Metabolism of Dichlorobiphenyls by Highly Purified Isozymes of Rat Liver Cytochrome P-450†

Laurence S. Kaminsky,* Margaret W. Kennedy, Stephen M. Adams, and F. Peter Guengerich

ABSTRACT: Hepatic mixed-function oxidase metabolism of the ubiquitous pollutant polychlorinated biphenyls (PCBs) is implicated in their toxification and detoxification. We used dichlorobiphenyls (DCBs) as models to investigate the effect of the chloro substituent sites on this metabolism experimentally and by molecular orbital calculations. Reconstituted, purified cytochrome P-450 PB-B and BNF-B, the major terminal oxidase isozymes of this system, from phenobarbital (PB)- and β-naphthoflavone (BNF)-induced rats were used to investigate this metabolism. Both isozymes are also induced by PCBs. High-performance liquid chromatography (HPLC) was used to detect, quantify, and isolate metabolites. Metabolite structures were identified by mass spectrometry, dechlorination to identifiable hydroxybiphenyls, and HPLC retention times. All DCBs yielded 3- and 4- but no 2-monohydroxylated metabolites (3,3′-DCB also yielded a dihydroxy metabolite). Di-*o*-chloro-substituted DCBs were metabolized primarily by cytochrome P-450 PB-B, mono-*o*-chloro substituted DCBs by both isozymes approximately equivalently, and DCBs without *o*-chloro substituents by BNF-B primarily. Thus PB-B preferentially metabolizes noncoplanar DCBs and BNF-B coplanar DCBs. The cytochrome isozymes exhibited differing regioselectivities for DCB metabolism—PB-B hydroxylated unchlorinated phenyl rings and BNF-B chlorinated rings. Incorporation of epoxide hydrolase yielded DCB dihydrodiols, and hydroxy metabolite patterns were consistent with those calculated from ring-opened arene oxide intermediates. Thus the rates and regioselectivities of metabolism and thus possibly the toxicity and carcinogenicity of DCBs are dependent on the cytochrome P-450 isozymes induced.

Mammalian metabolism of PCBs[1] is catalyzed primarily by the hepatic microsomal mixed-function oxidase system. The extent to which this metabolism affects the toxicity (Kimbrough, 1974; Yoshimura & Ikeda, 1978), mutagenicity (Wyndham et al., 1976), and carcinogenicity (Kimbrough et al., 1975) of these widely distributed environmental pollutants has been only partially elucidated. PCBs occur in the environment as complex mixtures of many of the 209 possible congeners, and evaluation of the human health related effects of this contamination is dependent on a knowledge of the properties of the individual congeners.

Cytochrome P-450 isozymes, the terminal oxidases of the hepatic mixed-function oxidase system, metabolize PCBs, particularly those with low chloro substitution to monohydroxy metabolites via epoxide intermediates (Forgue et al., 1979; Preston & Allen, 1980). Metabolism possibly via epoxide formation leads to mutagenic effects of PCBs (Wyndham et al., 1976) and to covalent binding of PCBs to cellular proteins with consequent toxicity (Shimada & Sato, 1978). Some hydroxylated metabolites of PCBs exhibit greater toxicity than the parent compounds: human erythrocyte membranes have a greater susceptibility to hemolysis from some hydroxylated PCB metabolites than do the parent PCBs (Miller, 1978), the monohydroxylated metabolite of 2,4,3′,4′-tetrachlorobiphenyl has a significantly lower LD_{50} in mice than the parent compound (Yamamoto & Yoshimura, 1973), and in a cultured cell system, both the monohydroxylated metabolite and its epoxide precursor were more toxic than 2,5,2′,5′-tetrachlorobiphenyl (Stadnicki & Allen, 1979).

PCBs induce the synthesis of hepatic cytochromes P-450 (Litterst et al., 1972). Depending on the specific PCB congeners present in the mixture, the resulting isozymes can be of the form induced either by PB or by the polycyclic aromatic hydrocarbons (Goldstein et al., 1977). It was originally reported that PCB congeners chlorinated symmetrically in the meta and para positions lead to polycyclic aromatic hydrocarbon inducible forms and chlorination in the para and ortho positions of both phenyl rings leads to induction of PB-inducible forms of the enzyme (Goldstein et al., 1977). Some PCB congeners, chlorinated at one ortho position and at meta and para positions of both phenyl rings, induce both classes of cytochromes P-450 (Parkinson et al., 1980a), and some hexachlorobiphenyls with two *o*-chloro substituents exhibit a similar mixed induction (Parkinson et al., 1980b). A recent study indicates that rules for predicting which PCBs are PB-like inducers cannot be clearly defined (Parkinson et al., 1980c).

