Cytochrome P-450 Mediated Reductive Dehalogenation of the Perhalogenated Aromatic Compound Hexachlorobenzene†

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ABSTRACT: Hexachlorobenzene (HCB) elicits concentration-dependent and saturable type 1 binding spectra when added to oxidized (Fe^{3+}) cytochrome P-450 (CYT P-450) in control, phenobarbital- (PB) induced, and β-naphthoflavone- (BNF) induced male Sprague-Dawley rat liver microsomes. The spectral binding constants (K_s) for HCB in control and PB-induced microsomes are 180 μM and 83 μM, respectively, and correlate inversely with the specific content of CYT P-450 (0.9 and 2.1 nmol/mg) in the two microsomal preparations. BNF-induced microsomes show type 1 interaction only at low HCB concentration. Overall biotransformation of HCB, monitored by loss of [^{14}C]HCB from the reaction medium, is dependent on NADPH and intact microsomes. Dimethyl sulfoxide (Me_2SO), a potent hydroxyl radical scavenger and the solvent used for HCB dissolution, does not affect the biotransformation of HCB in aerobic reactions. Pentachlorobenzene (PCB) appears to be the initial and major isolatable CYT P-450 mediated dechlorination product of HCB with NADPH-fortified rat liver microsomes. Trace levels of pentachlorophenol (PCP) and an unidentified metabolite are also observed. PCB formation is enhanced under anaerobic conditions but is inhibited by metyrapone and carbon monoxide. PCB formation is also inhibited with aerobic reaction conditions, while PCP formation is observed. The data indicate that CYT P-450 in hepatic microsomes supports the reductive dechlorination of HCB to PCB.

Mechanistic studies of the CYT[1] P-450 monooxygenase system have focused mainly on the nature of the activated oxygen species (Guengerich & McDonald, 1984). However, with organic substrates that have nonhydroxylatable carbon or heteroatom positions, reductive activation of the substrate has been observed (Mansuy et al., 1974; Wolf et al., 1977; Gillette et al., 1968; Nastainczyk et al., 1982; Uehleke et al., 1973; Castro et al., 1985; Ruf et al., 1984). This group of substrates has been limited to polyhalogenated aliphatic, nitroso, hydroxylamine, and nitro compounds.

The role of the CYT P-450 monooxygenase system in the metabolism of hexachlorobenzene (HCB), a hepatoporphyrinogen (Courtney, 1979) and suspect carcinogen (Cabral et al., 1977), has been postulated but never thoroughly demonstrated. Dehalogenation of this perhalogenated aromatic compound would be expected to go through a reductive mechanism, as observed with the analogous polyhalogenated aliphatic hydrocarbons, or, because of its aromaticity, to undergo oxidative dehalogenation via an arene epoxide intermediate (Knight & Young, 1958; Sato et al., 1963). In vivo and in vitro experiments have shown that HCB undergoes very slow biotransformation (Debets & Strik, 1979). Metabolism studies using ^{14}C-radiolabeled HCB, TLC, GC/ECD, and/or GC/MS have shown trace levels of pentachlorobenzene (PCB) and tetrachlorobenzenes, higher levels of oxidative products (e.g., chlorinated phenols, quinones, and their glucuronide conjugates), and thiol and methyl thioether analogues of the oxidative products. These studies were conducted with subacute or chronic HCB exposure allowing detectable amounts of metabolites to accumulate and by induction of the CYT P-450 system. CYT P-450 inducers, such as phenobarbital (PB) and β-naphthoflavone (BNF), enhanced HCB-induced porphyria, which is thought to be due to a reactive intermediate or metabolite. Hence, the CYT P-450 monooxygenase system was implicated in the oxidative dechlorination of HCB. Long sample collection times also allowed secondary biotransformation of the initial, putatively CYT P-450 mediated dechlorinated products of HCB. The only microsomal-based HCB metabolism study that we are aware of (Mehendale et al., 1975) reported qualitatively that oxidative products occur when noninduced rat liver microsomes are fortified with NADPH. However, nonenzymatic (NADPH-independent) production of chlorinated benzenes was observed. It was concluded by these investigators and other investigators that the reductive dechlorination of HCB is a minor pathway.

The experiments described in this paper test the hypothesis that CYT P-450 monooxygenase mediates the dechlorination of HCB. Rat liver microsomes are used as the enzyme source because the effects of the different CYT P-450 isozyme families and accompanying electron-transfer components induced by PB and BNF can be compared and the components (NADPH-CYT P-450 reductase, lipid, and CYT P-450 hemoproteins) are in approximately in vivo stoichiometries and spatial arrangements. This paper reports for the first time evidence indicating that CYT P-450 catalyzes the reductive

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[1] Abbreviations: CYT, cytochrome; HCB, hexachlorobenzene; PCB, pentachlorobenzene; PCP, pentachlorophenol; TtCB, tetrachlorobenzene; TtCP, tetrachlorophenol; *p*-TCBQ, tetrachloro-*p*-benzoquinone; DCDMS, dichlorodimethylsilane; *k'*, capacity factor $[(t_s - t_0)/t_0$, t_0 = retention time of *p*-TCBQ and t_s = retention time of analyte]; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; GC/ECD, gas chromatography/electron capture detection; FAB, fast atom bombardment.

