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DEPENDENCE OF BENZO[*a*]PYRENE METABOLIC PROFILE ON THE CONCENTRATION OF CUMENE HYDROPEROXIDE WITH UNINDUCED AND INDUCED RAT LIVER MICROSOMES

ALLAN K. L. WONG, ERCOLE CAVALIERI* and ELEANOR ROGAN
The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68105, U.S.A.

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Abstract—The effect of cumene hydroperoxide (CHP) in microsomal metabolism of benzo[*a*]pyrene (BP) was studied using liver microsomes from mature male Wistar rats induced with phenobarbital (PB), 3-methylcholanthrene (MC), Aroclor 1254 or olive oil (uninduced). In contrast to NADPH-supported metabolism, these inducers did not increase the CHP-dependent metabolism. Total BP metabolism was dependent on CHP concentration and was maximal at 0.15 mM, except for PB-induced microsomes, which had a maximum at 0.5 mM CHP. At 0.05 mM CHP, the major metabolites were phenols. However, increasing CHP concentration enhanced the formation of dihydrodiols, quinones and protein-bound BP but reduced phenol production. At and above 0.15 mM CHP, the profile of BP metabolites was essentially constant, with at least 66% quinones but no more than 10% phenols. The effect of CHP on inhibition of phenol formation and enhancement of quinone formation was reversed by preincubation of microsomes with BP or by increasing BP concentration. These results suggest that CHP-dependent metabolism of BP is selectively mediated by constitutive cytochrome P-450 isozyme(s) and that two forms of BP binding sites exist in cytochrome P-450 isozymes and are responsible for the hydroxylation of BP at C-3 and C-6.

Organic hydroperoxides can replace NADPH and oxygen in supporting metabolism of xenobiotics by liver microsomes and purified cytochrome P-450 [1–6]. Although in both cases a highly oxidized form of iron is involved in activation of substrates, it is not yet certain whether the hydroperoxide-supported reactions occur by a classical peroxidase-type reaction [7, 8]. Metabolism of benzo[*a*]pyrene (BP†) supported by NADPH and oxygen and by cumene hydroperoxide (CHP) [5] shows a marked difference in the profiles of products. In fact, under peroxidatic conditions the predominant metabolites are the BP quinones, which are formed by an initial one-electron oxidation of BP [9]. When lipid hydroperoxides are generated, BP quinones are the major metabolites [10]. To provide evidence on some of the factors involved in the hydroperoxide-supported cytochrome P-450 catalytic process, a study of the effect of CHP concentration on BP metabolism was conducted with uninduced and with phenobarbital (PB)-, 3-methylcholanthrene (MC)- and Aroclor-induced rat liver microsomes.

MATERIALS AND METHODS

Chemicals. Aroclor 1254 was purchased from Analabs Inc. (North Haven, CT). BP and MC were purchased from the Aldrich Chemical Co. (Milwaukee, WI), and CHP was obtained from the Sigma Chemical Co. (St. Louis, MO). BP was purified by chromatography on silica gel with hexane–benzene (8:2) and recrystallization from benzene–methanol (m.p. 176–178°). [^{14}C]BP (sp. act. 58.5 μCi/mole) was purchased from Amersham (Arlington Heights, IL), diluted 100-fold with unlabeled BP, and dissolved in dimethyl sulfoxide to give a 1.6 mM solution. The mixture was checked periodically for oxidized product by high pressure liquid chromotography (HPLC) and stored frozen under argon.

Pretreatment of animals and preparation of liver microsomes. Male Wistar rats (8- to 10-weeks-old, Eppley Colony) were injected intraperitoneally with Aroclor dissolved in olive oil (100 mg/ml) at 100 mg/kg on days 1 and 3 and killed on day 6. MC was dissolved in olive oil (20 μmoles/ml) and injected on two consecutive days at 100 μmoles/kg. PB was dissolved in saline (40 μmoles/ml) and injected on five consecutive days at 400 μmoles/kg. Both MC- and PB-treated animals were killed 24 hr after the last injection. Uninduced animals received injections of olive oil. All animals were fasted overnight before being killed by cervical dislocation.

Preparation of liver microsomes was performed at 0–4°. The liver was perfused with 10 ml of 0.9% NaCl–0.1 mM EDTA, pH 7.5, via the portal vein, minced, and homogenized in 30–40 ml of 0.25 M sucrose–0.1 mM EDTA, pH 7.5. The homogenate was 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

* Author to whom all correspondence should be addressed.

