhibitory effect of CHP is specific. When microsomes were preincubated with 0.080 mM pyrene and 0.15 mM CHP (Fig. 2D), the metabolic profile resembled that with 0.05 mM CHP (Fig. 2A), in which 3-hydroxyBP was the predominant metabolite and quinones were low. This alteration in BP metabolism by pyrene was also observed at 2 mM CHP (data not shown).

The modulation of BP metabolic profile was further investigated by using a series of PAH with different numbers of aromatic rings condensed in various geometric configurations (Fig. 1). This study was concerned mainly with the variation in the two major metabolites, 3-hydroxyBP and BP quinones (Table 1).

At 2 mM CHP, when quinones were the predominant metabolites, preincubation with unlabeled BP resulted in a similar apparent reduction in total metabolism and quinone formation due to dilution of the radiolabeled BP (Table 1). Naphthalene, with two aromatic rings, exhibited no effect, whereas phenanthrene was no more effective than BP. Although benz[*a*]anthracene has four aromatic rings, it showed only slightly more inhibitory effect than BP on total BP metabolism and failed to modulate the BP metabolic profile as pyrene did. On the other hand, pyrene inhibited quinone formation by 87% and increased 3-hydroxyBP formation 10-fold. These data show that the geometric arrangement of the four condensed aromatic rings is critical in the inhibition of BP quinone formation. Similar results were observed at 0.15 mM CHP (data not shown).

At 0.05 mM CHP, when 3-hydroxyBP was the major metabolite, unlabeled BP apparently reduced the total metabolites and 3-hydroxyBP formation by dilution (Table 1). Naphthalene was more effective than BP and resulted in 36% inhibition of both total BP metabolism and 3-hydroyBP formation. Both phenanthrene and benz[*a*]anthracene inhibited total BP metabolism and 3-hydroxyBP formation by 50–56%. The 31% reduction of BP metabolism due to pyrene resulted mainly from inhibition of quinones and dihydrodiols. On the other hand, pyrene was the least effective in inhibiting 3-hydroxyBP formation. These data again emphasize the importance of the pyrene ring structure in the selective inhibition of BP quinone formation.

With the concentration of CHP at 0.1 mM (Fig. 2B), BP quinones became the predominant metabolites, but a substantial amount of 3-hydroxyBP was still formed. Under these conditions the inhibitory effects of phenanthrene and pyrene on total BP metabolism, as well as quinone and 3-hydroxyBP formation, were found to be dose dependent (Table 2). Formation of metabolites decreased, with the exception of 3-hydroxyBP found in the presence of pyrene as inhibitor. In this case the amount of 3-hydroxyBP was larger than in the control experiment at all concentrations of pyrene. Moreover covalent binding of BP to microsomal protein correlated with quinone and not with 3-hydroxyBP formation. In fact, the dose-dependent decrease in quinone formation by phenanthrene reduced BP binding to microsomal protein, while the substantial inhibition of quinone formation by pyrene abolished BP–protein binding.

To rule out binding of BP quinones to proteins, covalent binding of BP and BP quinones to microsomal protein was compared. At 2 mM CHP the predominant metabolites were quinones, and approximately 12 nmoles BP quinones were formed per incubation in this experiment. When microsomal protein was incubated with 0.080 mM BP, the specific binding was $2.54 \pm 0.21$ nmoles BP/mg protein. When microsomal protein was incubated with 0.020 mM BP quinones, the specific binding was $0.85 \pm 0.21$ nmoles/mg protein. Therefore, from these results and those in Table 2, it is evident that reactive precursors to quinones are responsible for binding of BP to microsomal protein.

To gain some preliminary insight on the nature of the various isozymes, CHP-dependent BP metabolism by liver microsomes from male and female rats was compared (Fig. 3). BP metabolism was

