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METABOLISM OF DICHLOROBIPHENYLS BY HEPATIC MICROSOMAL CYTOCHROME P-450*

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Abstract—*In vitro* rat hepatic microsomal metabolism of ten individual dichlorobiphenyls (DCBs) has been investigated as part of a major study of the role of metabolism in the toxicity of polychlorinated biphenyl (PCB) pollutant mixtures. The DCBs were metabolized to monohydroxy and dihydrodiol metabolites and unstable metabolites of intermediate polarity. DCBs with both chloro substituents on the same ring, one or both of which were ortho substituents, were susceptible to the same regioselectivities for hydroxylation by control, phenobarbital (PB)- or β-naphthoflavone (BNF)-induced cytochromes P-450 (principally in the 4-position), with the greatest rates of hydroxylation arising with PB-induced cytochrome P-450. In contrast, DCBs with no ortho chlorosubstituents had regioselectivities for hydroxylation by control and PB-induced cytochrome P-450 which differed from that of BNF-induced cytochromes P-450; the greatest rates of hydroxylation were with BNF-induced systems. DCBs with one chloro substituent on each ring were metabolized, with the site of hydroxylation being under the electronic influence of the chloro substituent. With 4,4′-DCB, 60 per cent of the hydroxylated DCB metabolite underwent an NIH shift [G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop and S. Udenfriend, *Science* **157**, 1524 (1967)]. The BNF-induced system produced the highest rates of dihydrodiol fomation that were eliminated by an epoxide hydratase inhibitor. The results indirectly prove that arene oxides are intermediates in DCB metabolism and are possibly the source of DCB mutagenicity. The PCBs 2,4,2′4′- and 3,4,3′,4′-tetrachlorobiphenyl induced the same effects as PB and BNF respectively. Thus, PCBs differentially affect the metabolism of their individual components and are, possibly, responsible for enhancing their own toxicity by inducing enhanced rates of formation of arene oxide intermediates.

The widespread contamination of the environment by polychlorinated biphenyls (PCBs), first recognized over 10 years ago [1–3], has provided the major impetus for determination of the toxicity of these compounds. A voluminous literature has appeared documenting the toxicity of PCBs in man and animal [4–6]. However, because PCBs occur in the environment as mixtures of the 209 possible congeners and because relatively more toxic dibenzofuran impurities occur in environmental samples [7, 8], the mechanisms of PCB toxicity are not clearly understood. Thus, evaluation of toxicity of individual purified PCB congeners and the influence of individual PCBs on the toxicity of others are essential prerequisites for an understanding of environmental PCB toxicity.

Many PCBs undergo hepatic microsomal mixed function oxidase metabolism [9–11], which influences the diversity of toxic effects of the compounds. Thus, highly chlorinated PCBs are resistant to metabolism and, because of their lipophilicity, remains as persistent contaminants in man and animal. In contrast, the less chlorinated PCBs are metabolized by hepatic cytochromes P-450 to activated intermediates which may be responsible for toxicity, including mutagenic and carcinogenic effects. Thus, 4-chlorobiphenyl is metabolized to an arene oxide intermediate which produces mutagenic effects as detected with the Ames test [12]. 2,2′-Dichlorobiphenyl covalently binds to protein following microsomal metabolism *in vitro*, and induction of the microsomal mixed function oxidases enhances the binding [13]. Secondary metabolism of PCB metabolites is required to produce the binding which, if it occurs *in vivo*, could produce toxic effects. Monohydroxylated metabolites of a series of PCBs have a markedly greater hemolytic potency for human erythrocyte membranes than do the parent PCBs, which suggests that for these PCBs in this system the metabolites may be more toxic than the parent compounds [14]. In the case of 2,4,3′,4′-tetrachlorobiphenyl, a monohydroxylated metabolite (LD_{50} = 0.43 g/kg) is five times as toxic to mice as is the parent compound (LD_{50} = 2.15 g/kg) [15]. In an *in vitro* cultured cell system, the metabolites of 2,5,2′5′-tetrachlorobiphenyl, 2,5,2′,5′-tetrachloro-4-biphenylol, and 2,5,2′,5′-tetrachlorobiphenyl-3,4-oxide are more toxic than the parent compound [16].

Elucidation of the metabolic pathways of PCBs and of the roles of the numbers and positions of chloro substituents in this metabolism is necessary for the evaluation of PCB toxicity. Investigations of the metabolism of purified individual PCBs *in vitro*

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using microsomal mixed function oxidase systems provides the most facile method for gaining such metabolic information. A recent review of PCB metabolism indicates that the *in vitro* metabolism of individual PCBs has been investigated to only a limited extent [17]. The parent compound, biphenyl, is metabolized primarily to hydroxybiphenyl, and the regioselectivity of metabolism is dependent on the form of cytochrome P-450 catalyzing the metabolism. Thus, microsomes from control rats yield primarily 4-hydroxybiphenyl, whereas those from phenobarbital (PB)-induced and β-naphthoflavone (BNF)-induced rats also yield significant quantities of 3- or 2-hydroxybiphenyl respectively [18–20]. In contrast, the regioselectivity of different forms of cytochrome P-450 for biphenyl hydroxylation disappears when the 2-, 3- and 4-chlorobiphenyls are substrates, and 4′-hydroxylation is the predominant hydroxylation site [10–12, 21]. Of the dichlorobiphenyls (DCBs), 2,3-DCB yields 2,3-dichloro-4′-biphenylol [22] and 2,2′- and 2,4′-DCB yield four and two monohydroxylated compounds respectively [23]. 2,5,2′-Trichlorobiphenyl, 2,5,2′,5′-tetrachlorobiphenyl, and 2,3,4,3′,4′-pentachlorobiphenyl all yield monohydroxylated metabolites with rat liver microsomes [24], whereas with PB-induced rabbit liver microsomes 2,2′-dichloro- and 2,5,2′,5′-tetrachlorobiphenyl yield two monohydroxy metabolites each, and 2,4,6,2′,4′,6′-hexachlorobiphenyl is not metabolized but uncouples electron transport from monooxygenation [25]. With monkey liver microsomes, 2,5,2′,5′-tetrachlorobiphenyl is metabolized to activated intermediates that bind covalently to microsomal macromolecules [26]. Bacterial metabolism of PCBs apparently proceeds to phenyl ring cleaved products [27]. The *in vivo* metabolism of 4-chlorobiphenyl, dichloro-, trichloro-, tetrachloro-, pentachloro- and hexachlorobiphenyls has been investigated more extensively and reviewed recently [17].