† Abbreviations: BP, benzo[*a*]pyrene; MC, 3-methylcholanthrene; PB, phenobarbital; CHP, cumene hydroperoxide; and HPLC, high pressure liquid chromatography.

microsomal pellets were resuspended in the same sucrose solution at 1 ml/g liver and stored at −80°.

Metabolism of [^{14}C]BP by rat liver microsomes. The assay for BP metabolism was performed in a 1 ml volume with 1 mg microsomal protein, 50 mM Tris–HCl, 150 mM KCl and 5 mM $MgCl_2$ at pH 7.5. The mixture was preincubated for 3 min at 37°. Unless otherwise specified, the reaction was initiated by adding 80 nmoles [^{14}C]BP, immediately followed by the appropriate amount of CHP. Blanks were samples without CHP or microsomal protein. After incubation for 10 min the reaction was terminated by addition of 1 ml acetone. BP metabolites were extracted three times with 1.5 ml ethyl acetate and stored at −10° in the dark before analysis. Protein in the aqueous phase was precipitated with 12 ml acetone, redissolved in 3 ml warm 1% sodium dodecyl sulfate, and extracted with 3 ml ethyl acetate. Aliquots (0.5 ml) of the protein solution were counted for radioactivity. In NADPH-dependent metabolism of BP, the microsomal protein was preincubated with an NADPH-generating system (4.3 μmoles NADP, 12.9 μmoles glucose-6-phosphate and 0.43 units of glucose-6-phosphate dehydrogenase) for 5 min followed by incubation with [^{14}C]BP for 30 min.

Inhibition of [^{14}C]BP metabolism was performed by preincubation with various inhibitors for 5 min. For carbon monoxide and argon treatment, 4-ml reaction mixtures in 50-ml Erlenmeyer flasks sealed with serum caps were flushed with the gas at 0° for 5 min. After preincubation at 37° for 3 min, appropriate amounts of [^{14}C]BP and CHP were injected through the serum cap, and the mixtures were incubated for another 10 min. Aliquots (1 ml) were mixed with 1 ml acetone and then extracted with ethyl acetate, as described above.

Analysis of [^{14}C]BP metabolites by HPLC. Samples of ethyl acetate extract were processed immediately before HPLC analysis. After evaporation to dryness under reduced light with a stream of oxygen-free argon, they were reconstituted in 100 μl dimethylsulfoxide–methanol (1:1). A 10-μl aliquot was counted for radioactivity, and the remainder was injected onto an Altex Ultrasphere 5 μm ODS reverse phase column on a SpectraPhysics SP8700 solvent delivery system. The column was eluted with 60% methanol for 10 min followed by a 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, which was monitored with a continuous flow system using a RAMONA radiometric detector (IN/US, Fairfield, NJ) with a 2.5-ml liquid cell. Liquid scintillation counting fluid had a flow rate of 2.5 ml/min. Radioactivity data were processed with an automatic data integration system. The recovery of radioactivity was greater than 95%. Major metabolites were identified by their u.v. absorbance spectra.

Other assays. Cytochrome P-450 content was measured by the technique of Omura and Sato [11], and protein concentration was determined according to Lowry *et al.* [12]. The radiometric assay of DePierre *et al.* [13] was also used to determine total microsomal metabolism of [^{14}C]BP. In this procedure, the enzymatic reaction was terminated by adding 1 ml of 0.5 M NaOH–80% ethanol to the sample, and the radioactivity in the aqueous phase was measured after extraction with 2 ml hexane.

RESULTS

To establish the optimal incubation time for CHP-supported metabolism, assays were performed with 0.05 and 2 mM CHP for different periods of time. The initial reaction was rapid at 2 mM CHP, and the amount of BP metabolites formed with 2 mM CHP was about 2.5 times that observed with 0.05 mM. Maximal metabolism of BP with 2 mM CHP was obtained after 6 min of incubation. With 0.05 mM CHP the rate of metabolism began to plateau at about 10 min. Therefore, 10 min was chosen as the standard incubation time when CHP was used as cofactor.