Table 2. Effect of phenanthrene and pyrene on the cumene hydroperoxide (0.1 mM)-dependent [$^{14}$C]benzo[*a*]pyrene metabolism by rat liver microsomes

| Inhibitor | | [$^{14}$C]BP metabolites* (nmoles) | | | |
|---|---|---|---|---|---|
| | | Ethyl acetate extractable metabolites | Quinones | 3-HydroxyBP | Protein bound BP |
| Solvent (control) | | 11.6 | 6.8 (58)† | 1.6 (14) | 1.19 |
| Phenanthrene | 0.08 mM | 7.1 | 3.3 (46) | 1.4 (20) | 0.69 |
| | 0.16 mM | 4.7 | 2.6 (55) | 0.7 (15) | 0.49 |
| | 0.24 mM | 4.0 | 1.9 (48) | 0.3 (8) | 0.39 |
| Pyrene | 0.08 mM | 5.8 | 1.5 (26) | 2.5 (43) | <0.01 |
| | 0.16 mM | 4.5 | 1.1 (24) | 1.9 (42) | <0.01 |
| | 0.24 mM | 4.2 | 0.8 (19) | 1.8 (43) | <0.01 |

* Average of two experiments.
† Percentage of total extractable metabolites.

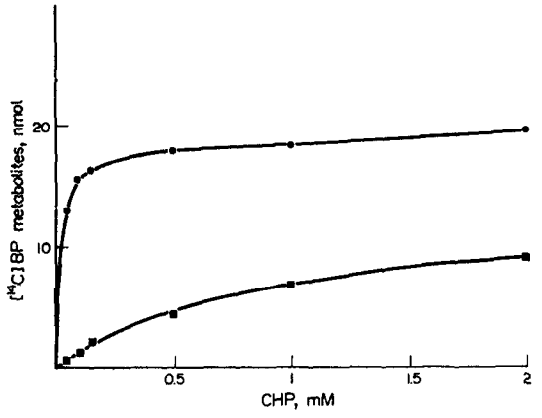


Fig. 3. Total ethyl acetate-extractable BP metabolites at various concentrations of CHP. Key: (●) male rat liver microsomes, and (■) female rat liver microsomes.

dependent on CHP concentration in both cases, but male rat liver microsomes catalyzed much more metabolism. In contrast to the male, female rat liver microsomes metabolized BP predominantly to quinones at both low and high CHP concentrations (Fig. 4). At 0.05 mM, CHP, BP metabolism was 0.9% and the distribution of metabolites was 61% quinones and 17% 3-hydroxyBP (Fig. 4A). At 2 mM CHP, BP metabolism increased to 7.8% with 74% quinones and 5.4% 3-hydroxyBP (Fig. 4B). Preincubation of microsomes with 0.080 mM pyrene reduced BP metabolism in the presence of 2 mM CHP to 1.2% (Fig. 4C) and resulted in a metabolic profile similar to that obtained with 0.05 mM CHP (Fig. 4A).

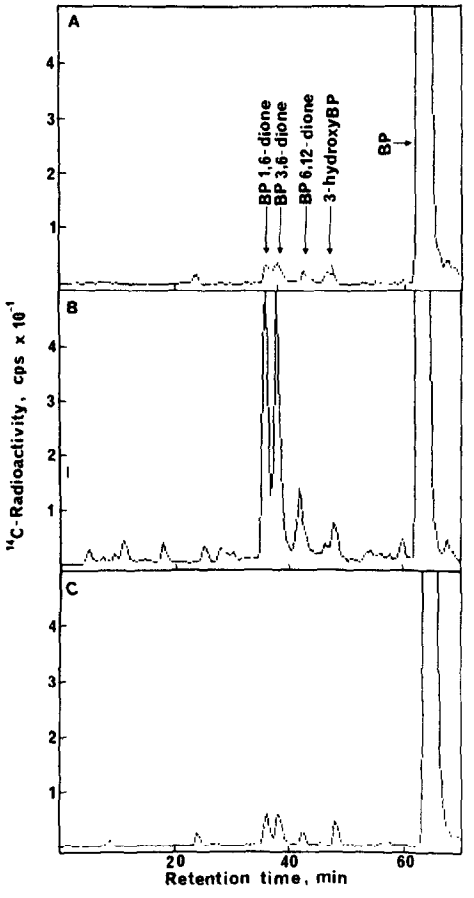


Fig. 4. Effect of CHP concentration and pyrene on HPLC profile of BP metabolites formed by female rat liver microsomes. (A) 0.05 mM CHP; (B) 2 mM CHP; and (C) 2 mM CHP plus 0.080 mM pyrene.