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dechlorination of HCB to PCB. Detailed C_{18} reverse-phase HPLC methodology, which was used to purify the [^{14}C]HCB and resolve the metabolites of HCB isolated from a lipid matrix, is also described.

Materials and Methods

Chemicals. All reagents were of ACS or HPLC quality and purchased from commercial sources. HCB and PCB (Aldrich Chemical Co., 97–98% purity) and PCP (Sigma Chemical Co.) were purified by recrystallization after treatment with activated charcoal. [^{14}C]HCB (New England Nuclear, 35.3 μCi/μmol) was purfied by subjecting 5 μCi of [^{14}C]HCB to C_{18} reverse-phase HPLC and collecting and extracting the radioactivity eluting at the (k') value of unlabeled HCB standard.

Liver Microsomes. Male Sprague-Dawley rats were induced with PB (75–100-g rats, 80 mg/kg in saline, ip, twice a day for 3 days) and BNF (50–75-g rats, 80 mg/kg in corn oil, once a day for 3 days). Liver microsomes were prepared by differential centrifugation as previously described (Dignam & Strobel, 1977).

Spectrophotometric Measurements. The Lowry assay (Lowry et al., 1951) and reduced CYT P-450–CO difference spectroscopy (Omura & Sato, 1964) were used to quantitate protein and CYT P-450, respectively. A Beckman Acta MVI double-beam recording spectrophotometer with turbid sample compartment was used to quantitate CYT P-450 and HCB/CYT P-450 binding interactions. Difference spectroscopy was used to study HCB binding interactions with oxidized (Fe^{3+}) CYT P-450 in microsomal suspensions (Remmer et al., 1966). Use of the turbid sample compartment and 3-mL, wide-faced, cuvettes reduce wavelength-dependent light scattering to give a flat base line.

HPLC/Liquid Scintillation Spectrophotometry (LSS). A C_{18} reverse-phase, 10-μm, 8-mm i.d. Radical PAK cartridge in a RCM-100 radial compression module with accompanying Waters Assoc. Models 6000A and 450 solvent delivery systems, 660 solvent programmer, and 440 UV absorbance (254 nm) detector was used to resolve and detect HCB metabolites. HCB resolution (k' = 3.24) from PCB (k' = 2.14) and PCP (k' = 0.99) is attained with a 100:700:0.16 water–methanol–acetic acid (v/v/v) to 100% methanol linear gradient over 20 min at 2 mL/min. HPLC effluent fractions of 0.4 mL are collected, and 3.5 mL of Liquiscint (National Diagnostics) is added per fraction. ^{14}C radioactivity is quantitated in a Packard Tri Carb LSS. Counting efficiency is approximately 50%. Better resolution of the metabolites eluting before PCP can be attained by lowering the solvent strength of the initial conditions. The high initial solvent strength gives fast analysis time and minimizes column pressure build-up due to very hydrophobic matrix elements. SEP PAK precolumn sample clean-up was used for the 5-mL incubation extracts.

NADPH-Dependent HCB Disappearance. Silanized glassware (1% DCDMS in CH_2Cl_2) was used for all HCB metabolism experiments. Stock solutions of HCB were prepared in Me_2SO. Aerobic reactions consisting of 4.2 mL of 4 μM PB CYT P-450 microsomes, 100 mM KP_i, and 1.5 mM $MgCl_2$, pH 7.4, are incubated with 21 μL of 3.52 mM HCB (6 μCi/μmol) and 42 μL of 7.04 mM HCB (1.5 μCi/μmol) resulting in final concentrations of 17.6 μM HCB/70.5 mM Me_2SO and 70.4 μM HCB/141 mM dimethyl sulfoxide (Me_2SO), respectively. NADPH is added to a final concentration of 2.5 mM, and the reaction is performed at 37 °C with shaking. Control samples consisted of native microsomes incubated with [^{14}C]HCB at 37 °C. NADPH is added prior to acidification and extraction. Aliquots of 1 mL are removed at 0 (right after addition of NADPH), 15, 40, and 60 min. A total of 0.5 mL of 2 N HCl is added to the aliquots, and hexane–toluene, 80:20 v/v, extraction is performed twice. A final toluene–2-propanol, 80:20 v/v, extraction is done. The organic extracts are pooled and allowed to evaporate in a vacuum hood. A total of 100 μL of CH_2Cl_2–methanol, 50:50 v/v, is used to dissolve the residue, and 25 μL is subjected to HPLC. Radioactivity is quantitated by LSS.