The effect of induction on microsomal metabolism of BP was compared using CHP and NADPH as cofactor. The results in Table 1 show that induction with PB and MC increased cytochrome P-450 levels by about 2.5 times, while Aroclor induced the level about 4.5 times. All three inducers increased the amount of NADPH-dependent BP metabolism substantially. In contrast, the amount of CHP-dependent BP metabolism was independent of induction. To assure that CHP-dependent metabolism was catalyzed by cytochrome P-450, several inhibitors were used, with inhibition ranging from 49 to 92% (Table 2). Carbon monoxide was a more effective inhibitor of cytochrome P-450 than argon, suggesting that the action of carbon monoxide was more than that of anaerobiosis. These data demonstrate the involvement of cytochrome P-450 in the metabolism of BP.

NADPH-supported microsomal metabolism of BP yielded dihydrodiols, quinones and phenols (Fig. 1). The metabolic profile with PB-induced microsomes

Table 1. Ethyl acetate-extractable products from BP metabolism by induced and uninduced rat liver microsomes in the presence of CHP or NADPH-generating system

Inducer	Cytochrome P-450 content (nmoles/mg protein)	[^{14}C]BP metabolized (nmoles)	
		NADPH	CHP*
Olive oil	0.77	29.9	13.0
PB	1.91	41.1	12.0
MC	1.72	73.1	12.6
Aroclor	3.53	65.7	13.6

* CHP concentration was 2 mM.

Table 2. Inhibition of CHP-supported [^{14}C]BP metabolism by uninduced rat liver microsomes

Inhibitor	[^{14}C]BP metabolized (nmoles)
None	15.43 ± 0.60
Carbon monoxide	4.01 ± 0.53
Argon	7.92 ± 0.14
Metyrapone, 1.5 mM	1.67 ± 0.75
2-(4,6-Dichloro-*o*-biphenyloxy)ethylamineHBr (DPEA) (0.5 mM)	1.26 ± 0.02
N-Bromosuccinimide, 0.8 mM	3.08 ± 0.40

CHP concentration was 2 mM. Each value is the mean ± S.D. of at least three incubations.

(Fig. 1B) was characterized by an increase in BP 4,5-dihydrodiol at the expense of BP 9,10-dihydrodiol, BP 7,8-dihydrodiol and 3-hydroxyBP. With MC-induced microsomes (Fig. 1C), the profile was similar to that of uninduced microsomes (Fig. 1A), whereas with Aroclor-induced microsomes (Fig. 1D) the profile was intermediate between PB-induced and uninduced.

Typical HPLC profiles of BP metabolites obtained with uninduced microsomes and various levels of CHP are presented in Fig. 2. MC- and Aroclor-induced microsomes gave similar profiles. At 0.05 mM CHP the major metabolites were phenols (49–54%) with 3-hydroxyBP as the major component (Fig. 2A). Substantial amounts of quinones were formed (28–35%). Dihydrodiols were minor metabolites (16–18%), and BP 9,10-dihydrodiol was the major component. Increased CHP concentration enhanced the formation of quinones and dihydrodiols, but reduced phenol production (Fig. 2B). At 0.15 mM CHP, quinones became the predominant metabolites (66–74%), with BP 1,6- and 3,6-dione as major components (Fig. 2C). With PB-induced microsomes, similar profiles of BP metabolites were observed, except that quinones were always the major metabolites. Incubation of BP with CHP or microsomal protein alone produced no detectable metabolites.

Formation of total BP metabolites was dependent on CHP concentration (Fig. 3). The optimal CHP concentration was 0.15 mM for uninduced, MC- and Aroclor-induced microsomes and 0.5 mM for PB-