## DISCUSSION

Multiple forms of cytochrome P-450 metabolize a wide variety of endogenous and foreign compounds, including fatty acids, steroids, prostaglandins, drugs, insecticides and various carcinogens. Cytochrome P-450 isozymes vary somewhat in substrate specificity [9–12], regiospecificity [10–16] and stereospecificity [10, 14, 15, 17]. These effects have been observed for various substrates, including warfarin [14, 15], testosterone [11], alicyclic compounds [12] and BP [10, 13, 16, 17]. The fact that CHP is utilized by one or a few constitutive isozymes [6] provides a means of examining regiospecificity in the metabolism of BP. In this study we have investigated modulation of BP metabolism by PAH such as naphthalene, phenanthrene, benz[*a*]anthracene and pyrene.

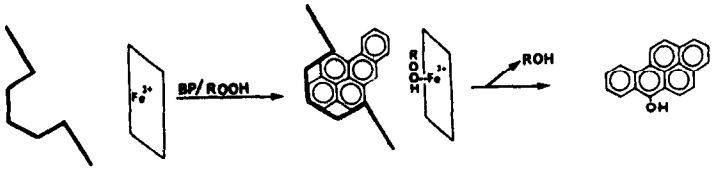


Fig. 5. Proposed interaction of BP with cytochrome P-450 binding site to form BP quinones.

Naphthalene, phenanthrene and benz[*a*]anthracene nonspecifically inhibited BP metabolism at 0.05 and 2 mM CHP (Table 1). However, pyrene showed a specific effect by eliminating the formation of quinones and increasing the formation of 3-hydroxyBP (Tables 1 and 2, Fig. 2D). Therefore, inhibition of quinone formation depends on the molecular geometry of the inhibitors: compounds with a pyrene region inhibit quinone formation.

BP must bind to the cytochrome P-450 apoprotein through the pyrene region to form quinones, because only pyrene effectively inhibited formation of these metabolites. This binding exposes C-6 to form 6-hydroxyBP, which by autoxidation leads to the three quinones [18, 19]. The hypothetical binding and catalytic sites are illustrated in Fig. 5.

In this regard, substantial evidence has been provided that formation of quinones occurs via an initial one-electron oxidation of BP, with formation of its radical cation with charge mainly localized at C-6 ([19, 20], manuscript in preparation). It has also been demonstrated that quinone formation and BP binding to protein increase with CHP concentration [6]. In the dose–response study reported here (Table 2), pyrene effectively inhibited not only formation of BP quinones, but also binding of BP to microsomal protein. Furthermore, the BP quinones formed in the metabolic incubations could not account for the level of protein-bound BP observed. These results strongly indicate that the BP radical cation is the reactive intermediate responsible for binding of BP to protein, as well as for quinone formation.

In the presence of 2 mM CHP, the inhibition of quinones by pyrene was accompanied by a 10-fold increase in 3-hydroxylation of BP (Table 1). This observation suggests that hydroxylations of BP at C-3 and C-6 were alternative pathways, depending on the availability of binding sites. It is clear that cytochrome P-450 isozyme(s) had different binding sites, and it seems that the binding site catalyzing formation of 3-hydroxyBP was the preferred one, since the predominant metabolite with CHP at low concentrations was 3-hydroxyBP (Fig. 2A). At higher CHP concentrations the cofactor interfered with 3-hydroxyBP formation. This effect rendered quinone formation predominant (Fig. 2C). The presence of pyrene as a competitor with BP for the pyrene region site restored formation of 3-hydroxyBP (Fig. 2D and Table 1).

When compared to males, female microsomes metabolized BP less effectively (Fig. 3). In this case quinones were the major metabolites, even at low CHP concentrations (Fig. 4A). High CHP concentrations (Fig. 4B) increased quinone formation dramatically, and when the microsomal preparation was incubated with pyrene (Fig. 4C), the profile of BP metabolites was restored to that obtained with low CHP concentration (Fig. 4A). Thus, metabolism of BP by female microsomes differed from that of the male in two ways. First, the level of BP metabolism was much less with females (Fig. 3), and second, 3-hydroxylation of BP was very ineffective (Fig. 4).

These experimental results allow us to distinguish between the binding sites for formation of 3-hydroxyBP and BP quinones in the CHP-dependent metabolism of BP. Further studies could provide evidence on the number of binding sites and cytochrome P-450 isozymes involved in BP metabolism.

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