HCB Metabolite Production. A final reaction volume of 5 mL containing either 2 or 4 μM CYT P-450 microsomal suspensions in the same buffer system described above is incubated with 50 μL of a 5.28 mM stock solution of [^{14}C]-HCB/Me_2SO (2 μCi/μmol). All samples were kept on ice while they were purged with a slow stream of humidified N_2 or CO for 15 min. NAD(P)H was added to a final concentration of 2.5 mM and the reaction was incubated at 37 °C. The aerobic reaction samples were left open to the atmosphere after purging with N_2. The saturated N_2 and CO samples were sealed and incubated as indicated. Two milliliters of 2 N HCl was added to the 5-mL reaction volumes for termination, and the extraction is the same as described for the HCB disappearance experiments, except 10-mL volumes of solvent are used. Once the organic extracts dry down to 0.2–0.3 mL, they are applied to CH_2Cl_2-equilibrated SEP PAK silica gel cartridges to remove polar lipids. The first 4 mL eluting with a CH_2Cl_2 wash is collected and allowed to slowly evaporate to dryness. A total of 50 out of 100 μL is fractionated and quantitated by HPLC/LSS. The overall recovery of HCB, PCB, and PCP was 98–100% with Me_2SO as the solvent for additions and the extraction procedure described.

Structure Verification. Anaerobic, 20 mL, 4 μM PB CYT P-450 microsomal reaction mixtures were incubated with 52.8 μM HCB (final concentration) added with 0.2 mL of Me_2SO. NADPH was added to 2.5 mM final concentration, and the reaction was run for 1 h at 37 °C. The reactions were extracted as previously described, and SEP PAK precolumn sample clean-up and HPLC of the residue was performed. Fractions eluting at the k' of PCB standard were collected and extracted. Toluene was used to redissolve the residue. A Finnigan 3200 GC/MS interfaced to an INCOS Data System with the following instrument parameters were used: column, 2 mm i.d. × 1.5-m glass column packed with 5% SP-2401 (Supelco); carrier gas, He; injector temperature, 225 °C; column operating temperature, 100 °C for 2 min and then 10 °C/min up to 250 °C; mass analyzer, 70-eV EI mode.

Results and Discussion

HCB is a unique polyhalogenated aromatic hydrocarbon. This perchlorinated molecule can be viewed as the aromatic analogue of carbon tetrachloride, a perchlorinated aliphatic CYT P-450 substrate. Oxidative or reductive CYT P-450 mediated dechlorination of HCB would be expected to yield PCP or PCB, respectively. HPLC and LSS was used to monitor production of these potential initial CYT P-450 mediated metabolites isolated from microsomal reactions with [^{14}C]HCB. HCB is a reported suspect human carcinogen, hepatoporphyrinogen, "mixed-type" CYT P-450 inducer, and environmental pollutant. Its interaction with the ubiquitous CYT P-450 mixed-function oxidase system requires clarification.

HCB-Dependent Type I Interaction with Oxidized (Fe^{3+}) CYT P-450 Hemoproteins in Microsomal Fractions. HCB-dependent and saturable type I difference spectra are generated in liver microsomal suspensions from PB-induced and control (noninduced) male Sprague-Dawley rats, as shown in Figure 1. Microsomes from BNF-induced rats show type I inter-

