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METABOLISM-RELATED SPECTRAL CHARACTERIZATION AND SUBCELLULAR DISTRIBUTION OF POLYCHLORINATED BIPHENYL CONGENERS IN ISOLATED RAT HEPATOCYTES*†

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Abstract—The disposition and biotransformation of 4,4′-dichlorobiphenyl (4-DCB), 2,2′,3,3′,6,6′-hexachlorobiphenyl (236-HCB), and 2,2′,4,4′,5,5′-hexachlorobiphenyl (245-HCB) were studied in isolated rat hepatocyte suspensions. The polychlorinated biphenyls (PCBs) were taken up rapidly by the cells but incompletely metabolized. Metabolism followed first-order Michaelis–Menten kinetics for 20 min and plateaued by 60 min, at which point only 32% of 4-DCB (0.005 to 100 μM) and 60% of 236-HCB (0.001 to 100 μM) were metabolized, while metabolism of 245-HCB was not detected (0.1 to 200 μM). Kinetic studies revealed that both 4-DCB and 236-HCB were metabolized by two Michaelis–Menten processes, displaying high- and low-affinity binding. Readdition of congener once metabolism plateaued resulted in a reinitiation of metabolism with the same proportion of metabolites produced. The termination of metabolism was not due to destruction of the mixed-function oxidases or to depletion of cofactors. The metabolism of PCB congeners is influenced by the affinity of the congener for cytochrome P-450 and partitioning of the congener within the hepatocyte. Analysis of absorbance differences (Δ absorbance 390–240 nm) of equimolar concentrations of congener (100 μM) revealed that 236-HCB displayed the greatest affinity of binding to cytochrome P-450 followed by 4-DCB, while 245-HCB showed virtually no binding. Microsomal preparations demonstrated equivalent but greater absorbance values. Subcellular distribution of ^{14}C-labeled congener and its metabolites showed that the majority of radioactivity appeared in the cytosolic fraction, representing 70% of the dose added for each congener. Cytosolic binding of congener and metabolites may influence both the availability of congener to cytochrome P-450 and the excretion rate of metabolites from the cell.

Polychlorinated biphenyls (PCBs) comprise a large class of persistent environmental contaminants which are readily absorbed into biological systems. Their propensity for bioaccumulation and long biological half-life have been reported for *in vivo* [1–8] and *in vitro* [9–12] systems. The consequences of the long-term accumulation of PCBs in human and animal tissues are unknown.

Metabolism, an important factor in the distribution and excretion of PCBs, is greatly affected by congener structure since less than 10% of a PCB dose administered to animals is excreted unmetabolized [1]. Both the position and degree of chlorine substituents on the biphenyl nucleus may affect the extent and rate of metabolism of a particular PCB congener [13, 14]. The ability of the liver enzymes to biotransform the various PCBs to excretable products becomes rate limiting, decreasing toward very low levels for the highly chlorinated compounds [15]. Differences in rates of metabolism may explain the accumulation of some isomers and contribute to the potential toxicities of others.

Several attempts have been made to find common properties among heterogeneous substrates, including the PCBs, which bind to the cytochrome P-450 system. PCB congeners are of relatively similar lipophilicity, favoring their dissolution in membranes and interaction with cytochrome P-450 [16–21]. Congener structure, however, does influence which form of cytochrome P-450 catalyzes the metabolism of a particular PCB [22–24] as well as which cytochromes are induced by PCBs [25, 26].

In the present investigation, PCB congener structure was evaluated in terms of congener binding to cytochrome P-450, congener biotransformation and the subcellular distribution and partitioning of both congener and metabolites within the hepatocyte. The PCB congeners selected, 4,4′-dichlorobiphenyl (4-DCB), 2,2′,3,3′,6,6′-hexachlorobiphenyl (236-HCB) and 2,2′,4,4′,5,5′-hexachlorobiphenyl (245-HCB) vary dramatically in rates of elimination from the body as demonstrated by *in vivo* studies [4, 6]. Evidence presented in this paper suggests that congener structure affects the affinity of the compound

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for cytochrome P-450 and its subcellular distribution. Partitioning of congener within the hepatocyte could influence the amount of congener accessible to the cytochrome P-450 enzyme system for metabolism. Furthermore, our results demonstrate that isolated hepatocyte suspensions are a useful model for assessing the metabolism and disposition of PCB congeners.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats, 200–250 g, were bred and raised in the University of Arizona College of Medicine Division of Animal Resources. All animals were housed in temperature-controlled rooms on a cycle of 12 hr of light and 12 hr of darkness and maintained on a standard Wayne Lab Blox diet.

The 4-DCB[U-^{14}C] (58 mCi/mmole), 236-HCB[U-^{14}C] (24 mCi/mmole), and 245-HCB[U-^{14}C] (23 mCi/mmole) were purchased from California Bionuclear (Sun Valley, CA). Unlabeled congeners were obtained from Analabs, Inc. (North Haven, CT). Congeners were shown to be greater than 98% pure by gas chromatography–mass spectroscopy. Collagenase, type II, was purchased from Worthington (Freehold, NJ) and albumin fraction V from Sigma (St. Louis, MO). Medium 199 and buffer salts were obtained from Gibco (Grand Island, NY). Dimethyl sulfoxide (DMSO) was purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ), and dichloromethane from the Eastman Kodak Co. (Rochester, NY). All other chemicals and solvents were of the highest grades commercially available.