Different forms of microsomal cytochrome P-450 exhibit varying regioselectivities for biphenyl metabolism (Billings & McMahon, 1978; Halpaap et al., 1978; Burke & Mayer, 1975), but with monochlorobiphenyls, this variation in regioselectivities is apparently suppressed (Wyndham et al., 1976; Wyndham & Safe, 1978a,b; Kennedy et al., 1980). Recently we have demonstrated with DCBs that different isozymes of microsomal-bound cytochrome P-450 do exhibit differing extents of hydroxylation and regioselectivities for hydroxylation of some of the congeners (Kennedy et al., 1981). Thus PCBs can affect the extent and type of their own metabolism through induction of hepatic cytochrome P-450.

In the present study, we have investigated the metabolism both experimentally and theoretically of a series of DCBs by reconstituted systems with highly purified forms of hepatic

† From the Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201 (L.S.K., M.W.K., and S.M.A.), and the Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (F.P.G.). *Received June 19, 1981.* This research was supported by Grants ES 01554 (L.S.K.) and ES 01590 and ES 00267 (F.P.G.) from the National Institutes of Health, U.S. Public Health Service, Department of Health and Human Services. F.P.G. is the recipient of Research Career Development Award ES 00041 from the National Institutes of Health.

[1] Abbreviations used: Pb, phenobarbital; BNF, β-naphthoflavone; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine; HPLC, high-performance liquid chromatography; PCB, polychlorinated biphenyl; DCB, dichlorobiphenyl; Tris, tris(hydroxymethyl)aminomethane.

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cytochrome P-450 from PB- and BNF-induced rats. The objective of the study was to determine clearly the manner in which the situation of chloro substituents affect the metabolism of PCBs by different cytochrome P-450 isozymes for ultimate comparison with microsomal metabolism.

Materials and Methods

Materials. The 10 DCBs and the few dichlorobiphenylols available were purchased from RFR Corp., Hope, RI, and Analabs, North Haven, CT, and were purified to homogeneity by HPLC as previously described (Kennedy et al., 1981). 2,4′- (13 mCi/mmol), 2,2′- (13 mCi/mmol), and 4,4′-DCB-*U*-^{14}C (58 mCi/mmol) were purchased from California Bionuclear Corp., Sun Valley, CA, and were purified by HPLC. Sodium dihydrobis(2-methoxyethoxy)aluminum hydride and NADPH were obtained from Pfaltz & Bauer, Stamford, CT, and from Sigma, St. Louis, MO, respectively. A 0.1% (w/v) aqueous suspension of dilauroyl-GPC (Serdary Research Laboratories, London, Ontario) was prepared by ultrasonication for five 15-s periods separated by 30-s delays between each ultrasonication. Water was deionized and glass distilled and was also filtered through a 0.22-μm membrane before use in the HPLC.

Purification of Enzymes. The cytochromes P-450 were purified from livers of male Sprague–Dawley rats (100–125 g) from Harlan Industries, Indianapolis, IN. Rats were induced with PB [0.1% (w/v) in drinking water for 5 days prior to killing] or BNF [intraperitoneal injections of 25 mg (kg of body weight)$^{-1}$ day^{-1} for 3 days prior to killing]. Livers were removed immediately following the death of the rats, and microsomes were prepared as previously described (Van der Hoeven & Coon, 1974).

Cytochromes P-450 were isolated from the microsomes and purified as previously described (Guengerich, 1977, 1978a; Guengerich & Martin, 1980). Preparations used in this study were the major fractions (PB-B and BNF-B) from PB- and BNF-induced rats and a minor fraction (PB-C) from PB-induced rats. Specific contents of cytochrome P-450 are reported in the figure legends. Cytochromes P-450 PB-B and BNF-B were homogeneous, as judged by polyacrylamide gel electrophoresis in six different systems (Guengerich, 1978a), while cytochrome P-450 PB-C was not completely homogeneous (Guengerich, 1977).

NADPH–cytochrome P-450 reductase was isolated and purified from hepatic microsomes from PB-induced rats by octylamino-Sepharose 4B and 2′,5′-ADP–agarose chromatography by the method of Yasukochi & Masters (1976) as subsequently modified (Guengerich, 1978b). The reductase was homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (M_r 74000), had an A_{455}/A_{380} ratio of 1.10, and catalyzed the reduction of 43 μmol of cytochrome *c* min^{-1} (mg of protein)$^{-1}$ at 30 °C in 0.3 M potassium phosphate buffer, pH 7.7 [43 units (mg of protein)$^{-1}$].