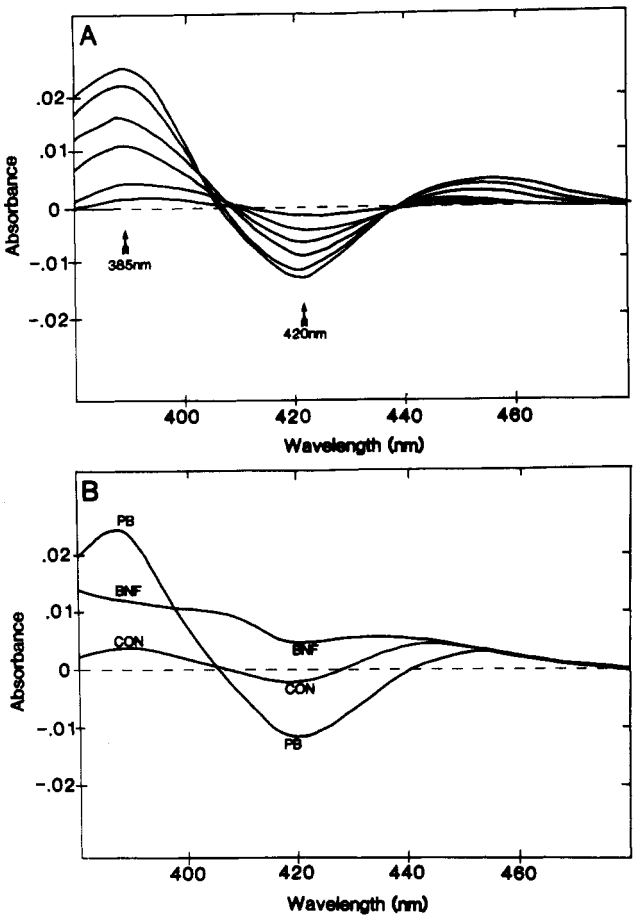

FIGURE 1: HCB-dependent and saturable type I difference spectra (A) generated with PB-induced rat liver microsomes (0.5 mg/mL) containing 1 μM oxidized CYT P-450. Six 2-μL aliquots of a 7.0 mM stock solution of HCB in Me_2SO were added to a 2-mL assay volume. Comparison (B) of HCB-induced (42.2 μM) difference spectra with equimolar (1 μM) oxidized CYT P-450 in PB- and BNF-induced and noninduced microsomes.

actions only at low HCB concentrations, but at high HCB concentrations, atypical HCB-dependent spectral perturbations occur. Thus, HCB exhibits specific binding to CYT P-450 hemoproteins. Type I interactions are observed with many of the typical assay substrates and effectors of the CYT P-450 monooxygenase system (Schenkman et al., 1982). The type I spectral transition is the result of an effector-induced conformational change or direct interaction at the heme iron center or porphyrin ring system in the CYT P-450 hemoprotein that shifts the low-spin to high-spin equilibria of the (Fe^{3+}) d-orbital electrons (Schenkman et al., 1982). The shift in spin equilibrium manifests itself as a hypsochromic shift in the heme γ-Soret band, observed as a peak at 385 nm and a trough at 420 nm with difference spectroscopy.

Qualitative and quantitative differences in the HCB-induced type I interactions exist between PB- and BNF-induced and noninduced rat liver microsomes. The three different microsomal preparations represent three different CYT P-450 isozyme profiles (Hildebrandt et al., 1968; Sladek & Mannering, 1969a,b; Thomas et al., 1981) and give three different HCB-dependent spectral phenomena, as shown in Figure 1B. The data in Table I indicate that at equimolar CYT P-450 the spectral binding constant (K_S) values for control and PB-induced rat liver microsomes are 180 and 83 μM, respectively, and correlate inversely with the specific contents of CYT P-450 in these microsomal preparations. With PB-induced microsomes, the maximum peak-to-trough absorbance difference is proportional to CYT P-450 concentration (0.125–2 μM),

Table I: HCB-Induced Type I Binding Constants (K_S) and A_{max} Values Generated with Different Rat Liver Microsomal Sources[a]

microsome source	specific content of CYT P-450 (nmol/mg of protein)	K_S (μM)	A_{max} (absorbance units)
control (noninduced)	0.9	180	0.006
PB induced	2.1	83	0.038
BNF induced	1.4	*b*	*b*

[a] K_S was determined from double-reciprocal analysis of the peak – trough absorbance difference ($A_{385nm} - A_{420nm}$) vs. HCB concentration with 1 μM oxidized microsomal CYT P-450. A_{max} was determined directly from the spectra. [b] HCB-induced type I interaction only at low concentrations.

but the apparent K_S remains constant (data not shown). However, highly hydrophobic substrates do not adhere to simple Michaelis–Menton kinetic interpretation (Nebert & Gelboin, 1968). Nonspecific binding of HCB to extraneous proteins and lipids may increase the apparent K_S values determined in control and PB-induced microsomes. The inverse correlation between K_S and specific content could also be due to PB induction of CYT P-450 isozymes that bind HCB with a low K_S (Waxman & Walsh, 1983).

Our data indicate that HCB specifically binds to CYT P-450 hemoproteins present in rat liver microsomal fractions, but such a phenomenon may or may not be catalytically relevant, because binding does not necessitate metabolism. HCB binds to (Fe^{3+}) CYT P-450 in a typical type I interaction but may react in an atypical fashion because of its perhalogenated aromatic structure.

CYT P-450 Mediated Reductive Dechlorination of HCB. Typical CYT P-450 monooxygenase activity requires NADPH, and O_2, and is inhibited by CO and (often by) metyrapone. The overall metabolism of HCB in a PB-induced microsomal reaction system is NADPH-dependent and linear over a 60-min aerobic incubation at 37 °C. The loss of [^{14}C]HCB (monitored by HPLC/LSS) from the reaction medium increased from 21 to 201 pmol of HCB min^{-1} (mg of microsomal protein)$^{-1}$ as the HCB concentration increased from 18 to 70 μM, respectively. The NADPH-dependent biotransformation of HCB is dependent on the initial substrate (HCB) concentration even when the concentration of Me_2SO [a potent hydroxyl radical scavenger (Winston & Cederbaum, 1983) and heavy metal chelator (Meek et al., 1960)] is increased from 70 to 141 mM. Thus, under aerobic conditions hydroxyl radicals produced by redox cycling and Fenton chemistry of oxygen at flavin, heme, non heme iron, and contaminating heavy metals in solution (Trager, 1982) appear not to be responsible for the NADPH-dependent HCB disappearance. However, other activated oxygen species in solution and enzyme bound and enzyme-bound hydroxyl radicals cannot be ruled out at this time. Under the assay conditions employed, radioactivity corresponding to potential metabolites was not detected.