Isolation and incubation of hepatocytes. The liver perfusion and hepatocyte isolation followed the methods of Berry and Friend [27], Seglen [28], Hayes and Brendel [29] and Moldeus *et al.* [30]. In brief, the rats were anesthetized with ether and the livers were perfused *in situ* through the portal vein with Ca^{2+}-free Hanks' buffer containing 0.5 mM ethyleneglycolbis (amino - ethylether) tetra - acetate (EGTA). Following excision of the liver from the abdominal cavity, the liver was placed in a thermostated perfusion apparatus and perfused with Krebs–Henseleit buffer (KH) containing collagenase (0.05%, w/v) and calcium (4 mM). The perfusate was recirculated at a flow rate of 10 ml/g/min. Following gentle disruption, the hepatocytes were pelleted by centrifugation at 40 *g* for 2 min. The supernatant fraction containing the nonparenchymal cells was discarded, and the washing procedure was repeated twice. The final hepatocyte pellet was resuspended in Medium 199 and filtered through a 64 μm sieve (Nitex; Tobler, Ernst & Traber, Inc., Elmsford, NY) to disperse the cells and remove any remaining undissociated cell clumps. The final suspension was diluted to a cell density of 4×10^6 cells/ml wet weight. The hepatocyte yield was about 60% of the initial liver weight. Cell viability was initially 90–95% and decreased at a rate of 2%/hr during the incubation period (0–4 hr) as assessed by the trypan blue exclusion method.

Hepatocyte suspensions were incubated in 50-ml Erlenmeyer flasks with conically-raised centers. The reaction flasks were maintained under carbogen atmosphere at 37° and swirled at 120 rpm in a New Brunswick gyratory shaker. Each ^{14}C-radiolabeled and/or unlabeled PCB congener was dissolved in DMSO and resuspended in 10% albumin for addition to the hepatocyte suspensions. The final amount of DMSO did not exceed 10 μl/ml of the incubation volume.

Uptake and metabolism. Hepatocyte suspensions were incubated with various concentrations of ^{14}C-labeled 4-DCB, 236-HCB, and 245-HCB (0.01 to 200 μM). At various time intervals (15 sec to 2 hr) 1-ml aliquots were separated into medium and cellular fractions by centrifugation using a Beckman/Spinco model 152 microfuge. Each fraction was treated with 1 N KOH, sonicated, and extracted with dichloromethane–ethanol (4:1) to separate parent PCB from the metabolite. A portion of the aqueous phase (0.5 ml), containing the metabolites, was counted in an Omnifluor-Triton X-100 scintillation fluid using a Searle Analytic model 81 liquid scintillation counter. Cellulose powder was added to the remainder of the sample to absorb any portion of the remaining aqueous layer. The mixture was then filtered by gravity through a Whatman type I filter. The organic phase containing parent PCB was measured and evaporated to dryness in a counting vial. Scintillation fluid was added directly to the vial and the sample counted.

The cytochrome P-450 content of each suspension was calculated from the CO-difference spectra of reduced hepatocytes according to Omura and Sato [31], using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. Difference spectra in whole cells were recorded using a Beckman Acta V spectrophotometer according to the method of Schenkman [32]. Warm gelatin, 200 μl of a 10% (w/v) 37° solution, was added to each 1 ml hepatocyte suspension, then cooled, to avoid settling of the cells.

Kinetics. Rates of metabolite production were determined from hepatocyte suspensions of 4-DCB and 236-HCB at final concentrations of 0.05, 0.1, 1.0, 3, 5, 10, 30, 50, 100 and 300 μM. After 20 min, 200-μl aliquots of hepatocyte suspension (4×10^6 cells/ml) were sonicated and extracted as described previously to quantify total metabolite production. The apparent K_m and V_{max} values were determined from a nonlinear least squares computerized curve fitting program modified for an Apple II computer.

Binding spectra. Affinities for the hepatic cytochrome P-450 of 4-DCB, 236-HCB, and 245-HCB were determined with hepatocytes, microsomes, and microsomes in the presence of 150 μM albumin by the difference spectral method of Schenkman [32]. To facilitate determination of cytochrome P-450 spectral binding in intact hepatocytes, warm gelatin, 200 μl of a 10% (w/v) 37° solution, was added to each 1-ml hepatocyte suspension. The cells were briefly cooled and then rewarmed to room temperature so that the cells would remain suspended. The cytochrome P-450 content (0.26 nmole/ml) was made equivalent for each suspension: hepatocytes (1×10^6 cells/ml) or microsomes (2 mg protein/ml), as calculated from the carbon monoxide difference spectrum described by Omura and Sato [31].

After a baseline had been recorded, a PCB–

DMSO–albumin solution (10–200 μM final concentrations) was added to the sample cell. Corresponding volumes of the DMSO–albumin mixture were added to the reference cell. The contents of the cuvettes were mixed well, and the difference spectra were recorded between 500 and 370 nm. No noticeable sedimentation of cells occurred during the scan, and both DMSO and albumin in the concentrations used were shown to be without influence on the spectral data. For all suspensions the extent of spectral change was measured by the difference between the wavelengths of minimum (420 nm) and maximum (390 nm) absorption. Spectral dissociation constants (K_s) were obtained from double-reciprocal plots of PCB congener concentration and spectral change.