Epoxide hydrolase was isolated and purified from hepatic microsomes from PB-induced rats as described previously (Guengerich et al., 1979). Form A, which was homogeneous as judged by electrophoretic techniques (Guengerich et al., 1979), was used in this study.

Cytochrome P-450 concentrations were determined from reduced CO vs. reduced difference spectra (Omura & Sato, 1964). Protein concentrations were determined by the method of Lowry et al. (1951).

DCB Metabolism. The metabolism of DCBs was investigated in reconstituted systems which were optimized for maximum rates and comprised of 1.0 μM cytochrome P-450 PB-B, PB-C, or BNF-B, 1.0 μM NADPH–cytochrome P-450 reductase, 31 μM dilauroyl-GPC, 50 mM Tris-HCl (pH 7.4), 0.2 mM dichlorobiphenyl (added as a suspension in 2.5% carboxymethylcellulose), and 1.0 mM NADPH. When epoxide hydrolase was added to the incubate, the concentration was 0.8 μM.

All components (except NADPH) were shaken together at 37 °C for 5 min, and the reaction was initiated by addition of the NADPH. Reactions were terminated after 5 min by shaking the reaction tubes for 10 s in a boiling water bath. Metabolites were extracted with 1,2-dichloroethane. The extract was evaporated to dryness with gentle heating to minimize volatilization of the metabolites. The residue was taken up in 40 μL of tetrahydrofuran and was analyzed by HPLC with a μBondapak C_{18} column as previously described (Kennedy et al., 1980) and with a μBondapak NH_2 column using the method reported for hydroxybiphenyl analysis (Burke & Prough, 1977). Products were quantitated by using an automated recording integrator. When ^{14}C-labeled substrates were used, the compounds eluting under the chromatographic peaks were collected and the ^{14}C counts determined in a liquid scintillation counter with internal standards.

Metabolites were identified by using a Finnigan 4000 mass spectrometer/data system. A Finnigan HPLC/mass spectrometer interface was used to introduce metabolites into the mass spectrometer, as previously described (Kennedy et al., 1980).

Metabolites were dechlorinated with sodium dihydrobis(2-methoxyethoxy)aluminum hydride (Goto et al., 1974), and the resultant monohydroxybiphenyls were identified by HPLC as previously described (Burke & Prough, 1977).

Molecular orbital calculations were performed with the semiempirical molecular orbital method MNDO (Thiel, 1978) with a program obtained from the Quantum Chemistry Program Exchange. Calculations were performed on the New York State Health Department VAX 11/780 computer.

Calculations on DCBs were performed at an idealized geometry with bond lengths of the following: single C–C, 1.54 Å; aromatic C–C, 1.4 Å; C–H, 1.1 Å; C–Cl, 1.7 Å. All bond angles were set at 120°. For biphenyl, calculated molecular properties at the idealized and fully optimized geometries were not significantly different. It was thus assumed that idealized geometries for the DCBs would also provide accurate results without the need for obtaining fully optimized geometries. Calculated electronic properties were not markedly affected by the inter-ring dihedral angle of the DCBs. In general, DCBs containing one or two *o*-chloro substituents were assumed to have an inter-ring dihedral angle of 90° and those without *o*-chloro substituents were assumed to be planar.

Calculations for the carbocations corresponding to DCB oxide ring-opened structures assumed a tetrahedral geometry about the hydroxylated carbon and a planar geometry about the carbon carrying the positive charge. The hydroxy hydrogen was assumed to be in an s-trans conformation relative to the ring. Each carbocation was geometry optimized completely to ensure consistency of the results. All calculations for the carbocations assumed a closed shell singlet electronic structure. In the transition from the DCB protonated epoxide to the ring-opened carbocation, the reaction path was approximated by a series of CCO epoxide ring angles whose variation corresponded to opening of the epoxide ring. At each value of the CCO angle, a MNDO calculation was performed and the energy and electronic structure obtained.