In vivo and in vitro metabolism studies on HCB, PCB, and PCP reveal large differences in their rates of clearance and dechlorination and follow the order HCB $\ll$ PCB $<$ PCP (Koss & Koransky, 1977; Ahlborg, 1977; Engst et al., 1976). If consecutive metabolism of HCB forms PCP through PCB, then the steady-state and, hence, isolatable levels of PCB (and PCP) might depend on the conditions of the reaction (e.g., concentration of enzyme, time of incubation, etc.). PCP is reported to be dechlorinated faster by BNF- than PB-induced rat liver microsomes fortified with NADPH (Ahlborg, 1977). Therefore, the isolatable amount of PCP produced from dechlorination of HCB is expected to vary depending on the

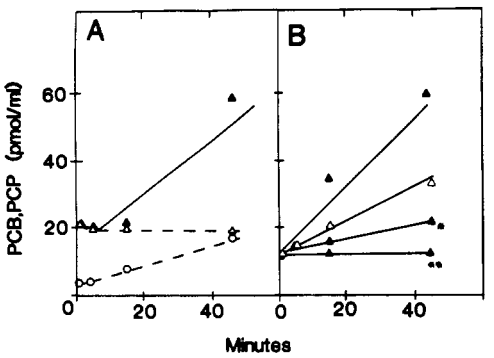

FIGURE 2: PCB and PCP production from [^{14}C]HCB incubated with PB-induced (A) and BNF-induced (B) rat liver microsomes. Reactions consisted of 2 μM microsomal CYT P-450 and 52.8 μM HCB (141 mM Me_2SO) fortified with 2.5 mM NADPH in 5.0 mL under aerobic (open symbols) and anaerobic (shaded symbols) conditions for 1 h at 37 °C: (triangles) PCB; (circles) PCP; (*) intact microsomes minus NADPH; (**) boiled microsomes plus NADPH.

microsome source. A comparison of activities between isozyme pools (BNF- and PB-inducible families) of CYT P-450 with HCB and PCB as substrates has not been reported.

NADPH-dependent PCB production occurs with microsomes under anaerobic and aerobic conditions, see Figure 2. PB-induced microsomes produce PCB above controls only at early time points (controls depicted in Figure 2B) under aerobic conditions. PCB production is enhanced with anaerobic reactions, see Figure 2A. PCP formation occurs under aerobic reaction conditions at the expense of PCB production compared to anaerobic reactions, Figure 2A. BNF-induced microsomes produce PCB under both anaerobic and aerobic conditions, Figure 2B. Aerobic conditions decrease the rate of PCB production by approximately 50% compared to anaerobic conditions, with no observable PCP formation. Since PCB formation occurs under both anaerobic and aerobic conditions, it appears to be the initial metabolite, and PCP is produced by secondary metabolism of PCB when oxygen is available. Observable PCP formation is dependent on the type of microsomes used. BNF-induced microsomes fortified with NADPH under anaerobic conditions produce PCB at the same rate as PB-induced microsomes under anaerobic conditions. The comparison is made at equimolar CYT P-450 and supports the argument that PCB production is CYT P-450 mediated. Alternatively, if non CYT P-450 systems are involved, then PCB production is subject to a common rate-limiting step in the different microsomal preparations and is enhanced by NADPH.

As shown in Figure 2B, a basal nonenzymatic (NADPH-independent) rate of PCB production occurs, which requires native/intact microsomes. We are not able to explain fully the NADPH-independent PCB production, but it may be a function of HCB's susceptibility to nucleophilic carbon–chlorine bond cleavage (Renner et al., 1978; Betts et al., 1955) coupled with redox active thiols, phenols, heme, and/or flavins present in native microsomal proteins.

Perturbation of the rat liver microsomal CYT P-450 system affects the formation of PCB, the initial reductive dechlorination product from HCB, as shown in Table II and in Figure 3B–D. The similar behavior observed between PCB production and product formation from substrates known to undergo reductive activation (Nastainczyk et al., 1982; Uehleke et al., 1973) indicates that HCB, a perhalogenated aromatic compound, also undergoes CYT P-450 reductive activation.