PCBs induce hepatic microsomal cytochromes P-450 [28] and, although initially results indicated the induction of novel forms of cytochrome P-450 [29], subsequent studies involving purification of the induced cytochromes indicated that PCB mixtures induce forms of cytochrome that are equivalent to mixtures of those induced by PB and 3-methylcholanthrene [30]. The positions of chloro substituents on the PCB govern the extent and forms of cytochrome P-450 that are induced [31]. Thus, 2,4,2′,4′-tetrachlorobiphenyl induces forms of cytochrome equivalent to those induced by PB, whereas 3,4,3′,4′-tetrachlorobiphenyl induces forms equivalent to those induced by polycyclic aromatic hydrocarbons [31]. PCB mixtures can, through induction of the mixed function oxidase enzymes, alter their own constituent metabolism and thus possibly affect the toxification and detoxification of the mixture.

We are systematically investigating the metabolism of individual PCBs to determine the role of number and position of chloro substituents in the metabolism, and the manner in which metabolism of PCBs ultimately affects their toxicity. We have reported on monochlorobiphenyl metabolism [11], and the present paper describes our investigations of the metabolism of DCBs by rat liver microsomes.

MATERIALS AND METHODS

The ten DCBs, 2,4,2′-4′- and 3,4,3′,4′-tetrachlorobiphenyl, and the dichlorobiphenylols were purchased from the RFR Corp., Hope, RI, and Analabs, North Haven, CT, and were purified by preparative high-performance liquid chromatography (h.p.l.c.). NADPH was obtained from the Sigma Chemical Co., St. Louis, MO. Acetonitrile was from Waters Associates, Milford, MA. BNF and 1,2-epoxy-3,3,3-trichloropropane were purchased from the Aldrich Chemical Co., Milwaukee, WI. Sodium dihydrobis (2-methoxyethoxy) aluminum hydride was purchased from Pfaltz & Bauer, Stamford, CT. PB was obtained from Mallinckrodt, St. Louis, MO. 2,4′-Dichlorobiphenyl[U-^{14}C] (13 μCi/mmole) and 4,4′-dichlorobiphenyl[U-^{14}C] (58 μCi/mmole) were purchased from the California Bionuclear Corp., Sun Valley, CA, and purified by h.p.l.c. Water was deionized and glass distilled. All other chemicals were of the highest grades commercially available.

Preparation of microsomes

Male Wistar rats (200–250 g body weight) from a colony maintained in this Division were acclimatized for 1–2 weeks at 21° with a 12-hr light cycle. They were killed by cervical dislocation after being fasted for 12 hr, and the livers were removed immediately, weighed, minced, and washed as free of blood as possible with 0.02 M Tris–HCl/0.15 M KCl, pH 7.4. The livers were homogenized at 4° in the same buffer, using a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 14,000 *g* and the supernatant fraction was chromatographed on a Sepharose CL 2B column (20 cm × 7 cm) by a modification of published methods [32, 33]. Protein concentration of the microsomal suspension was determined by the method of Schacterle and Pollack [34] and cytochrome P-450 by the difference spectral method [35].

Rats were induced by i.p. injection of 2,4,2′-4′- or 3,4,3′,4′-tetrachlorobiphenyl in corn oil [41 mg (0.14 mmole) · (kg body weight)$^{-1}$ · day^{-1}, for 3 days], and were killed 2 days after the final administration, or with PB in saline (100 mg · kg^{-1} · day^{-1} for 2 days) or BNF in corn oil (80 mg · kg^{-1} · day^{-1} for 2 days), and were killed 2 and 1 days, respectively, after the final administration.

High-performance liquid chromatography

A Waters Associates liquid chromatograph model 244 fitted with a μBondapak C_{18} reversed phase column (4 mm i.d. × 30 cm) for analytical studies or (7.8 mm i.d. × 30 cm) for preparative studies and a 254 nm detector filter was used to purify substrates and metabolites and to analyze reaction products. Results were calculated using a Hewlett–Packard 3385A automation system (recording integrator). The methods used were previously applied to studies of the monochlorobiphenyls [11].

Microsomal metabolites were identified using a Finnigan 4000 mass spectrometer/data system. A Finnigan liquid chromatograph interface was used to introduce compounds into the mass spectrometer as described previously [11].

2-, 3- and 4-Hydroxybiphenyls were analyzed by

h.p.l.c. on a Waters μBondapak NH_2 column using a slight modification of a published method [36]. The isomers 4,4′-dichloro-3-biphenylol and 3,4′-dichloro-4-biphenylol were also separated and quantitated using this system.