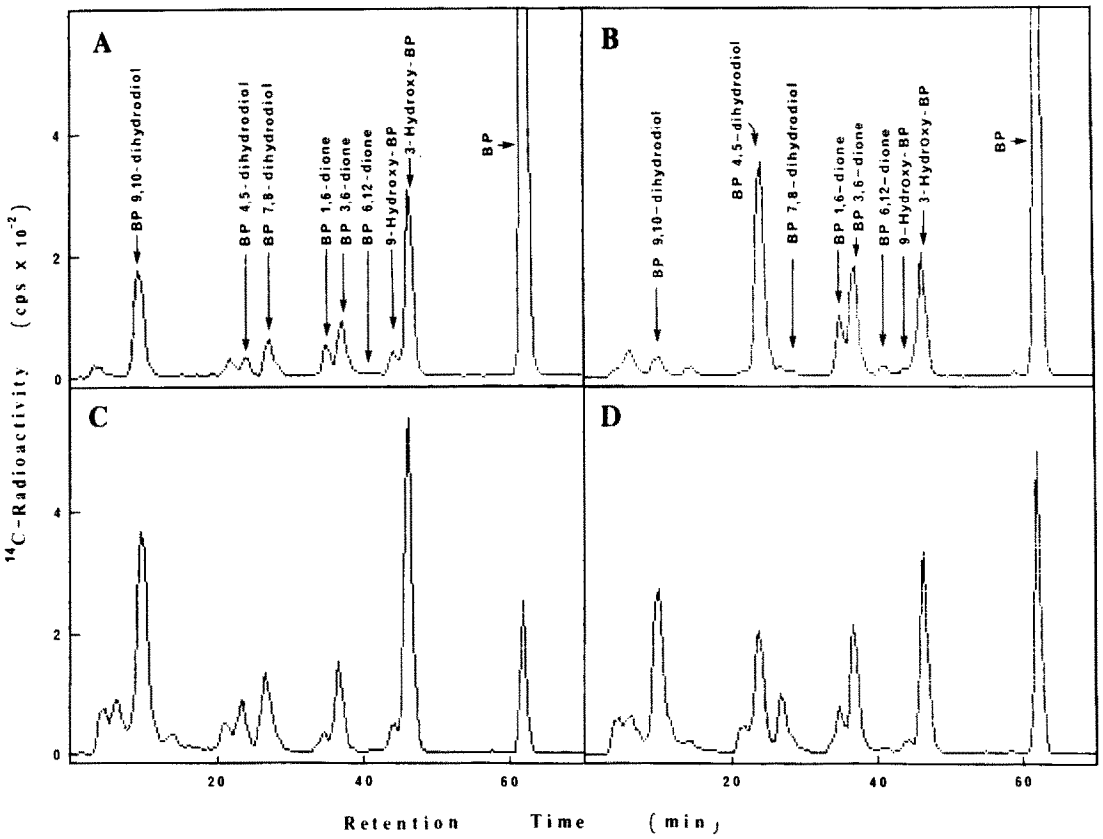

Fig. 1. HPLC profiles of [^{14}C]BP metabolites formed in NADPH-supported microsomal incubations. Key: (A) uninduced, (B) PB-induced, (C) MC-induced, and (D) Aroclor-induced.

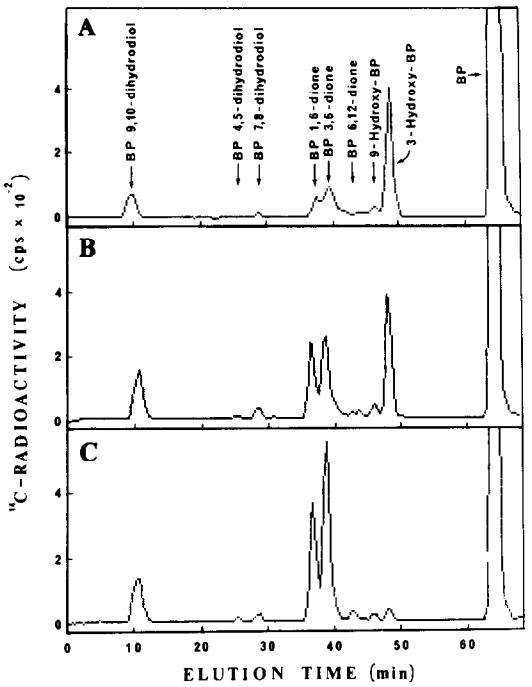

Fig. 2. HPLC profiles of [^{14}C]BP metabolites from uninduced rat liver microsomal incubations with various CHP concentrations. Key: (A) 0.05 mM, (B) 0.10 mM, and (C) 0.15 mM.

induced microsomes. BP metabolism was independent of induction within the range of CHP concentration tested, except that BP metabolism by PB-induced microsomes was lower at CHP concentrations below 0.5 mM.