Subcellular fractionation. Subcellular fractions were prepared at 4° from isolated hepatocytes essentially by the methods of Berman *et al.* [33], Yamamoto *et al.* [34], and Procaccini and Klunk [35]. In brief, hepatocyte incubations (10 ml) were disrupted with minimum homogenization (20 strokes) in a Potter–Elvehjem homogenizer (0.15 mm clearance) to break about 98% of the cells, as systematically monitored in each experiment by light microscopy. The homogenate was filtered through a 15 μm sieve to remove unbroken cells and then centrifuged at 1600 *g* for 20 min. The pellet (crude nuclei) was washed with Hanks' buffer and resuspended in 0.25 M sucrose–0.05 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer, pH 7.5, and stored at −20°.

The resultant supernatant fractions were combined and centrifuged at 4,500 *g* for 10 min to pellet the mitochondria; at 17,300 *g* for 10 min to obtain the crude lysosomal pellet; and at 105,000 *g* for 1 hr to obtain the microsomal and cytosolic fractions. The cytosol was decanted and frozen. The pelleted subcellular fractions were resuspended in 1 ml of Krebs–Henseleit buffer and frozen at −20° for future analysis. The nuclei suspension was added to a discontinuous sucrose gradient composed of, from bottom to top, 10 ml 45%, 10 ml 41%, 8 ml 37%, and 5 ml 31% sucrose. It was centrifuged at 67,000 *g* for 2 hr at 4° to yield a partially purified plasma membrane fraction at 31–37% sucrose and a nuclear pellet. Both the total radioactivity as well as the proportion of congener to metabolites were determined by extraction with dichloromethane–ethanol (4:1) for all subcellular fractions. Protein was determined by the method of Bradford [36]. The total radioactivity and protein were expressed as a proportion and defined as the relative specific activity (RSA) for each subcellular fraction [35].

RESULTS

Uptake and release of PCB congeners by cells. The congeners were rapidly absorbed by the cells such that 85% of the congeners were taken up within 30 sec. Less than 10% of the congener remained in the medium by 5 min. This is illustrated in Fig. 1 for each PCB congener. The amount of radioactivity within the medium reflected the changes observed in the cells: an initial rapid fall as a result of the uptake of the PCB congener into the cells followed by an increase due to the release of metabolites from the cells. Complexing the PCB congeners with albumin aided in the uptake of congener by the cells. Addition of the congeners to the incubation medium as a DMSO solution resulted in the formation of micelles in the medium and poor uptake of congener by the cells.

Metabolites were slowly eliminated by the cells with only 25–30% of the total metabolites produced found in the medium by 2 hr. Maximal levels of the water-soluble metabolites within the extracellular medium were 300 pmoles/10^6 cells and 260 pmoles/10^6 cells for 4-DCB and 236-HCB (10 μM) respectively (Table 1). Intracellular levels reached

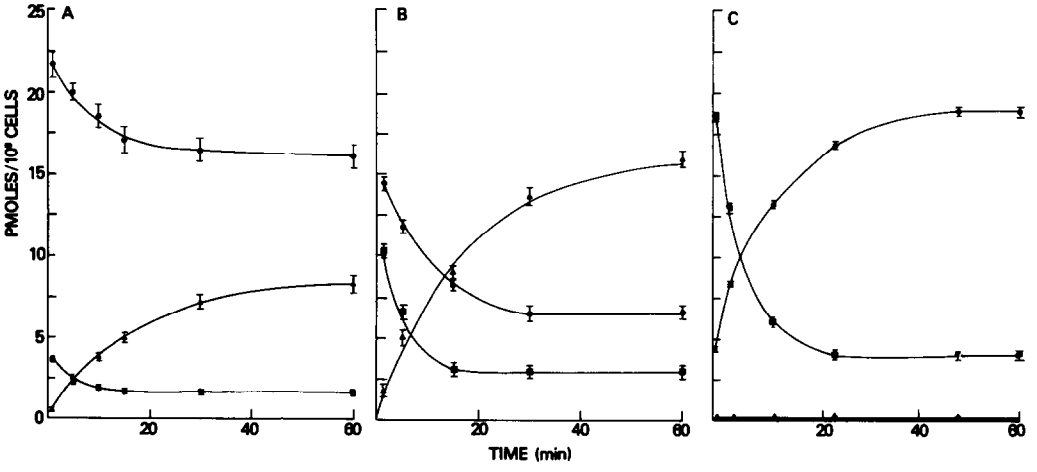

Fig. 1. Metabolism of 4-DCB, 236-HCB and 245-HCB in isolated hepatocyte suspensions. Hepatocytes (4×10^6 cells/ml) were incubated in Medium 199 with albumin bound congeners, 0.1 μM, for 60 min at 37°. Aliquots were removed, and the congener and metabolites were determined by extraction as described in Materials and Methods. Values are means ± S.E. of four determinations. (A) Hepatocyte suspensions incubated with 4-DCB; (B) hepatocyte suspensions incubated with 236-HCB; and (C) hepatocyte suspensions incubated with 245-HCB. Key: (●---●) intracellular congener; (■---■) extracellular congener; and (▲—▲) metabolites.

Table 1. Distribution of PCB congeners and metabolites between hepatocytes and medium

	Medium		Hepatocytes	
Addition	Congener	Metabolites	Congener	Metabolites
4-DCB*	212†	300	1380	575
236-HCB	250	260	1240	750
245-HCB	250	ND‡	2250	ND

Values are means of triplicate determinations differing by less than 10% from each other.

* PCB congeners were added to hepatocyte suspensions (4×10^6 cells/ml) with incubation concentrations of 10 μM.

† Amounts are expressed as pmoles produced/10^6 cells in 3 hr.

‡ Metabolite products were not detected.