In vitro *metabolism*

DCB suspensions were prepared for use as substrates in microsomal metabolism as follows: an appropriate volume of a standard solution in tetrahydrofuran was pipetted into the incubation vessel and taken to dryness under a gentle stream of air in a warm water bath. The residue, dissolved in 20 μl of acetone and 100 μl of buffered 2.5% (w/v) carboxymethyl cellulose (type 7LF, Hercules, Inc., Wilmington, DE), was added and mixed well by swirling. The acetone was removed by brief exposure to a strong stream of air in a warm water bath. This method yielded a finer suspension than was obtained by the ultrasonication method used previously [11]. Microsomal suspension (0.8 ml, 2 mg protein) was added and the mixture was allowed to stand for 10 min at room temperature with periodic mixing, to allow uptake of the DCB by the microsomes. Reaction was initiated by the addition of 0.1 ml (2 mg) of NADPH in buffer, and the reaction was incubated in a shaking water bath at 37° (100 strokes/min) for variable times. The reactions were terminated by swirling the tube for 15 sec in a boiling water bath. Diethyl ether (3 ml) was added to each tube and mixed well, and the aqueous portion was frozen out in an ice–salt bath. The extraction was repeated. The combined ether fractions evaporated, and the residue dissolved in tetrahydrofuran (200 μl).

Products from the incubation were quantitated and identified via h.p.l.c. and h.p.l.c.–mass spectrometry as described previously [11]. Dechlorination with sodium dihydrobis (2-methoxyethoxy) aluminum hydride and identification of resultant hydroxybiphenyls were performed as described previously [11, 37].

Binding to microsomal cytochrome P-450

The affinities of the DCBs for control and PB-, BNF-, 2,4,2′,4′- or 3,4,3′,4′-tetrachlorobiphenyl-induced cytochromes P-450 were determined using the difference spectral method over the range 350–500 nm [38]. Details have been reported previously [11].

Partition coefficients

The partition coefficients of the DCBs between aqueous buffer and microsomal phospholipid were determined as follows. To hepatic microsomes from control rats (2 mg protein/ml, 2 ml) were added 10–20 μg of unlabeled and 5–10 × 10^6 dpm of labeled 2,2′-, 2,4′- or 4,4′-dichlorobiphenyl[U-^{14}C] in 25 μl acetone. The suspensions were mixed well, allowed to stand at room temperature for 15 min, and centrifuged to sediment undissolved DCB. Part of the supernatant suspension (0.8 ml) was removed, and the ^{14}C-content was determined by scintillation counting. Carboxymethyl cellulose (2.5%) was added to the remainder which was centrifuged to sediment the microsomes. The supernatant fraction (0.96 ml) was decanted for ^{14}C-determination. Calculations of the partition coefficients between aqueous buffer and microsomal phospholipid were performed as described previously [11].

RESULTS

Preliminary studies on all aspects of this investigation were performed with microsomes from control and PB-, BNF-, 2,4,2′,4′- or 3,4,3′,4′-tetrachlorobiphenyl-induced rats. Results with PB and 2,4,2′,4′-tetrachlorobiphenyl induction were essentially identical within experimental error, as were results with BNF and 3,4,3′,4′-tetrachlorobiphenyl induction. All further studies were therefore performed with PB- or BNF-induced systems, and only results with these inducers will be presented. Results with these systems are probably applicable to the corresponding tetrachlorobiphenyl-induced systems.

DCB binding to microsomal cytochrome P-450

The difference spectra arising from the interactions of three representatives DCBs (2,2′-, 3,3′- and 4,4′-DCB) with control and PB- or BNF-induced forms of microsomal cytochrome P-450 are shown in Figs. 1–3. All ten DCBs yielded type I difference spectra with control and induced microsomal cytochrome P-450, with absorbance peaks at approximately 390 nm and troughs at approximately 420 nm. The extent of absorbance difference between peak and trough is an indication of the binding affinity of the DCB for the microsomal cytochrome P-450. For 2,2′-DCB (Fig. 1), BNF-induced cytochromes P-450

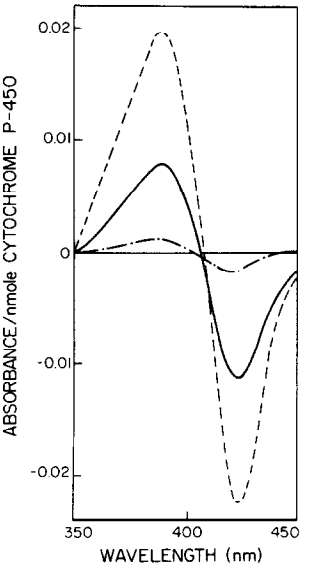


Fig. 1. Difference spectra of 2,2′-dichlorobiphenyl with hepatic microsomes from control rats (0.88 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (—), PB-induced rats (2.26 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (——), and BNF-induced rats (1.64 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (— · —). 2,2′-Dichlorobiphenyl was added as a suspension in carboxymethyl cellulose (50 μM). An equivalent volume of carboxymethyl cellulose was added to the reference cuvette. Spectra were recorded at 25° in 0.02 M Tris–HCl buffer, pH 7.4.

bound less than controls, whereas PB-induced cytochromes bound to a markedly greater extent. 4,4'-DCB had the opposite specificity, exhibiting the greatest affinity for BNF-induced cytochromes P-450, whereas PB-induced cytochromes P-450 bound only slightly more than control cytochromes (Fig. 2). 3,3'-DCB bound similarly to 4,4'-DCB but to a greater extent with all three types of microsomal cytochromes P-450 (Fig. 3).

Partition coefficients of DCBs

The partition coefficients for the microsomal suspension–aqueous buffer system (N = 3) were: for 2,2'-DCB, $1.62 \pm 0.23 \times 10^4$; for 2,4'-DCB, $1.98 \pm 0.50 \times 10^4$; and for 4,4'-DCB, $1.31 \pm 0.28 \times 10^4$. There is no significant difference ($P < 0.05$) between these values, indicating that differences in metabolism did not arise from differing solubilities in the microsomes.