O_2 and CO bind to the reduced (Fe^{2+}) CYT P-450 hemoprotein at a common binding site (Estabrook et al., 1970). Activation of O_2 can occur at this time or after another

Table II: Effects of Different Perturbations of CYT P-450 System on PCB Production

conditions[a]	PCB (pmol min^{-1} mg^{-1})[b]	% activity[c]
N_2 + NADPH	1.57 ± 0.19	100
air + NADPH	0.63 ± 0.02	15.3
CO + NADPH	0.69 ± 0.02	20.7
N_2 + NADHP + metyrapone[d]	0.79 ± 0.15	29.7
N_2 + NAD(P)H[e]	1.10 ± 0.30	57.6
N_2 (−NADPH)	0.46 ± 0.03	0

[a] Five milliliter reaction volume containing 4 μM PB-induced microsomal CYT P-450, 52.8 μM HCB, 141 mM Me_2SO, and 2.5 mM NAD(P)H final concentrations incubated for an hour at 37 °C. [b] Mean ± standard deviation (n = 3). [c] Percent activity is calculated by using values corrected for nonenzymatic PCB production, N_2 (−NADPH). For example, percent activity for air + NADPH = [(0.63 − 0.46)/(1.57 − 0.46)] ×]00. [d] A 1 mM metyrapone final concentration. [e] NADH was added at a 1:9 (NADH:NADPH) molar ratio while keeping the final total reducing equivalence concentration at 2.5 mM.

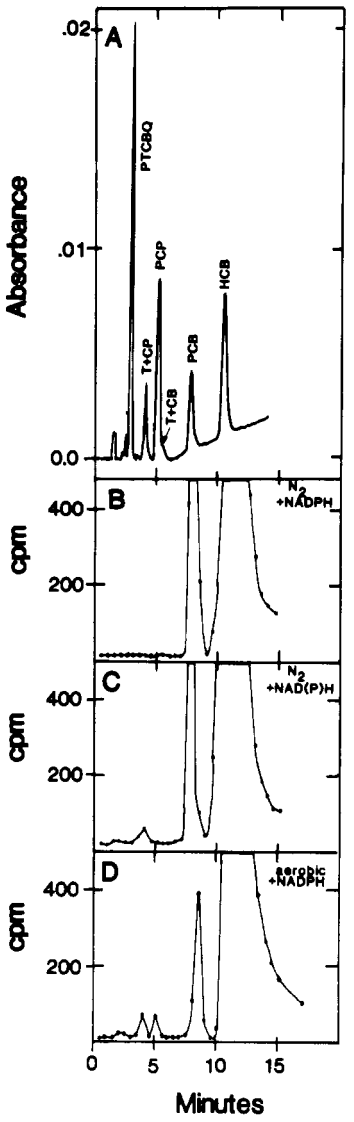

FIGURE 3: Resolution of potential metabolites of HCB by C_{18} reverse-phase HPLC (A). HCB; hexachlorobenzene, PCB; pentachlorobenzene, PCP; pentachlorophenol, TtCB; tetrachlorobenzene, TtCP; tetrachlorophenol; p-TCBQ; p-tetrachlorobenzoquinone. Standards of 0.5 μg were injected in 20 μL of 50:50 (v/v) CH_2Cl_2–CH_3OH; 254-nm UV absorbance; 0.02 absorbance scale. Initial conditions: 100:300:0.08 (v/v/v) H_2O–CH_3OH–acetic acid; 20-min linear gradient up to 100% CH_3OH, 2 mL/min. Combination HPLC/LSS analysis of [^{14}C]HCB metabolites isolated from PB microsomal reactions (B–D). Reaction conditions correspond to those given in Table II.

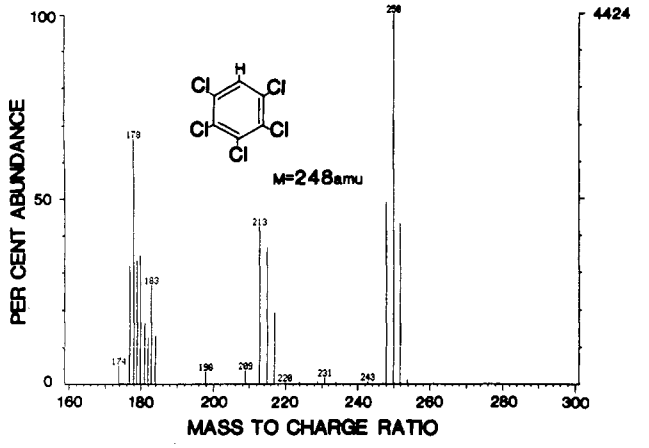

FIGURE 4: GC/MS structure verification of PCB isolated from PB microsomal reactions after HPLC resolution. Mass spectra of PCB metabolite.

electron is pushed through the heme iron center (Guengerich & MacDonald, 1984). CO binds to reduced CYT P-450 and forms a dead-end complex but is O_2-reversible and 450 nm light reversible. As shown in Table II, both air (O_2) and CO inhibited PCB recovery to the highest extent (84 and 80%), when compared to anaerobic conditions, suggesting that these molecules compete with HCB at the heme-iron center. We also recognize the role of secondary metabolism (described earlier) and its effects on PCB levels, especially under aerobic conditions. The apparent air-sensitive PCB recovery may be due to secondary metabolism of PCB to form PCP and another unidentified product (Figure 3D). However, when reaction mixtures are saturated with CO, a condition that is also anaerobic, PCB recovery is inhibited to the same extent as observed under aerobic conditions. We hypothesize that CO and O_2 bind to reduced (Fe^{2+}) CYT P-450, therefore decreasing the amount of reduced CYT P-450 that can reductively activate HCB. However, we also acknowledge that different mechanisms of dechlorination by the CYT P-450 system may be operating in the presence or absence of O_2.