Results

The cytochrome P-450 Pb-B reconstituted system with 2,5-DCB as substrate was used as a model for optimizing the system for DCB metabolism. Rates of metabolite formation

Table I: Monohydroxy Metabolites of Dichlorobiphenyls Catalyzed by Purified Reconstituted Cytochromes P-450 PB-B and BNF-B from Rat Liver

DCBs[a]	monohydroxy-DCBs	
	cytochrome P-450 PB-B	cytochrome P-450 BNF-B
2,3-DCB	2,3-DCB-3'-ol; 2,3-DCB-4'-ol[b] (2,3-DCB-4-ol)[c]	2,3-DCB-4-ol (2,3-DCB-3'-ol)
2,4-DCB	2,4-DCB-3'-ol; 2,4-DCB-4'-ol	2,4-DCB-4'-ol; 2,4-DCB-3-ol or -5-ol
2,5-DCB	2,5-DCB-4'-ol; 2,5-DCB-3'-ol	2,5-DCB-4-ol
2,6-DCB	2,6-DCB-3'-ol; 2,6-DCB-4'-ol	
3,4-DCB	3,4-DCB-3'-ol; 3,4-DCB-4'-ol	3,4-DCB-4'-ol
3,5-DCB	3,4-DCB-4'-ol	3,5-DCB-4-ol (3,5-DCB-4'-ol; 3,5-DCB-3'-ol)
2,2'-DCB	2,2'-DCB-3- or -5-ol (2,2'-DCB-4-ol; 2,2'-DCB-5- or -3-ol)	
3,3'-DCB	3,3'-DCB-4-ol (3,3'-DCB-5-ol)	3,3'-DCB-4-ol (3,3'-DCB-5-ol)
4,4'-DCB	4,4'-DCB-3-ol; 3,4'-DCB-4-ol	4,4'-DCB-3-ol; 3,4'-DCB-4-ol
2,4'-DCB	2,4'-DCB-3- or -5- or -3'-ol; 2,4'-DCB-4-ol (2,4'-DCB-3'- or -5'-ol)	2,4'-DCB-3- or -5- or -3'-ol; 2,4'-DCB-4-ol

[a] Abbreviation used: DCB, dichlorobiphenyl. [b] Major metabolites. [c] Minor metabolites in parentheses.

were linear for only 5 min, and major deviations from linearity were observed by 10 min of reaction. Maximal rates were obtained with 0.2 mM 2,5-DCB, and marked substrate inhibition was observed at 0.5 mM and higher concentrations of substrate. Optimal rates of metabolism were obtained at a 1:1 ratio of reductase to cytochrome P-450, and at concentrations of cytochrome P-450 exceeding 1 μM, rates of metabolism were markedly diminished. Dilauroyl-GPC produced maximum enhancement of metabolic rates at 30 μM and in the absence of the lipid rates were approximately 29% of maximal.

Nine of the ten DCBs investigated yielded monohydroxylated products as the only metabolites detectable by HPLC, under the conditions of the cytochrome P-450 PB-B and BNF-B-reconstituted systems used. The exception was 3,3'-DCB in the cytochrome P-450 BNF-B system where sufficient metabolite was generated to compete with residual substrate for further metabolism to the dihydroxy product (see below).

The metabolites were isolated by HPLC and identified as monohydroxy-DCBs by mass spectrometry with parent ions at m/e 238. In all cases, dechlorination of the metabolites yielded 3- or 4-hydroxybiphenyl with no detectable 2-hydroxybiphenyl. In some cases, because of specific chloro substituent patterns, identification of the hydroxylation site in the dechlorinated molecule was sufficient to identify unambiguously the monohydroxy-DCB metabolite, in other cases synthetic standards were available, and in yet other cases retention times provided an indication of possible metabolite structures.

The major and some minor metabolites of the DCBs are presented in Table I. For 2,6- and 2,2'-DCBs, the relatively very slow rates of metabolism catalyzed by cytochrome P-450 BNF-B precluded the determination of the structures of the metabolites. Of the remaining eight DCBs, 2,3-, 2,4-, 2,5-, 3,4-, and 3,5-DCBs yielded different major metabolites when metabolized by either cytochrome P-450 PB-B or BNF-B, and 2,4'-, 3,3'-, and 4,4'-DCBs yielded the same major metabolites when metabolized by the two isozymes.

The overall apparent rates of formation of monohydroxylated metabolites catalyzed by cytochromes P-450 PB-B and BNF-B are shown in Figure 1. Rates are based on integrated areas of chromatographic peaks representing all monohydroxylated metabolites. Areas are corrected for detector response differences based on relative responses of DCBs. Comparison of the ratios of the rates of formation of total monohydroxylated metabolites catalyzed by cytochromes P-450 PB-B and BNF-B separates the DCBs into three groups:

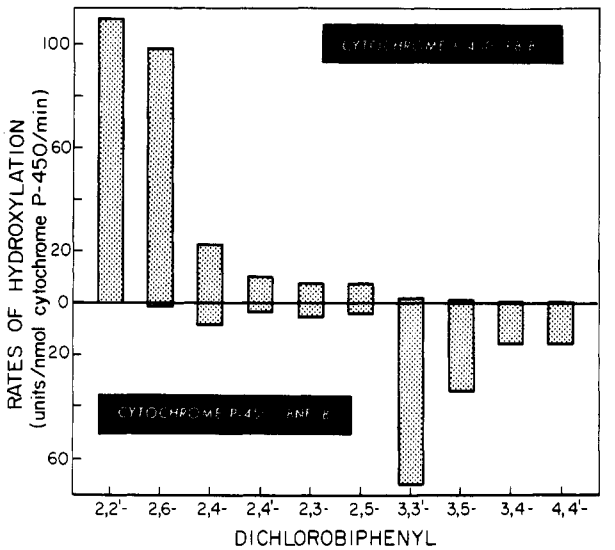

FIGURE 1: Rates of hydroxylation of an isomeric series of dichlorobiphenyls catalyzed by cytochrome P-450 PB-B and BNF-B in reconstituted systems. Rates are based on units of integrated areas of chromatographic peaks representing all of the monohydroxylated metabolites. These areas have been corrected for differences in detector responses based on the relative responses of the parent DCBs. Metabolites were analyzed by HPLC, and the analytical procedures are described under Materials and Methods. Specific contents of cytochromes P-450 PB-B and BNF-B were 16.0 and 12.2 nmol (mg of protein)$^{-1}$.

4,4'-, 3,3'-, 3,5-, and 3,4-DCB with ratios of 0.02–0.03, 2,4-, 2,5-, 2,6-, and 2,4'-DCB with ratios of 1.4–3.3, and 2,2'- and 2,6-DCB with ratios of 73–>100. Thus DCBs with no o-chloro substituent are better substrates for cytochrome P-450 BNF-B than for cytochrome P-450 PB-B by a factor of approximately 50, DCBs with a single o-chloro substituent are metabolized to approximately the same extent by both isozymes, and DCBs with two o-chloro substituents are metabolized to an approximately 100-fold greater extent by cytochrome P-450 PB-B.

Apart from the major contribution of o-chloro substituents to the determination of relative rates of metabolism of the various DCBs by either cytochrome P-450, no other factor, such as number of chloro substituents on each ring or the presence of a 4-substituent, apparently affected the rates of metabolism in a consistent manner.

The rates of hydroxylation of 2,2'-, 2,4'-, and 4,4'-DCB based on rates of biotransformation of ^{14}C counts from substrate to hydroxylated product were 2.62, 0.23, and 0.00 nmol (nmol of cytochrome P-450)$^{-1}$ min^{-1}, respectively, with cyto-

chrome P-450 PB-B and 0.03, 0.05, and 0.25 nmol (nmol of cytochrome P-450 BNF-B)$^{-1}$ min^{-1}, respectively, with cytochrome P-450 BNF-B. The relative rates of cytochrome P-450 PB-B to BNF-B catalyzed metabolism and the relative rates of metabolism of the DCBs by each cytochrome are in close agreement with the relative rates of hydroxylation (Figure 1) calculated on the basis of integrated HPLC peak areas. With the ^{14}C-labeled DCBs, trace quantities of other ^{14}C-labeled products were observed in the chromatograms.

The cytochrome P-450 PB-C catalyzed metabolism of 2,2′-, 2,4′-, 2,5-, and 3,5-DCB was investigated. In each case the extent of metabolism was lower than that catalyzed by cytochrome P-450 PB-B by factors of 0.16, 0.21, 0.09, and 0.79, respectively. On the basis of relative retention times, the major monohydroxy metabolites were apparently the same as those produced by cytochrome P-450 PB-B.

In some reactions of those DCBs which were metabolized by both cytochrome isozymes at similar rates (2,3-, 2,5-, and 2,4-DCB), epoxide hydrolase was added. In each case, a new chromatographic peak was obtained which was more polar than that of the monohydroxy metabolites and had a mass spectral parent ion corresponding to that of a PCB dihydrodiol (*m/e* 256). The quantity of monohydroxylated metabolite was diminished when epoxide hydrolase was added, and the extent to which the monohydroxylated metabolite was diverted to dihydrodiol was greater for the cytochrome P-450 BNF-B than for the PB-B catalyzed reaction. Similar results have been reported in a preliminary communication (Kaminsky et al., 1981).