Microsomal metabolism

Preliminary metabolic studies were performed with ^{14}C-labeled, 2,4'-DCB and microsomes from BNF-induced rats to determine which peaks on the resultant chromatogram arose from the substrate. In Fig. 4 the ^{14}C-counts corresponding to the chromatographic peaks are displayed. Fractions were collected at 30-sec intervals for the first 8 min, and at 1-min intervals from 8 through 16 min; an additional fraction was collected from 16 min to the start of the substrate peak. All three major peaks contained ^{14}C, but the peak eluting at approximately 12 min, which represents a monohydroxylated DCB, contained the least. The relative heights of the absorbance peaks are similar to those of the ^{14}C-counts, indicating that absorbance at 254 nm provided a good estimate of the relative amounts of the metabolites.

Since not all of the hydroxylated DCBs were available, it was not possible to determine the absolute rates of metabolism of the DCBs. However, from the relative detector responses at 254 nm of the DCBs and the commercially available dichlorobiphenylols (Table 1), a number of relationships can be derived for predicting the responses of metabolites for which standards are not available: (1) DCBs with ortho chloro substituents have diminished responses, and with two ortho chloro substituents the response is further diminished; (2) hydroxylation

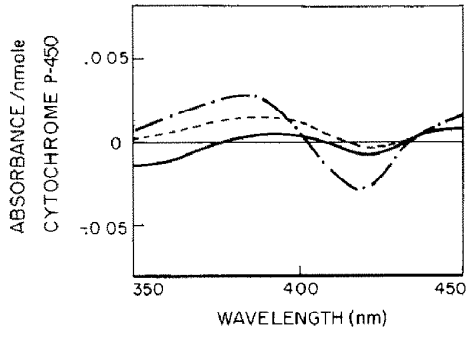


Fig. 2. Difference spectra of 4,4'-dichlorobiphenyl with hepatic microsomes from control rats (1.03 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (—), PB-induced rats (2.27 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (——), and BNF-induced rats (1.64 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (— · —). 4,4'-Dichlorobiphenyl was added as a suspension in carboxymethyl cellulose (50 μM). Other conditions were as described in Fig. 1.

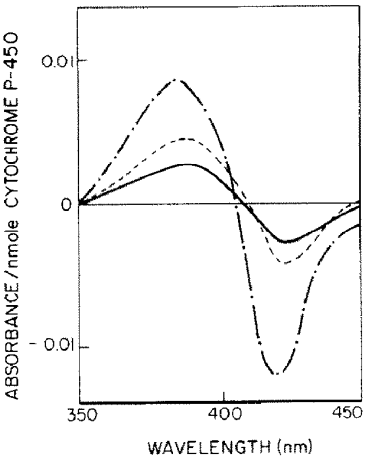


Fig. 3. Difference spectra of 3,3'-dichlorobiphenyl with hepatic microsomes from control rats (0.88 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (—), PB-induced rats (2.27 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (——), and BNF-induced rats (0.94 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (— · —). 3-3'-Dichlorobiphenyl was added as a suspension in carboxymethyl cellulose (50 μM). Other conditions were as described in Fig. 1.

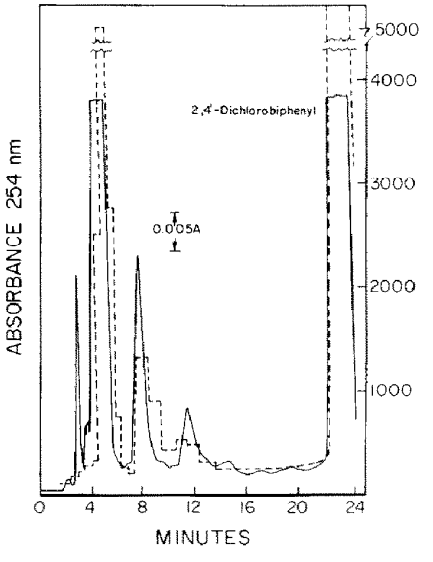


Fig. 4. High-performance liquid chromatogram of the products of 2,4'-dichlorobiphenyl[U-^{14}C] catalyzed by hepatic microsomes (2 mg/ml) from BNF-induced rats. Key: (—) absorbance at 254 nm continuously recorded; (– – –) cpm of collected fractions. Reactions were performed at 37° for 30 min with rapid shaking.

Table 1. High-performance liquid chromatographic u.v. spectral responses for dichlorobiphenyls and dichlorobiphenylols at 254 nm

Compound	Relative response (relative to 4-chlorobiphenyl = 1.00)
4-Chlorobiphenyl	1.00
3,4-Dichlorobiphenyl	1.19
4,4′-Dichlorobiphenyl	0.99
3,3′-Dichlorobiphenyl	0.81
3,5-Dichlorobiphenyl	0.61
2,3-Dichlorobiphenyl	0.59
2,4′-Dichlorobiphenyl	0.54
2,5-Dichlorobiphenyl	0.51
2,4-Dichlorobiphenyl	0.31
2,6-Dichlorobiphenyl	0.06
2,2′-Dichlorobiphenyl	0.04
2′,5′-Dichloro-4-biphenylol	0.38
2′,5′-Dichloro-3-biphenylol	0.29
2′,5′-Dichloro-2-biphenylol	0.10
3,4′-Dichloro-4-biphenylol	0.67
3,5-Dichloro-4-biphenylol	0.56
3,5-Dichloro-2-biphenylol	0.26
4,4′-Dichloro-3-biphenylol	0.58

of DCBs diminishes the detector response; and (3) hydroxy groups diminish DCB detector responses in the order ortho hydroxy > meta hydroxy > para hydroxy.