Metyrapone is a nonmetabolized substrate and can be considered as a functional CO analogue from its effect on the reduced (Fe^{2+}) CYT P-450 γ-Soret band (Estabrook et al., 1970). Metyrapone is thought to bind to the same site as O_2 and CO but either enhances or inhibits the metabolism of substrates in microsomal systems. Metyrapone inhibits PCB production by 70% in PB-induced microsomes, as shown in Table II. Metyrapone at 1 mM generates a strong type II difference spectrum with 1 μM PB CYT P-450 suspensions. Dithionite reduction and exposure to CO, of the sample used to generate the type II spectrum, produce a peak at 454 nm (data not shown). Thus, metyrapone definitely affects the PB-induced microsomal CYT P-450 monooxygenase system and inhibits the reductive dehalogenation of HCB.

Inhibition of PCB formation from HCB by metyrapone under anaerobic conditions provides convincing evidence that cytochrome P-450 mediates the reductive dechlorination of HCB. This observation and the demonstration of inhibition of PCB production by incubation under a CO atmosphere, taken together, strongly support our hypothesis.

Flavin- (FAD-) mediated dechlorination of HCB and the effects of CYT b_5 on the NADPH-dependent CYT P-450 mixed-function oxidase system can be tested by including NADH in microsomal reactions. FAD is a prosthetic group found in both NADPH-CYT P-450 reductase and NADH-CYT b_5 reductase and is essential for electron transfer. Addition of NADH and NADPH to microsomes increases electron flux through both reductases' flavin centers. Increased reductive dechlorination of HCB would be observed if flavin-mediated events occur. The effect of CYT b_5 on the CYT P-450 system and HCB dechlorination is unpredictable. However, addition of both NADPH and NADH to microsomal reactions with HCB provides further characterization of the systems involved in HCB biotransformation and simulates in vivo conditions. NADH, in a 1:9 molar (NADH:NADPH) ratio, under anaerobic conditions inhibits PCB production by 40% as shown in Table II. This apparent inhibition may be due to secondary metabolism of PCB, since a more polar metabolite was observed, see Figure 3C. The inhibition may also be due to competition between NADH and NADPH at the NADPH-CYT P-450 reductase. FAD-mediated dechlorination of HCB is probably not occurring, since increased PCB production was not observed.

GC/MS was used to verify the production of PCB in large-scale microsomal reactions with unlabeled HCB. The PCB fraction isolated after HPLC resolution gave a similar GC retention time, parent and base mass ion, and five chlorine ion cluster compared to those of authentic PCB (Figure 4).

The number of classes of chemicals that undergo reductive activation by the CYT P-450 "monooxygenase" system is expanding. The role of the CYT P-450 system as an elimination mechanism for this group of chemicals is maintained, since reductive activation produces products that are themselves, or eventually become, subject to hydroxylation. Monooxygenase activity has been the most obvious function of the CYT P-450 system, but these substrates reveal CYT P-450 hemoproteins' basic function as an electron transferase or terminal reductase.

We feel that the experiments presented indicate that HCB, a unique member of the polyhalogenated aromatic hydrocarbons, is reductively dehalogenated by the CYT P-450 mixed-function oxidase system to produce PCB, as an initial metabolite. Multiple CYT P-450 isozymes present in microsomal fractions hinder any mechanistic interpretations. A purified, reconstituted CYT P-450 system should increase the specific activity of PCB production and facilitate evaluation of the stoichiometry between NAD(P)H oxidation, HCB disappearance, and product formation. Mechanism-based and catalytic site accessible trapping reagents coupled with the use of more versatile and informative analytical techniques, such as GC/MS and/or, possibly, FAB/MS, may be used to elucidate the activated HCB intermediate, precursor to PCB.

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Registry No. HCB, 118-74-1; CYT P-450, 9035-51-2; PCB, 608-93-5; PCP, 87-86-5; TtCB, 12408-10-5; TtCP, 25167-83-3; *p*-TCBQ, 118-75-2.

REFERENCES

Ahlborg, U. G. (1977) in *Pentachlorophenol Chemistry, Pharmacology, and Environmental Toxicology* (Rao, K. R., Ed.) pp 131–137, Plenum, New York.

Betts, J. J., James, S. P., & Thorpe, W. V. (1955) *Biochem. J. 61*, 611.

Cabral, J. R. P., Shubik, P., Mollner, T., & Raitano, F. (1977) *Nature (London) 269*, 510–511.