575 pmoles/10^6 cells for 4-DCB and 750 pmoles/10^6 cells for 236-HCB. No aqueous extractable metabolites were observed in the medium or cells derived from 245-HCB suspensions, although uptake of this congener by the cells was identical to that of 4-DCB and 236-HCB.

Extent of metabolism. PCB congeners were not completely metabolized by the isolated hepatocytes. Figure 1 depicts the metabolic profiles of 0.1 μM 4-DCB, 236-HCB, and 245-HCB. Following uptake, metabolite production reached a plateau by 60 min. Little, if any, metabolism occurred between 60 and 240 min (data not shown). The amount of unmetabolized congener remaining within the hepatocytes represented 60% of 4-DCB added, 35% of 236-HCB, and 90% of 245-HCB. The decline in congener concentration within the hepatocytes following uptake corresponded to metabolite production.

Metabolites, as aqueous soluble radioactivity, were extracted primarily from the cellular fraction. Less than 10% of the aqueous extractable counts were found in the medium within 1 hr. Total metabolite production accounted for 32% of the 4-DCB added and 60% of the 236-HCB added. No aqueous extractable counts were observed in extractions of boiled hepatocytes. Similar percentages of the congeners were metabolized for both 4-DCB and 236-HCB with congener concentrations ranging from 0.1 to 100 μM (data not shown). No metabolism, however, was detected for 245-HCB (1–200 μM) in this preparation.

The extent (%) of metabolism for 4-DCB and 236-HCB could not be altered by: adjusting the manner in which the congener was administered to the hepatocyte suspensions; varying the cell concentration; or by replacing the medium with fresh medium (after 1 hr) to stimulate mobilization of unmetabolized congener and further metabolism within the hepatocytes. Addition of 4-DCB (0.1 μM) as cumulative aliquots dispensed over 45 min yielded the same proportion of metabolites as that observed upon administration of the congener as a bolus (data not shown).

The plateau in the rate of metabolite production for both 4-DCB and 236-HCB cannot be explained easily. Determination of the cytochrome P-450 content at the plateau (1 hr) by the carbon monoxide difference spectra revealed no differences between PCB-exposed hepatocyte suspensions and unexposed suspensions. Addition of fresh congener to a pre-existing PCB incubation during this decreased metabolic phase yielded a similar type I binding spectra as observed with freshly isolated cells and resulted in a reinitiation of metabolite production with the same proportion of metabolites produced (Fig. 2). This phenomenon was also observed in hepatocyte suspensions which had been aged for 1 or 2 hr prior to PCB exposure (Fig. 2). Metabolism was reinitiated in each suspension. A decreased rate and extent of metabolite production were observed in the suspensions aged 2 hr prior to congener addition; however, the effects of cofactor and cytochrome P-450 depletion within the hepatocytes apparent at 2 hr did not account for the limited metabolism observed at 60 min. Moreover, the plateau in metabolite production was not the result of product inhibition. A soret peak indicative of the formation of a metabolite stable complex was not observed in the PCB suspensions. Metabolites extracted from PCB-hepatocyte incubations (10 μM) and added to unexposed hepatocytes were capable of binding to the cytochrome P-450; however, such binding spectra were not observed in hepatocyte preparations at the time metabolism plateaued.

Rate of metabolite production. The kinetics of 4-DCB and 236-HCB biotransformation were investigated over a range of congener concentrations from 0.05 to 300 μM in hepatocyte suspensions maintained at 37°. Metabolite production was linear for 20 min following first-order Michaelis–Mentan kinetics. The data are shown as Eadie–Hofstee kinetic plots (Fig. 3). The kinetic plots are nonlinear, indicating the presence of at least two separate reactions. Based on the assumption that each curve is the result of two reactions, the data were analyzed by a nonlinear least squares computerized curve fitting program. The results were compatible with the existence of a saturable, relatively high-affinity binding site plus a low-affinity, high-capacity binding site that was not clearly saturable. The kinetic constants were derived from the computer analysis of the data. The high-affinity reaction for 4-DCB exhibited an apparent K_m of 0.1 μM and a V_{max} of 1.3 pmoles/min/mg hepatocytes and for 236-HCB an apparent K_m of

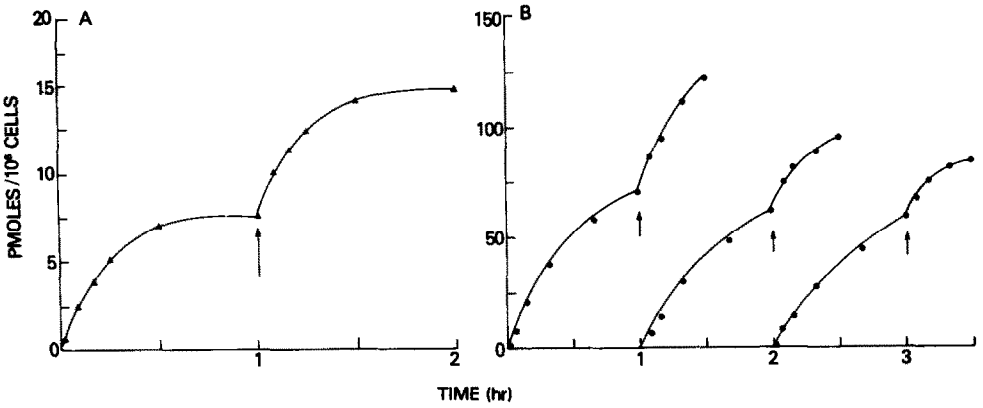

Fig. 2. Effect of 4-DCB metabolite production by the readdition of congener to hepatocyte suspensions. Cells (4×10^6 cells/ml) were incubated with 4-DCB for 1 hr at 37°. After an hour, fresh congener was added to pre-existing incubations. Metabolite production was determined by the extraction of aliquots as described in Materials and Methods. Values are means of three determinations differing by less than 10% from each other. Key: (A) hepatocytes incubated with 0.1 μM 4-DCB (▲—▲, 4-DCB metabolites); (B) hepatocytes aged 0, 1 and 2 hr prior to the addition of 1 μM 4-DCB (●---●, 4-DCB metabolites); and readdition of congener (↑) resulting in final incubation concentrations of 0.1 μM (A) and 1 μM (B).