The h.p.l.c. metabolite patterns, catalyzed by microsomes from control, and PB- or BNF-induced rats, of DCBs with both chloro substituents on the same phenyl ring (Fig. 5), or with one chloro substituent on each phenyl ring (Fig. 6), are presented. All DCBs yielded, to a greater or lesser extent, a

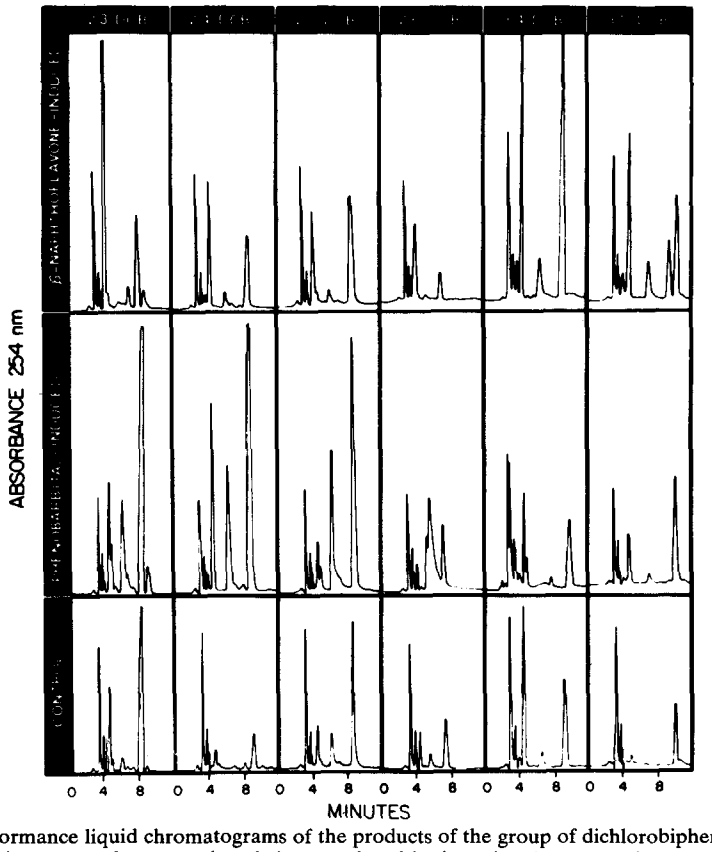


Fig. 5. High-performance liquid chromatograms of the products of the group of dichlorobiphenyls with both chloro substituents on the same phenyl ring, catalyzed by hepatic microsomes from control rats (0.85 nmole cytochrome P-450/mg protein, 2 mg protein/ml), PB-induced rats (2.99 nmoles cytochrome P-450/mg protein, 2 mg protein/ml), and BNF-induced rats (1.41 nmoles cytochrome P-450/mg protein, 2 mg protein/ml). All chromatograms were terminated prior to the elution of unreacted substrates. Reactions were performed at 37° for 10 min with rapid shaking.

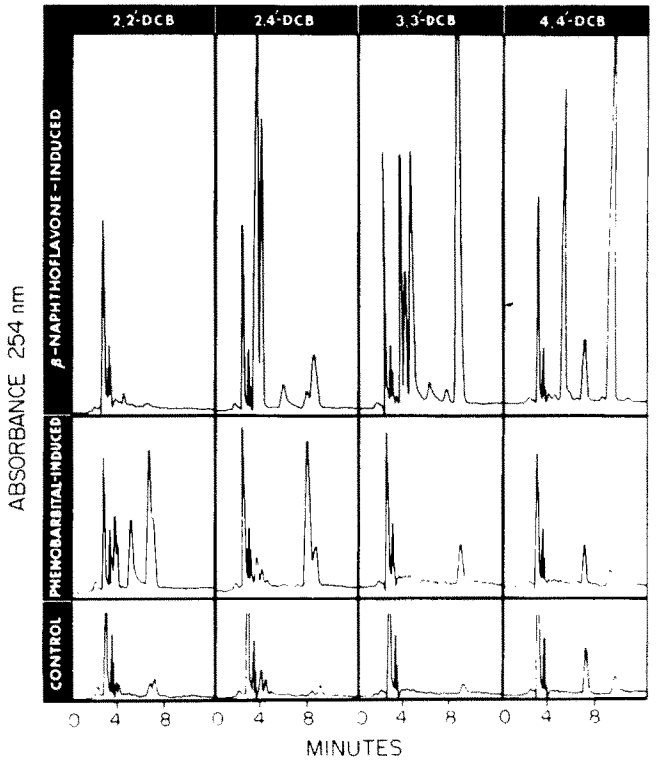


Fig. 6. High-performance liquid chromatograms of the products of the group of dichlorobiphenyls with one chloro substituent on each phenyl ring, catalyzed by hepatic microsomes from control rats (0.85 nmole cytochrome P-450/mg protein, 2 mg protein/ml), PB-induced rats (2.99 nmoles cytochrome P-450/mg protein, 2 mg protein/ml), and BNF-induced rats (1.41 nmoles cytochrome P-450/mg protein, 2 mg protein/ml). All chromatograms were terminated prior to elution of the substrates. Reactions were performed at 37° for 10 min with rapid shaking.

metabolite with retention time between 8 and 11 min (corresponding to the retention times for the monohydroxylated DCB standards), a more polar metabolite at a retention time of approximately 6 to 7.5 min, and a further even more polar metabolite at approximately 4 to 5 min. By analogy with the results with ^{14}C-labeled 2,4'-DCB, and the fact that none of the peaks were present when DCBs were eliminated from the incubation mixtures, it was concluded that these peaks all represented metabolites of the DCBs.

In a series of incubations of the DCBs with microsomes from BNF-induced rats, the uncompetitive epoxide hydratase inhibitor, 1,2-epoxy-3,3,3-trichloropropane [39], was added at a concentration equivalent to the DCB concentration (0.5 mM). The effect of the inhibitor on the metabolite pattern of 2,4-DCB is shown in Fig. 7. For most DCBs, the peaks eluting at approximately 4–5 min were eliminated, and peaks eluting in the region corresponding to that of monohydroxylated DCBs were increased. This suggests that peaks eluting at approximately 4 min represent dihydrodiols, which arise from epoxide hydratase catalyzed hydration of intermediate epoxide metabolites. Inhibition of this hydration would favor the alternative pathway of phenol formation.