In an effort to determine the structure of the apparent secondary metabolite of 3,3′-DCB, the hydroxylated metabolites were isolated from the HPLC eluant and added back to a cytochrome P-450 BNF-B reconstituted system as the substrate. The metabolite from this reaction had the same retention time as the minor product from 3,3′-DCB metabolism and had a parent ion in the mass spectrum indicating a dihydroxy metabolite (*m/e* 254).

MNDO molecular orbital calculations on DCBs were performed to facilitate the derivation of mechanisms of DCB metabolism. In particular calculations were designed to provide predictions of the directions of the ring opening of DCB oxide metabolic intermediates.

Calculations were performed on protonated biphenyl 3,4-oxide in an effort to determine the effect of phenyl-ring twist angles on the directions of arene oxide ring opening. A maximum in the barrier to ring opening was found at a CCO angle of 70° (either direction) at phenyl-ring twist angles of 90° and 45°. For coplanar phenyl rings, an activation energy was determined at a 70° C_4C_3O and 65° C_3C_4O angle. Energies at this angle were used to estimate barriers to ring opening. At phenyl-ring twist angles of 90°, 45°, and 0°, barriers to opening in the 4 direction were 1.7, 0.7, and 0.3 kcal/mol, respectively, and barriers to opening in the 3 direction were 2.4, 2.9, and 3.2 kcal/mol, respectively. The energy differences in favor of the 4-OH biphenyl carbocation relative to the 3-OH biphenyl carbocation at 90°, 45°, and 0° phenyl-ring twist angles were 2.3, 7.7, and 11.9 kcal/mol. From both the energy barriers and relative energy of the ring-opened carbocations, a preference for ring opening to the 4-OH carbocation is indicated. This carbocation would rearomatize to 4-hydroxybiphenyl. In particular, with coplanar phenyl rings, the barrier to ring opening in the 4 direction is negligible, and upon protonation, ring opening would occur without activation energy. This facile ring opening in the 4 direction is in agreement with the finding that biphenyl yields primarily the 4-hydroxy isomer in vivo in rats (Halpaap et al., 1978).

In view of the differences in energies calculated for arene oxide ring opening at various phenyl-ring twist angles, calculations on ortho chlorinated biphenyl oxides were performed with perpendicular phenyl rings and on non-ortho-substituted DCBs with coplanar phenyl rings. Apart from the twist angle of the phenyl rings, all internal coordinates were optimized.

2,4-DCB-3′,4′-oxide. Calculations were performed on this DCB to determine whether chloro substituents on one phenyl ring affect the opening of 3,4-oxides on the other ring.

Barriers for protonated oxide ring opening in the 3 and 4 directions were 2.6 and 2.5 kcal/mol, respectively. The 4′-OH carbocation was 1.3 kcal/mol more stable than the 3′-OH carbocation. The near equality of the activation energies and energies of the ring-opened carbocations suggests that both 3′-hydroxy and 4′-hydroxy derivatives would arise from 3′,4′-oxide, consistent with the experimental results. Thus, apart from the effect on the phenyl-ring twist angle, the chloro substituents have no apparent effect on the direction or energetics of arene oxide ring opening when the oxide is on the opposite, unchlorinated phenyl ring.

Calculations on 2,2′-, 3,3′-, and 4,4′-DCB-oxides were performed to evaluate the extent to which variously situated chloro substitutents affect the ring opening of oxides on the same phenyl ring.

2,2′-DCB-oxides. For 2,2′-DCB-3,4-oxide calculations with the phenyl-ring twist angle of 45°, which would be expected to approximate the actual preferred angle, indicated that formation of the 4-hydroxy carbocation would be favored. However, this contrasts with the experimental results where 3- or 5-OH metabolites were detected principally.

For the 2,3-oxide, activation energies for ring opening to the 2-OH and 3-OH carbocations were calculated to be 3.1 and 0.9 kcal/mol, respectively, at a 45° phenyl-ring twist angle. Thus formation of the 3-OH metabolite would be favored which is consistent with experimental results.

These calculations thus suggest that the 2,2′-DCB is possibly metabolized via a 2,3-oxide intermediate.

3,3′-DCB-oxides. Calculations were performed on the 3,4- and 4,5-oxides with coplanar phenyl rings. In both cases for the protonated epoxides, ring opening to the 4-OH carbocation was calculated to occur without any activation energy. This is consistent with the experimental results where the 4-OH metabolite was the principal product detected.