Formation of different classes of BP metabolites was similarly influenced by CHP concentration for uninduced and induced microsomes (Fig. 4). Higher CHP concentration enhanced the formation of quinones, dihydrodiols and protein-bound BP, but it reduced the formation of phenols. The critical CHP concentration was 0.15 mM, above which the profile of BP metabolites was essentially constant. At and above this concentration, the preponderant metabolites were quinones, which accounted for at least two-thirds of all metabolites. Dihydrodiols and protein-bound BP were formed in approximately equal amounts. BP phenols were minor metabolites and accounted for no more than 10% of total BP metabolism. BP metabolites were similarly produced in all microsomal preparations, with the exception of phenols, which were poorly formed by PB-induced microsomes even at low concentrations of CHP.

The effect of preincubation of microsomes with BP before addition of CHP was studied. Preincubation with BP for 3 and 30 sec markedly enhanced the formation of 3-hydroxyBP (Table 3). Although there was no significant difference in total BP metabolites, phenol formation increased from 2 to 13 and 19% after preincubation with BP for 3 and 30 sec respectively. Increased formation of 3-hydroxyBP was also observed with 2 mM CHP and preincubation of BP for 30 sec (data not shown). The amount of quinones was reduced, but that of dihydrodiols was unchanged, with BP 9,10-dihydrodiol remaining as the major component. When the BP concentration was doubled without preincubation, total BP metabolism was increased by 50%. This was the result of enhanced formation of both phenols (mainly 3-hydroxyBP) and dihydrodiols (mainly BP 9,10-dihydrodiol). On the other hand, the production of quinones was reduced by 45%.

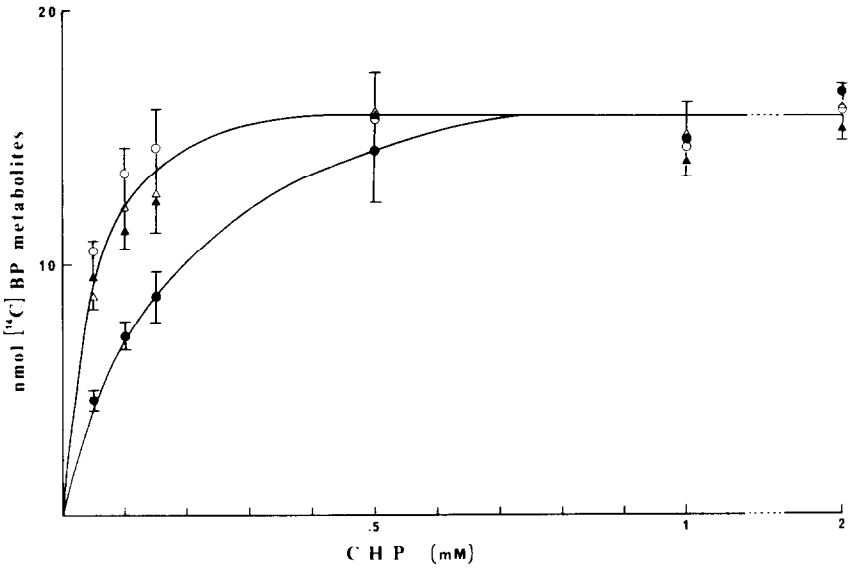

Fig. 3. Total [^{14}C]BP metabolite formation at various CHP concentrations in rat liver microsomal incubations. Key: (○) uninduced, (△) Aroclor-induced, (▲) MC-induced, and (●) PB-induced microsomes.

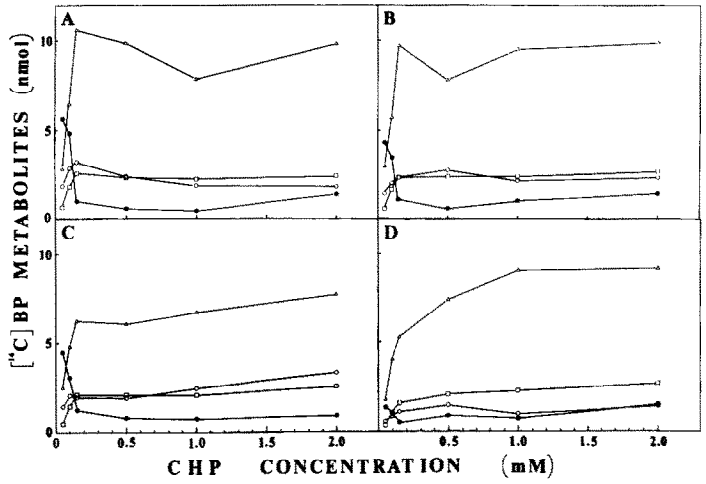

Fig. 4. Effect of CHP concentration on formation of [^{14}C]BP metabolites from rat liver microsomal incubations. Key: (A) uninduced, (B) Aroclor-induced, (C) MC-induced, and (D) PB-induced microsomes, (△) quinones, (●) phenols, (○) dihydrodiols, and (□) protein-bound BP.