Descriptions of the metabolism of each individual DCB follow. In those cases where dechlorination of a monohydroxy DCB metabolite results in ambiguity we have assumed that the most probable structure is that with the hydroxyl substitutent on the unchlorinated phenyl ring, based on results with the monochlorobiphenyls [11]. Rates of monohydroxy DCB formation were linear with time for 10 min with 2,5-dichlorobiphenyl as substrate, and we have assumed that the rates for all substrates are also linear for the same period.

2,3-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (8.74 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. This metabolite yielded 4-hydroxybiphenyl on dechlorination and is thus probably 2,3-dichloro-4'-biphenylol. A minor monohydroxylated metabolite (9.55 min) also yielded 4-hydroxybiphenyl and is probably 2,3-dichloro-4-biphenylol. A more polar metabolite (6.50 min) was produced with all three microsomal preparations but most prominently with PB-induced systems. The most polar metabolite (4.94 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system resulted in nearly complete elimination of the 4.94 min peak and a large increase in the amount of the major monohydroxy metabolite.

2,4-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (9.85 min) was

Table 2. Relative rates of formation of the monohydroxy metabolites of the dichlorobiphenyls catalyzed by hepatic microsomes from control, phenobarbital-induced or β-naphthoflavone-induced rats*

Dichlorobiphenyl		Relative rates of monohydroxy metabolite formation		
		Control	PB-induced	BNF-induced
2,3-	/nmole P-450	1.0	0.8	0.4
	/mg protein	1.0	2.7	0.6
2,4-	/nmole P-450	1.0	2.8	1.2
	/mg protein	1.0	9.9	2.0
2,5-	/nmole P-450	1.0	0.5	0.6
	/mg protein	1.0	1.8	1.1
2,6-	/nmole P-450	1.0	0.5	0.4
	/mg protein	1.0	1.8	0.6
3,4-	/nmole P-450	1.0	0.2	1.8†
	/mg protein	1.0	0.8	3.0
3,5-	/nmole P-450	1.0	0.3	0.8‡
	/mg protein	1.0	1.2	1.4
2,2′-	/nmole P-450	NR§	NR	NR
	/mg protein			
2,4′-	/nmole P-450	1.0, 1.0	8.7, 1.3	2.4, 4.3
	/mg protein	1.0, 1.0	30.5, 4.7	4.0, 7.1
3,3′-	/nmole P-450	1.0	1.0	29.7†
	/mg protein	1.0	3.6	49.5
4,4′-	/nmole P-450	1.0‖	0.2‖	10.2‖
	/mg protein	1.0	0.7	17.0

* All results are relative to control microsomes arbitrarily set at 1.0 and represent ratios of integrated peak areas. Data are calculated from the means of duplicate experiments which varied by a maximum of 5 per cent.

† Metabolite differs from that produced with the control and PB-induced systems.

‡ A second major monohydroxylated metabolite was also produced by this system.

§ Metabolites not resolved by h.p.l.c.

‖ Mixture of metabolites.

Table 3. Relative rates of formation of the dihydrodiol metabolites of the dichlorobiphenyls catalyzed by hepatic microsomes from control, phenobarbital-induced or β-naphthoflavone-induced rats*

Dichlorobiphenyl		Relative rates of dihydrodiol metabolite formation		
		Control	PB-induced	BNF-induced
2,3-	/nmole P-450	1.0	0.4	2.3
	/mg protein	1.0	1.3	4.3
2,4-	/nmole P-450	1.0	2.0	3.1
	/mg protein	1.0	7.3	5.2
2,5-	/nmole P-450	1.0	0.4	1.5
	/mg protein	1.0	1.0	2.2
2,6-	/nmole P-450	1.0	0.2	1.8
	/mg protein	1.0	0.6	3.1
3,4-	/nmole P-450	1.0	0.3	1.3
	/mg protein	1.0	0.6	2.1
3,5-	/nmole P-450	1.0	0.7	4.3
	/mg protein	1.0	2.0	6.1
2,2′-	/nmole P-450	NDM†	NDM	NDM
	/mg protein			
2,4′-	/nmole P-450	1.0, 1.0	0.3, 0.3	8.1, 8.3
	/mg protein	1.0, 1.0	1.1, 1.1	13.4, 13.8
3,3′-	/nmole P-450			>100
	/mg protein			>100
4,4′-	/nmole P-450			>25
	/mg protein			>25

* All results are relative to control microsomes arbitrarily set at 1.0 and represent ratios of integrated peak areas. Data are calculated from the means of duplicate experiments which varied by a maximum of 5 per cent.

† No detectable metabolite.

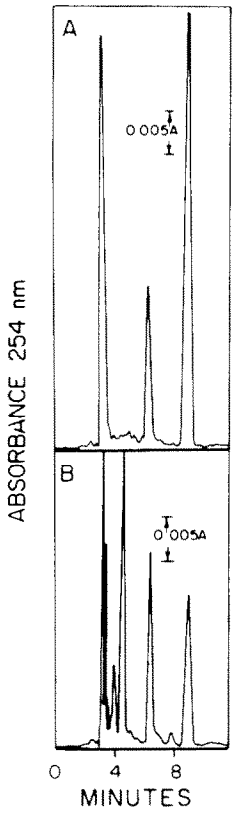


Fig. 7. High-performance liquid chromatograms of the products of 2,4-dichlorobiphenyl catalyzed by hepatic microsomes (2 mg/ml) from BNF-induced rats with (A) and without (B) 1,2-epoxy-3,3,3-trichloropropane (0.5 mM) added. Reactions were performed at 37° for 30 min with rapid shaking.

produced by microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. This metabolite yielded 4-hydroxybiphenyl on dechlorination and is thus 2,4-dichloro-4′-biphenylol. A minor monohydroxylated metabolite (8.80 min) yielded 3-hydroxybiphenyl. A more polar metabolite (7.09 min) was produced primarily by the PB-induced system. The most polar metabolite (5.25 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. The mass spectrum of this compound had a parent ion at *m/e* 256 consistent with a dihydrodiol of the DCB. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system resulted in the elimination of the 5.25 min peak and a large increase in the major monohydroxylated metabolite.