0.48 μM and a V_{max} of 3.7 pmoles/min/mg hepatocytes. The low-affinity reaction for 4-DCB exhibited an apparent K_m of 340 μM and a V_{max} of 56.8 pmoles/min/mg hepatocytes and for 236-HCB an apparent K_m of 645 μM and a V_{max} of 367 pmoles/min/mg hepatocytes.

Interaction of PCBs with cytochrome P-450. Addition of 4-DCB, 236-HCB, and 245-HCB to hepatocyte or microsomal suspensions yielded characteristic type I difference spectra with maxima at approximately 390 nm and minima at approximately 420 nm. The extent of absorbance difference

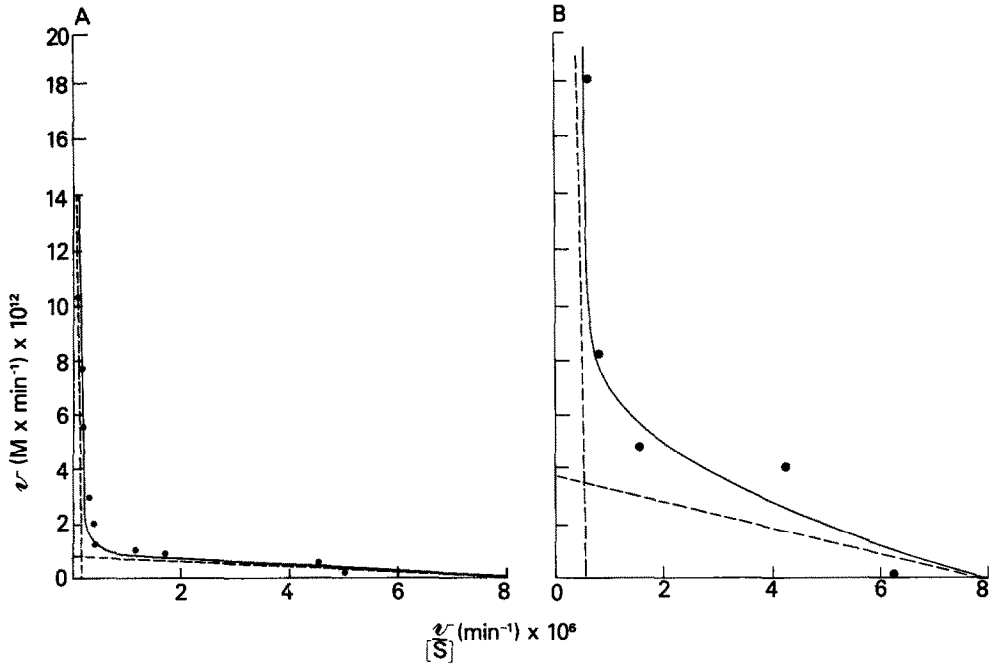

Fig. 3. Eadie–Hofstee plots of 4-DCB and 236-HCB. Rates of metabolite production were determined in hepatocyte suspensions (4×10^6 cells/ml) over 20 min for 4-DCB and 236-HCB at 0.05, 0.1, 1.0, 3, 5, 10, 30, 50, 100, and 300 μM. The mean rates of metabolite production were determined from three determinations as described in Materials and Methods. The values fit the theoretical curve drawn as predicted by computer analysis for a two-site model displaying a high-affinity and a low-affinity site. (A) Hepatocytes incubated with various concentrations of 4-DCB; and (B) hepatocytes incubated with various concentrations of 236-HCB. Key: (●---●) mean rates of metabolite production for each congener.

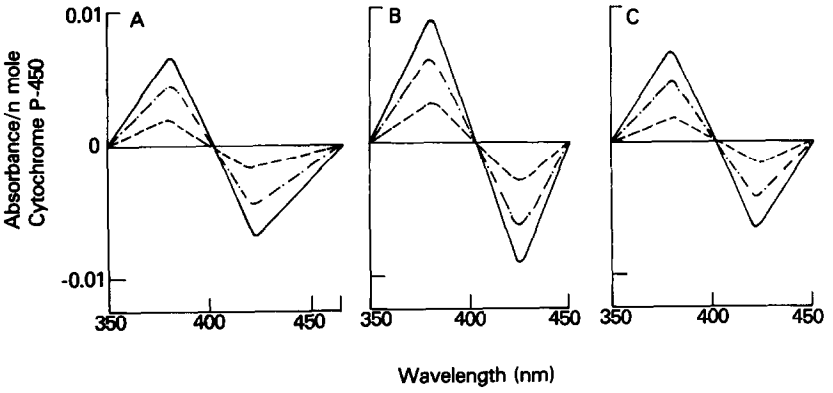

Fig. 4. Binding of PCB congeners to cytochrome P-450. Difference spectra of the PCB congeners were recorded with hepatocytes, microsomes, and microsomes in 10% albumin. All suspensions contained 0.26 nmole cytochrome P-450. Each congener was added as a suspension in DMSO–albumin (100 μM). An equivalent volume of DMSO–albumin was added to the reference cuvette. Spectra were measured between 350 and 500 nm at 25° in KH or 0.05 M phosphate buffer, pH 7.5. Values represent the means of three determinations differing by less than 10% from each other. (A) Hepatocyte suspension; (B) microsomal suspension; and (C) microsomal suspension containing 150 μM albumin. Key: (——) 236-HCB, (–·–·–) 4-DCB, and (– – –) 245-HCB.