DISCUSSION

At both high and low levels of CHP, formation of total BP metabolites was essentially complete in 10 min. The ability of PB, MC and Aroclor to induce isozymes of cytochrome P-450 which catalyze NADPH-supported reactions is evident in Table 1 and has been widely reported. However, the level of CHP-supported metabolism was not increased upon induction. In fact, total metabolism decreased markedly with PB-induced microsomes at low CHP concentrations (Fig. 3).

Metabolism in NADPH-supported reactions (Fig. 1) yielded the three classes of products, phenols (3- and 9-hydroxyBP), quinones BP 1,6-, 3,6- and 6,12-dione) and dihydrodiols (BP 4,5-, 7,8- and 9,10-dihydrodiol) plus trace amounts of more polar products. When compared to uninduced microsomes, the main features in PB-induced microsomal profiles were an increase in BP quinones and BP 4,5-dihydrodiol. With MC-induced microsomes, a major increase in 3-hydroxyBP and 7,8- and 9,10-dihydrodiol was observed. Aroclor-induced microsomes enhanced metabolites observed with PB- and MC-induced microsomes.

The profile of BP products obtained with 0.05 mM CHP (Fig. 2) showed the following metabolites: 3-hydroxyBP, 9-hydroxyBP, BP diones and BP 9,10-dihydrodiol, whereas the other two dihydrodiols (4,5- and 7,8-) were formed only in trace amounts. The singular feature of this metabolic profile was the sharp change when the concentration of CHP was raised from 0.10 to 0.15 mM. In the latter case, formation of 3-hydroxyBP became negligible. Our results are at variance with the observation of Capdevila *et al.* [5] that quinones are predominant in metabolism of BP by PB-induced microsomes at CHP concentrations of 0.012 to 0.12 mM.

The same metabolic profile at each concentration of CHP was observed with PB-, MC- and Aroclor-induced microsomes (data not shown). As CHP was increased from 0.05 to 2.0 mM, the amount of quinones, 9,10-dihydrodiol and protein-bound metabolites increased and then plateaued similarly with all microsomal preparations, while the amount of phenols decreased (Fig. 4). The lack of increased metabolism with induced microsomes and the similarity of the metabolic profiles at various CHP concentrations with uninduced and induced microsomes clearly indicate that constitutive isozyme(s) of cytochrome P-450 conducts the CHP-supported metabolism of BP.

A puzzling effect in these experiments is represented by the sharp decrease in 3-hydroxyBP in a narrow range of CHP concentrations. This suggests that the cofactor may interfere with formation of

Table 3. Effect of substrate preincubation on rat liver microsomal metabolism of [^{14}C]BP in the presence of 0.15 mM CHP

Preincubation time (sec)	[^{14}C]BP conc (μM)	(nmoles [^{14}C]BP metabolized)	% Metabolite	
			Quinones	3-hydroxyBP
0	80	12.2	78	2
3	80	13.2	69	13
30	80	13.2	60	19
0	160	19.5	29	52

this metabolite. This "interference" can be partially reversed by preincubation of BP with the microsomes or by increasing the concentrations of BP in the incubation mixture (Table 3). More detailed studies of this phenomenon are currently in progress to elucidate possible mechanisms by which CHP interferes with formation of 3-hydroxyBP.

This study shows that phenols are formed in CHP-supported metabolism of BP, in contrast to previously reported results [5]. The lack of phenols at high concentrations of CHP is apparently due to "interference" by the cofactor with the enzyme binding site required for formation of phenols. In contrast to NADPH-supported metabolism, CHP-dependent metabolism of BP is selectively mediated by cytochrome P-450 isozyme(s) which is not inducible by MC, PB or Aroclor. Two forms of BP binding site may exist in cytochrome P-450 and are responsible for hydroxylation of BP at C-3 and C-6. It is important to learn whether other hydroperoxides, especially the lipid hydroperoxides, behave similarly with regard to the mechanism of metabolic activation as well as selectivity in cytochrome P-450 isozyme(s).

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