2,5-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (9.39 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. Dechlorination of this metabolite yielded 4-hydroxybiphenyl and it is thus probably 2,5-dichloro-4′-biphenylol. A minor metabolite visible as a shoulder in the PB-induced metabolite pattern yielded 3-hydroxybiphenyl on dechlorination. A more polar metabolite (6.80 min) was produced primarily by the PB-induced system. The most polar metabolite (5.05 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system resulted in the elimination of the 5.05 min peak and a large increase in the major monohydroxylated metabolite.

2,6-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (7.93 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. This metabolite peak was resolved into a major and minor component, both of which produced 4-hydroxybiphenyl on dechlorination. The major metabolite is thus probably 2,6-dichloro-4′-biphenylol and the minor metabolite 2,6-dichloro-4-biphenylol. Multiple peaks were obtained particularly with the PB-induced system in the more polar range of 6–7 min. The most polar metabolite (4.82 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system resulted in the elimination of the 4.82 min peak and an increase in the major monohydroxy metabolite.

3,4-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (10.15 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. From the control and PB-induced systems this metabolite yielded 4-hydroxybiphenyl on dechlorination and is thus 3,4-dichloro-4′-biphenylol. From the BNF-induced system 3-hydroxybiphenyl was the dechlorinated product and the metabolite is thus 3,4-dichloro-3′-biphenylol. A more polar metabolite (7.43 min) was principally produced by the BNF-induced system. The most polar metabolite (5.32 min) was produced by microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced system resulted in the elimination of the 5.32 min peak and a large increase in the major monohydroxy metabolite.

3,5-*Dichlorobiphenyl (Fig. 5).* The major monohydroxylated metabolite (*m/e* 238) (11.06 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. With the BNF-induced system, another major monohydroxy metabolite (*m/e* 238) was found (10.26 min), while only a trace of this compound was produced with the PB-induced system. The 11.06 min metabolite yielded 4-hydroxybiphenyl on dechlorination and is thus probably 3,5-dichloro-4′-biphenylol, while the other product arising in the BNF-induced system (10.26 min) yielded 3-hydroxybiphenyl and is thus 3,5-dichloro-3′-biphenylol. A more polar metabolite (7.80 min) was produced in the PB-induced, and more markedly, in the BNF-induced system. The most polar metabolite (5.52 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. The mass spectrum of this metabolite was consistent with that of a dihydrodiol of a DCB (*m/e* 256). Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing

system resulted in elimination of the 5.52 min peak and an increase in both monohydroxy DCB metabolite peaks, with the 3,5-dichloro-3′-biphenylol metabolite increasing by the greatest extent.

2,2′-*Dichlorobiphenyl (Fig. 6).* Two major monohydroxylated metabolites (*m/e* 238) (7.58 and 7.94 min) were produced with microsomes from control and PB-induced rats and in trace quantities with BNF-induced rats. PB induction markedly enhanced rates of metabolite formation, but the products were not clearly resolved by h.p.l.c. Dechlorination of the two metabolites yielded 3-hydroxybiphenyl, and the metabolites are thus 2,2′-dichloro-3-biphenylol and 2,2′-dichloro-5-biphenylol. Dechlorination of the trace metabolite from the BNF-induced system appeared to yield 4-hydroxybiphenyl primarily. A more polar metabolite (6.02 min) was produced with the PB-induced system only. No significant quantities of the most polar metabolite were detected in the 4–5 min chromatographic region.

2,4′-*Dichlorobiphenyl (Fig. 6).* Two major monohydroxylated metabolites (*m/e* 238) (9.24 and 9.88 min) were produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. Dechlorination of the 9.24 min metabolite yielded a 4-hydroxybiphenyl and this metabolite is thus 2,4′-dichloro-4-biphenylol; the 9.88 min metabolite yielded 3-hydroxybiphenyl, and this metabolite could thus be 2,4′-dichloro-3′-biphenylol, 2,4′-dichloro-3-biphenylol, or 2,4′-dichloro-5-biphenylol. A more polar metabolite (7.16 min) was produced only with the BNF-induced system. The most polar metabolites (4.88 and 5.29 min) were produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 3. Both metabolites yielded mass spectra indicative of dihydrodiols of DCBs (*m/e* 256). Treatment of the isolated metabolites with acid followed by chromatographic isolation yielded compounds with mass spectra indicative of monohydroxy DCBs (*m/e* 238). Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system resulted in elimination of both polar metabolites and the appearance of two compounds with retentions times similar to those of the monohydroxy DCBs. These compounds were not the same as the monohydroxy DCBs produced in the absence of the epoxide hydratase inhibitor.

3,3′-*dichlorobiphenyl (Fig. 6).* The major monohydroxylated metabolite (*m/e* 238) (10.07 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. Dechlorination of the metabolite produced in the BNF-induced system, however, yielded 3-hydroxybiphenyl and is thus 3,3′-dichloro-5-biphenylol, whereas dechlorination of the control and PB-induced system metabolites yielded 4-hydroxybiphenyl and the metabolite is thus 3,3′-dichloro-4-biphenylol. A more polar metabolite (7.52 min) was produced only with the BNF-induced system. Only trace quantities of the most polar metabolites were produced with control and PB-induced systems, but with the BNF-induced system three metabolites were produced (5.12, 5.48 and 6.00 min). Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced system resulted in elimination of the 5.12 and 6.00 min peaks, along with large increases in the 5.48 min peak and in the major monohydroxy metabolite.