between peak and trough parallels the binding affinity of the PCB congener for the cytochrome P-450. At equimolar concentrations of congener (100 μM) 236-HCB displayed the greatest spectral change (Δ absorbance 390–420 μM) followed by 4-DCB. The 245-HCB congener showed little binding (Fig. 4). More congener was found to bind to the cytochrome P-450 in the microsomal preparation than in the hepatocyte preparation for this concentration of PCB. Addition of albumin to the microsomal preparations, however, decreased congener binding to the levels observed in intact hepatocyte suspensions.

Spectral dissociation constants were determined by Lineweaver–Burk plots of PCB concentration (10–100 μM) and spectral change in hepatocyte and microsomal suspensions for each congener (Table 2). The extent of metabolism for 236-HCB, 4-DCB, and 245-HCB was inversely proportional to the dissociation constant (K_s), a lower value being indicative of a higher affinity to cytochrome P-450 and a greater potential for biotransformation. For example, 236-HCB exhibited the lowest K_s value, 2.8×10^{-5} M, indicating a greater affinity to cytochrome P-450 than either 4-DCB or 245-HCB and was metabolized to the greatest extent, 60% for 236-HCB, 32% for 4-DCB and no metabolites formed for 245-HCB. The K_s value of 4-DCB, 8.7×10^{-5} M, was 3-fold higher while that of 245-HCB, 2.87×10^{-4} M, was nine times that of 236-HCB. A similar pattern in the K_s values was obtained in microsomes and suggests that the two systems are comparable. The greater K_s values observed in the hepatocyte suspensions could be mimicked by the addition of albumin to the microsomes.

Table 2. Spectral dissociation constants of PCB congeners in isolated hepatocytes and microsomes

Congener	K_s (μM)	
	Hepatocytes	Microsomes
4-DCB	87	71
236-HCB	28	24
245-HCB	287	245

Subcellular distribution of ^{14}C-labeled PCB and metabolites. The distribution of radioactivity among hepatocyte subcellular fractions following a 60-min incubation of hepatocytes with 10 μM 4-DCB, 236-HCB, or 245-HCB is shown in Fig. 5. The greatest proportion of radioactivity for each congener was found in the cytosolic fraction. This was reflected in the relative specific activity (RSA), which was about 3-fold greater for cytosol than the highest particulate fraction, the plasma membrane, which exhibited an RSA of 0.88 (Fig. 5).

In addition to the high proportion of congener associated with the cytosol, the major portion of metabolite was also associated with the cytosolic fraction. For example, 32% of 4-DCB added to an hepatocyte suspension was metabolized by 1 hr and 70% of the metabolites were present in the cytosol. For 236-HCB 60% of the dose added was metabolized and about 66% of the metabolites were associated with the cytosol at 1 hr. Essentially no metabolism was observed in the 245-HCB suspensions (0.34%). However, 65% of the total radioactivity added was associated with the cytosol. For 245-HCB the radioactivity corresponded to congener. In comparison, higher congener levels were found in the cytosol for 245-HCB (65%) than for 4-DCB (56%) or 236-HCB (30%).

Disrupted hepatocyte preparations did not metabolize the PCB congeners as demonstrated by the lack of metabolite production from such suspensions (Fig. 5, D–F). Disruption of hepatocytes by homogenization diluted the factors necessary for metabolism about 25-fold. Total radioactivity was more evenly distributed among the subcellular fractions derived from disrupted hepatocyte suspensions, but the cytosolic fraction still contained the highest pro-