4,4′-DCB-3,4-oxide. Calculations were performed with the 3,4-oxide with coplanar phenyl rings. The protonated epoxide was calculated to open spontaneously to the 4-OH carbocation, while 2.2 kcal/mol activation energy was required for opening to the 3-OH carbocation. The 3,4′-DCB-4-OH, a product of an NIH shift, was calculated to be 6.9 kcal/mol more stable than the 4,4′-DCB-4-OH carbocation. Thus the mechanistic pathway is apparently formation of the DCB-3,4-oxide, protonation, spontaneous ring opening to the 4,4′-DCB-4-OH carbocation, NIH shift, and rearomatization to 3,4′-DCB-4-OH. Experimentally 60% of the metabolites arise as a consequence of the NIH shift.

In Table II the calculated net atomic charges on the carbon atoms of each of the 10 DCBs is presented.

Discussion

The only DCB products detected in the reconstituted cytochrome P-450 metabolizing systems were monohydroxylated DCBs, with the one exception, 3,3′-DCB, which yielded a dihydroxy metabolite. Mass spectral analyses permitted the identification of the metabolites as monohydroxy-DCBs, but

Table II: Calculated Net Atomic Charges in the Carbon Atoms of Dichlorobiphenyls (Charges Were Calculated by the Semiempirical Molecular Orbital Method MNDO[b])

DCB[a]	carbon no.											
	1	2	3	4	5	6	1′	2′	3′	4′	5′	6′
2,3-	−2	+1	−1	−4	−5	−3	−5	−3	−6	−5	−6	−3
2,4-	−1	0	−3	−1	−4	−4	−5	−3	−6	−5	−6	−3
2,5-	−2	0	−4	−4	−1	−2	−5	−3	−6	−5	−6	−3
2,6-	0	0	−4	−5	−4	0	−5	−2	−6	−5	−6	−2
3,4-	−2	−3	−1	0	−4	−4	−5	−4	−6	−5	−6	−4
3,5-	−2	−2	−2	−3	−2	−2	−5	−4	−6	−5	−6	−4
2,2′-	−3	+1	−5	+5	−5	−3	−3	+1	−5	+5	−5	−3
3,3′-	−3	−3	−2	−4	−6	−4	−3	−3	−2	−4	−6	−4
4,4′-	−3	−4	−4	−1	−4	−4	−3	−4	−4	−1	−4	−4
2,4′-	−3	0	−4	−5	−5	−4	−4	−3	−5	−1	−5	−3
biphenyl	−4	−4	−6	−6	−6	−4	−4	−4	−6	−6	−6	−4

[a] Abbreviation used: DCB, dichlorobiphenyl. [b] In units of 0.01 electron.

sites of hydroxylation could not be elucidated. Sites of hydroxylation relative to the phenyl–phenyl bond were identified by dechlorination of the metabolites and identification of the resultant hydroxybiphenyls, and in some cases, this was sufficient to unambiguously identify the metabolite. In other cases, because of the chloro substitution patterns, identification was ambiguous. These ambiguities were resolved in some cases on the basis of our observation of major differences in HPLC retention times between DCBs with monohydroxy substituents on the chloro-substituted phenyl ring and those with hydroxy substituents on the unchlorinated phenyl ring (Kennedy et al., 1980, 1981). For DCBs where major metabolites form the cytochrome P-450 PB-B and BNF-B systems yielded the same dechlorinated products but had different retention times on the μBondapak NH_2 column, the retention times of BNF-B-related metabolites were markedly shorter.

In the absence of synthetic standards for all the metabolites, absolute rates of DCB metabolite formation could not be determined. However, estimates of the detector responses for each metabolite were made on the basis of corrections of actual absorbances utilizing responses of DCBs and available hydroxy-DCB standards (Kennedy et al., 1980, 1981). The apparent rates shown in Figure 1 are based on these corrected responses. In the case of 2,2′-, 4,4′-, and 2,4′-DCB, actual rates were determined with ^{14}C-labeled substrates. Relative metabolic rates calculated from these absolute rates were in close agreement with relative rates based on corrected HPLC detector responses, thus providing confirmatory support for the apparent rates in Figure 1.

These studies have demonstrated clear differences in the metabolism of DCBs by cytochrome P-450 PB-B and BNF-B. The major hepatic isozyme from PB-induced Sprague–Dawley rats preferentially metabolizes dichlorinated biphenyls with noncoplanar conformations while the major isozyme from BNF-induced rats preferentially metabolizes coplanar dichlorinated biphenyls. The conformational planarity occurs in the absence of *o*-chloro substituents, while the presence of two *o*-chloro substituents prevents a planar conformation (Robertson, 1961; Trotter, 1961; Bastiansen & Skancke, 1967; Farbrot & Skancke, 1970; Wilson, 1975). Since the rates of metabolism by both cytochrome P-450 isozymes of DCBs, with only one *o*-chloro substituent, are approximately equivalent, it is possible that these DCBs can be forced into a planar conformation on binding to the active site of cytochrome P-450 BNF-B.