Castro, C. E., Wade, R. S., & Belser, N. O. (1985) *Biochemistry 24*, 204–210.

Courtney, D. K. (1979) *Environ. Res. 20*, 225–266.
Debets, F. M. H., & Strik, J. J. T. W. A. (1979) in *Chemical Porphyria in Man* (Strik, J. J. T. W. A., & Koeman, J. H., Eds.) pp 181–208, Elsevier/North-Holland Biomedical Press, New York.
Dignam, J. D., & Strobel, H. W. (1977) *Biochemistry 16*, 1116–1123.
Engst, R., Macholz, R. M., Kujawa, M., Lewerenz, H. J., & Plass, R. (1976) *J. Environ. Sci. Health, Part B B11*, 95–117.
Estabrook, R. W., Franklin, M. R., & Hildebrandt, A. G. (1970) *Ann. N.Y. Acad. Sci. 174*, 218–232.
Gillette, J. R., Kamm, J. J., & Sasame, H. A. (1968) *Mol. Pharmacol. 4*, 541–548.
Guengerich, F. P., & MacDonald, T. L. (1984) *Acc. Chem. Res. 17*, 9–16.
Hildebrandt, A., Remmer, H., & Estabrook, R. W. (1968) *Biochem. Biophys. Res. Commun. 30*, 607–612.
Ichikawa, Y., Yamano, T., & Fujisawa, H. (1969) *Biochim Biophys. Acta 171*, 32–46.
Knight, R. H., & Young, L. (1958) *Biochem. J. 70*, 111–119.
Koss, G., & Koransky, W. (1977) in *Pentachlorophenol Chemistry, Pharmacology, and Environmental Toxicology* (Rao, K. R., Ed.) pp 131–137, Plenum, New York.
Kutz, F. W., Strassman, S. C., & Sperling, J. F. (1979) *Ann. N.Y. Acad. Sci. 320*, 60.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mansuy, D., Nastainczyk, W., & Ullrich, V. (1974) *Naunyn-Schmiedeberg's Arch. Pharmacol. 285*, 315–324.
Meek, D. W., Straub, D. K., & Drago, R. S. (1960) *J. Am. Chem. Soc. 82*, 6013–6016.
Mehendale, H. M., Fields, M., & Matthews, H. B. (1975) *J. Agric. Food Chem. 23*, 261–265.
Nastainczyk, W., Ahr, H. J., & Ullrich, V. (1982) *Biochem. Pharmacol. 31*, 391–396.
Nebert, D. W., & Gelboin, H. V. (1968) *J. Biol. Chem. 243*, 6242–6249.
Netter, K., Kahl, G., & Magnussen, M. (1969) *Naunyn-Schmiedebergs Arch. Exp. Path. Pharmakol. 265*, 205–215.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370.
Remmer, H., Schenkman, J. B., Estabrook, R. W., Sasame, H., Gillette, J., Cooper, D. Y., Narasimhulu, S., & Rosenthal, O. (1966) *Mol. Pharmacol. 2*, 187–190.
Renner, G., Richter, E., & Schuster, K. P. (1978) *Chemosphere 8*, 663.
Ruf, H. H., Ahr, H. J., Nastainczyk, W., Ullrich, V., Mansuy, D., Battioni, J. P., Montiel-Montoya, R., & Trautwein, A. (1984) *Biochemistry 23*, 5300–5306.
Sato, T., Fukuyama, T., Suzuki, T., & Yoshikawa, Y. (1963) *J. Biochem. (Tokyo) 53*, 23–27.
Schenkman, J. B., Remmer, H., & Estabrook, R. W. (1967) *Mol. Pharmacol. 3*, 113–123.
Sladek, N. E., & Mannering, G. J. (1969a) *Mol. Pharmacol. 5*, 174–185.
Sladek, N. E., & Mannering, G. J. (1969b) *Mol. Pharmacol. 5*, 186–199.
Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1981) *J. Biol. Chem. 256*, 1044.
Trager, W. F. (1982) *Drug Metab. Rev. 13*, 51–69.
Uehleke, H., Hellmer, K. H., & Tabarelli, S. (1973) *Xenobiotica 3*, 1–11.
Waxman, D. J., & Walsh, C. (1983) *Biochem. J. 22*, 4846–4855.
West, S. B., & Lu, A. Y. H. (1977) *Arch. Biochem. Biophys. 182*, 369–378.
West, S. B., Levin, W., Ryan, D., Vore, M., & Lu, A. Y. H. (1974) *Biochem. Biophys. Res. Commun. 58*, 516–522.
Winston, G. W., & Cederbaum, A. I. (1983) *J. Biol. Chem. 258*, 1508–1513.
Wolf, C. R., Mansuy, D., Nastainczyk, W., Deutschmann, G., & Ullrich, V. (1977) *Mol. Pharmacol. 13*, 698–705.