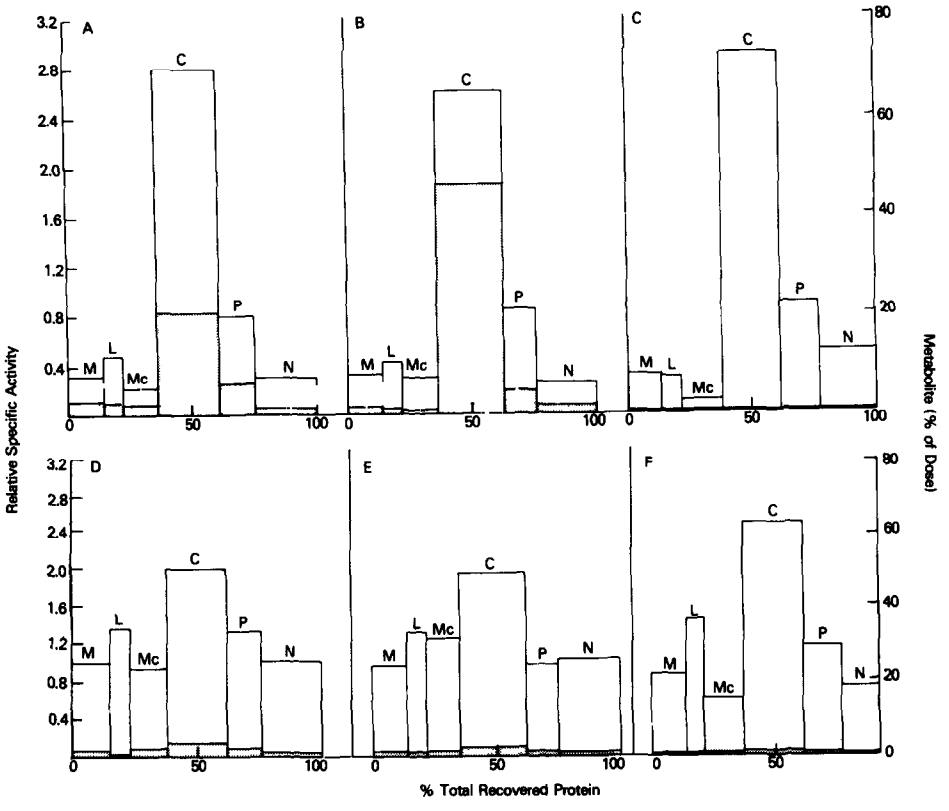

Fig. 5. Subcellular fractionation of ^{14}C-labeled PCB congeners and metabolites. Suspensions of intact and disrupted hepatocytes (4×10^6 cells/ml) were incubated at 37° for 1 hr with 10 μM 4-DCB, 236-HCB and 245-HCB. Subcellular fractions were prepared at 4° from each hepatocyte incubation as described in Materials and Methods. Protein and total radioactivity were determined for each subcellular fraction and expressed as a percentage of the total. The amount of congener and proportion of metabolite were determined for each subcellular fraction and expressed as a percentage of the dose added. Values represent means of three determinations differing by less than 10% of each other. (A–C) subcellular fractions derived from intact hepatocyte suspensions; and (D–F) subcellular fractions derived from hepatocyte suspensions that were disrupted prior to incubation with congener. (A,D) 4-DCB; (B,E) 236-HCB; and (C,F) 245-HCB. Key: (M) mitochondria; (L) lysosomes; (Mc) microsomes; (C) cytosol; (P) plasma membrane; (N) nuclei; (▭) proportion of congener in each subcellular fraction; and (▩) proportion of metabolite in each subcellular fraction.

portion of radioactivity with RSA values of about 1.6 for each congener. The RSA values for the other particulate fractions ranged from 0.6 to 1.0.

DISCUSSION

In these experiments, three polychlorinated biphenyls varying in metabolic potential, 4-DCB, 236-HCB, and 245-HCB, served as model compounds to examine the relationship of chlorine content and position with respect to cytochrome P-450 spectral interactions and subcellular distribution. PCB congener structure is proving to be an important determinant in the metabolic disposition of these compounds. Evidence presented in this paper demonstrates that congener structure influences both the subcellular partitioning and binding of PCB congeners to cytochrome P-450, thereby influencing the metabolic potential of the various PCB congeners within the liver.

Incubation of rat hepatocytes with the radiolabeled PCB congeners 4-DCB, 236-HCB and 245-HCB complexed with albumin demonstrated a rapid uptake of radioactivity into the cells. The uptake of PCB congeners by hepatocytes is compatible with a diffusional process in that uptake is rapid, nonsaturable, not significantly temperature dependent and based on the lipophilic nature of the congeners. Complexing the PCB congeners with albumin aided in the solubilization of the compounds in the medium and facilitated the uptake of congener by the hepatocytes. The mode of transport of PCBs *in vivo* is unknown but PCBs have been shown to bind effectively to both plasma proteins and lipoproteins [35, 37–39]. Binding to proteins such as albumin could expedite the uptake of such compounds by the

liver since receptors for albumin have been identified on the liver cell surface [40, 41]. The presence of such receptors may, in part, explain the more complete uptake of PCB congeners by hepatocytes in the presence of albumin.

Following the initial uptake of congener, the amount of congener in the medium did not change throughout the incubation period. The increase in radioactivity in the medium reflected the release of metabolites from the cells. Transport of the metabolites to the extracellular medium was slow. Only a small proportion of the total metabolites produced was released. The majority of the metabolites, as well as the parent compound, was retained within the cell. Even though the majority of each congener was taken up by the liver cells, only a portion of the congener was metabolized. Differences observed in the magnitude of the binding spectra among the congeners was a reflection of the extent of metabolism observed for each, 236-HCB being metabolized to the greatest extent followed by 4-DCB and then 245-HCB. This was also the order of binding to the cytochrome P-450. Similar differences have been obtained for the biliary excretion of PCBs as metabolites from the isolated perfused rat liver and from *in vivo* elimination rates. Using isolated perfused livers, Mehendale [9] showed that only 30% of 4-DCB and 1.3% of 245-HCB were excreted into the bile within 4 hr. Following intravenous administration to rats, only 34% of 4-DCB, 58% of 236-HCB, and 0.7% of 245-HCB [1] were excreted during the first 24 hr.

The rate of metabolite production of 4-DCB and 236-HCB resulted in nonlinear Eadie–Hofstee plots, which permitted calculation of two sets of kinetic constants. It is unlikely that the nonlinearity reflected the uptake of PCB congeners by the cells, since the labeled congeners had equilibrated with the surrounding medium in less than 5 min. The literature suggests that the nonlinearity observed in a kinetic study is more likely a reflection of multiple forms of cytochrome P-450 participating in the biotransformation rather than a single cytochrome P-450 producing two different products [42–44].