The two cytochrome P-450 isozymes also differed in the regioselectivity of formation of the major metabolites of DCBs which contain an unchlorinated phenyl ring. Cytochrome P-450 BNF-B exhibited a preference for hydroxylation of the DCBs at the chloro-substituted phenyl ring while cytochrome P-450 PB-B hydroxylated the nonchlorinated phenyl ring. There are no obvious features of the calculated net atomic charges on the carbons which provide insight into this difference (Table II). In fact, the net charges on the phenyl carbons provide no obvious clue for predicting the sites of metabolic attack, and mechanisms must be sought elsewhere.

It has recently been claimed (Preston & Allen, 1980) that the major metabolite of 2,5,2′5′-tetrachlorobiphenyl generated by microsomes from PB-induced rats does not arise from an arene oxide intermediate. The present results, however, provide a number of indications that arene oxide intermediates are involved: epoxide hydrolase incorporation into the reconstituted system produces dihydrodiols, an NIH shift was observed with 4,4′-DCB, and metabolite patterns are consistent with predictions based on calculations of arene oxide ring openings. The low (less than 3 kcal/mol) value of the calculated energy barrier to ring opening also explains the lack of detection of most arene oxides. Calculations indicated that the major metabolites produced by the cytochrome P-450 PB-B reconstituted system could be explained by an intermediate 3,4-oxide of the DCBs. However, for 2,2′-DCB, an intermediate 2,3-oxide would explain the experimentally determined metabolite. For 3,3′-DCB metabolism, the formation of the 4-OH metabolite exclusively is clearly explained by the calculated barrier to opening of the arene oxide.

While the experimental results clearly demonstrated the major role of *o*-chloro substituents in influencing rates of DCB metabolism, the molecular orbital calculations suggested that these substituents also play a role in the ultimate regioselectivity of metabolism by affecting the phenyl-ring twist angles and thus the direction of arene oxide ring openings. The calculated results indicate that this may be the major influence of the chloro substituents on the direction of ring opening. Deprotonation (aromatization) of the hydroxy carbocations arising from protonated arene oxide ring opening should be facile and independent of ring position.

In all cases, the calculations indicated that the positive charge on the ring-opened DCB hydroxy carbocations tends to be delocalized predominantly para to the hydroxy group. In the case of coplanar DCBs, 4-OH carbocations would be stabilized by additional resonance structures incorporating the other phenyl ring, but 3-OH carbocations would not. These resonance structures are dependent on the coplanarity of the phenyl rings and do not contribute in noncoplanar systems. Thus in non-*o*-chloro-substituted DCBs, there is an additional stabilizing factor for 4-OH over 3-OH substituents which is absent in *o*-chloro-substituted DCBs.

Our limited studies with cytochrome P-450 PB-C indicated that it is markedly less active than cytochrome P-450 PB-B for the metabolism of DCBs. In PB-induced Wistar rats, this cytochrome P-450 is the major liver isozyme (MacDonald et al., 1981; Fasco et al., 1980), and thus Wistar rats would probably be less efficient than Sprague–Dawley rats in metabolizing DCBs.

A feature of the metabolizing properties of hepatic microsomal and purified cytochrome P-450 from polycyclic aromatic hydrocarbon induced rats is the formation of 2-hydroxybiphenyl (Billings & McMahon, 1978; Burke & Mayer, 1975). In contrast no ortho-hydroxylation of any of the DCBs by reconstituted cytochrome P-450 BNF-B was detected. Our results are consistent with other studies on chlorinated biphenyls where no 2-hydroxylation was reported with micro-

somal preparations (Wyndham et al., 1976; Wyndham & Safe, 1978a,b; Kennedy et al., 1980, 1981). Clearly chloro substitution of the biphenyl nucleus suppresses the ability of the cytochrome P-450 isozyme to hydroxylate ortho to the phenyl–phenyl bridge. Since molecular orbital calculations demonstrated that a chloro substituent on biphenyl has minimal electronic inductive influence on the opposite phenyl ring, it is possible that suppression of 2-hydroxylation is steric in origin.

Thus in view of the ability of PCBs to induce the cytochrome P-450 isozymes studied here, it is clear that these pollutants can affect the metabolism of their constituent DCBs. Depending on the DCB, induction could alter (increase or decrease) the rates and regioselectivities of metabolism and thus possibly the toxicity and carcinogenicity of the DCBs.

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