The observations of rapid uptake and incomplete metabolism of PCB congeners in isolated hepatocyte suspensions may be explained by one of the following hypotheses. First, upon uptake of the PCBs by the cells, a certain proportion of the PCB interacts with the mixed-function oxidase system while the remaining congener binds nonspecifically to cytosolic protein and/or is partitioned into subcellular compartments. During this distributionary phase congener access to the cytochrome P-450 is sufficient to maintain first-order kinetic behavior. However, once the PCBs are partitioned into subcellular compartments, the PCBs are no longer available for metabolism at the initial rate. An alternative hypothesis is that the decreased metabolism observed is due to metabolite inhibition: the metabolites produced exert a negative feedback [45, 46] by interacting with the cytochrome P-450 system, preventing further binding of congener and decreasing the rate of metabolism of remaining PCB. Addition of fresh PCB would increase the congener to metabolite ratio, resulting in further metabolite production because of successful competition for the mixed-function oxidase system.

The results of this study support the hypothesis that the partitioning of PCB congeners with cellular macromolecules greatly influences the metabolic potential of a congener. First, the metabolites produced following the first dose of congener did not alter the amount or the half-life of additional congener metabolized following a second dose. If the plateau region were the result of metabolite inhibition, the metabolite pattern following re-addition of congener would be expected to be reduced. However, there was no evidence of an inhibitory influence on metabolite production. The hepatic cytochrome P-450 was not changed in concentration; the cytochrome P-450 complex did not lose its ability to catalyze further metabolism of additional congener nor was a Soret peak produced, indicative of a metabolite stable complex. Second, the subcellular distribution of each ^{14}C-labeled PCB congener and metabolites revealed that the majority of radioactivity appeared in the cytosolic fraction, with the plasma membrane fraction exhibiting the highest amount of radioactivity among the particulate fractions. It was interesting to find that the site of the metabolizing enzymes, the microsomal fraction, showed only a small degree of the total radioactivity. Third, the potential binding of PCB congeners to cytosolic proteins was also suggested by differences in the magnitude of spectral binding of cytochrome P-450 interactions. Increasing the protein content of the microsomal suspension by the addition of albumin decreased the congener spectral binding to the levels observed in hepatocyte suspensions. Together these findings suggest that the binding of congeners to hepatic cytosolic macromolecules may limit the availability of congener for binding to the cytochrome P-450 and that cytosolic binding of metabolites could influence the rate of metabolite elimination from the hepatocyte. Once produced, the phase I metabolites may also associate with cellular constituents, thereby hindering phase II metabolism and subsequent release of the conjugated products from the cell. Schwenk *et al.* [47] observed that conjugation of 2,2′-dichlorobiphenyl and release of the conjugates were twenty-four times slower than those of napthol in isolated hepatocyte suspensions, demonstrating that both cellular biotransformation and transport of PCB metabolites are slow in comparison to compounds of similar lipophilicity. For certain lipophilic carcinogens the cytosolic binding proteins have been shown to facilitate the oxidation of benzo[*a*]pyrene by the microsomal enzymes [48]. Several carcinogen binding proteins are localized in the cytosol [49–51]; however, the specificity of ligand-protein binding and transport destination within the cell remains to be understood. For the PCB congeners examined in this study, binding to the Ah receptor can be excluded to poor binding affinities [52].

It is known that metabolism of PCB congeners is greatly affected by congener structure. Metabolism appears to be structure dependent, while uptake is dependent on hydrophobicity. As a class of compounds, the PCBs are lipophilic; hence, the potential for interacting with cytochrome P-450 would, on that

basis, be considered both excellent and comparable. On the other hand, some of the PCB congeners, 245-HCB for example, are poorly metabolized [1] and, as shown here, bind poorly to cytochrome P-450. The key property of the PCB molecule in determining cytochrome P-450 affinity appears to be congener structure: the positions of the chlorine atoms on the biphenyl nucleus affect binding more than either the degree of chlorination or the lipid solubility, at least for these PCB congeners. This was apparent by comparing the binding spectra of 236-HCB and 245-HCB. Both congeners contain six chlorine substituents on the biphenyl ring but 236-HCB was found to exhibit the greater affinity for the cytochrome P-450. Binding of 246-HCB to rabbit hepatic cytochrome P-450 [16] displayed a similar binding affinity (K_s, 18 μM) as 236-HCB to rat hepatic cytochrome P-450 (hepatocytes, 28 μM and microsomes, 24 μM), suggesting that the degree of ortho chlorination may be a major structural determinant which modulates binding to cytochrome P-450.

The data obtained in this study utilizing isolated hepatocytes are consistent with those obtained in biological systems of higher organization (isolated perfused livers and whole animal studies). The ability of a PCB congener to be metabolized is determined by both its affinity for cytochrome P-450 and its partitioning within the subcellular compartments of the hepatocyte. Steric properties of the congener appear to govern the affinity of PCB congener interaction with cytochrome P-450, while the lipophilic nature of PCBs contributes to the partitioning within subcellular compartments. Such partitioning can influence the overall disposition and pharmacokinetics of PCBs. Similar phenomena occur *in vivo* except that there is a transfer of PCBs from hepatic storage sites to long-term storage sites present in adipose tissue. The results suggest that isolated hepatocytes are a valuable tool in predicting the biotransformation of PCBs and other environmental compounds.

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