4,4′-*Dichlorobiphenyl (Fig. 6).* The major peak representing monohydroxylated metabolite (*m/e* 238) (10.18 min) was produced with microsomes from control, PB-induced and BNF-induced rats in ratios provided in Table 2. Dechlorination of the components of the monohydroxylated metabolite peak yielded 3- and 4-hydroxybiphenyl. The two metabolites must thus be 4,4′-dichloro-3-biphenylol and 3,4′-dichloro-4-biphenylol, which are not resolved on the C_{18} h.p.l.c. column, but are readily resolved on the NH_2 column with retention times of 7.39 and 6.64 min respectively. The metabolites were identified and quantitated against standards using the NH_2 column—60 per cent of the total monohydroxylated metabolite was 3,4′-dichloro-4-biphenylol, and 40 per cent was 4,4′-dichloro-3-biphenylol. Thus, of the metabolites retaining the chloro substituent, 60 per cent undergo the NIH shift. A more polar metabolite (7.82 min) was produced with control, BP-induced and BNF-induced systems in approximately equivalent concentrations. The most polar metabolite (5.93 min) was virtually absent in control and PB-induced systems, but was a major metabolite in the BNF-induced systems. Addition of 1,2-epoxy-3,3,3-trichloropropane to the BNF-induced metabolizing system did not produce any change in the 5.93 min peak.

The metabolites represented by h.p.l.c. peaks at 6 to 7.5 min are unstable, and all attempts to isolate purified metabolites for mass spectrometry appeared to yield mixtures. The yields of these metabolites were unaffected by addition of 1,2-epoxy-3,3,3-trichloropropane to the microsomal reaction mixtures, which implies that arene oxides are not intermediates on the pathways of formation of these metabolites. Time course studies of the formation of these polar metabolites showed a lag of several minutes compared to those of monohydroxy metabolites, possibly indicating that these are secondary metabolites. Our subsequent studies (in preparation) on the microsomal metabolism of hydroxy DCBs support the suggestion that these polar metabolites are secondary metabolites.

DISCUSSION

The differing regioselectivities of the various forms of microsomal cytochromes P-450 for biphenyl metabolic hydroxylation [18–20] described in the introduction are undetectable when 2-, 3- or 4-chlorobiphenyls are used as substrates [11, 21]. In the present study, however, the various forms of cytochromes P-450 present in microsomes from PB- and BNF-induced rats again exhibited major differences in regioselectivities for the metabolism of some of the DCBs. Thus, with 3,5-, 3,4- and 3,3′-dichlorobiphenyls, microsomes from control and PB-induced rats yielded metabolites primarily hydroxylated in the 4′-position, whereas from BNF-induced rats major hydroxylation also occurred in the 3′-position. In contrast, with 2,3-, 2,4-, 2,5- and 2,6-DCB no differences in regioselectivities were detected. With 2,2′-DCB a possible difference in regioselectivities

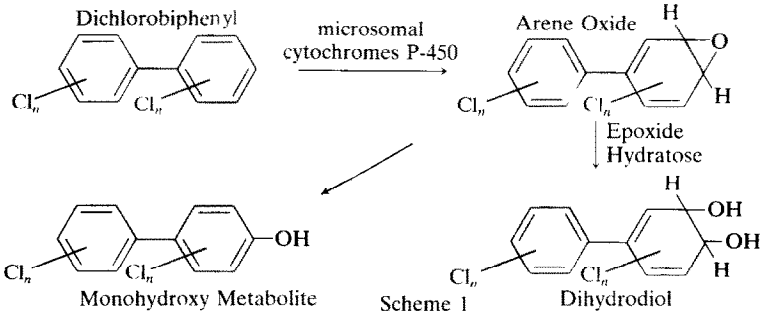
was detected, but metabolism catalyzed by the BNF-induced system was very low, and this result was uncertain. The metabolism of 2,4′-DCB also exhibited differences in regioselectivities, with microsomes from PB-induced rats yielding primarily 4-hydroxylation and from BNF-induced rats yielding primarily 3-hydroxylation. Thus, the group of DCBs with both chloro substituents on the same ring undergo differing regioselectivities for metabolism with different forms of cytochrome P-450 only when no ortho chloro substituent is present. When at least one ortho substituent is present there is no regioselectivity difference of metabolism by differently induced cytochromes. Since ortho chloro substituents are capable of preventing biphenyls from assuming planar configurations [40], the effect of such chloro substituents on regioselectivities of hydroxylation must be due to the differences in planarity between DCBs with and without ortho chloro substituents. In those DCBs with one chloro substituent on each ring the situation with respect to regioselectivity is more complex, presumably because electronic effects of the substitutents now play a role in the site of hydroxylation.

It is generally accepted that, with only very few exceptions, the extent of absorbance differences in type I difference spectra is an indication of the affinity of the microsomal cytochromes P-450 for the substrate [38]. Thus, the greater the absorbance difference the greater the affinity of the cytochrome P-450 for the substrate [38]. The results shown in Figs. 1–3 thus indicate that the affinity of microsomal cytochromes P-450 for 4,4′-DCB is relatively low, with 3,3′-DCB having an intermediate affinity and 2,2′-DCB having the greatest affinity. In general, those DCBs with at least one ortho chloro substituent have the greatest affinity for PB-induced cytochromes P-450, whereas DCBs with no ortho chloro substituent have the greatest affinity for BNF-induced cytochromes P-450. The results of the binding studies indicate that an unoccupied 4-position facilitates binding to cytochromes P-450.

The relative rates of formation of monohydroxy DCBs as a function of inducing agent are consistent with results of the binding studies. Thus, DCBs with at least one ortho chloro substituent are metabolized to monohydroxy products to a greater extent by microsomes from PB-induced rats than from BNF-induced rats, while DCBs without ortho chloro substituents are preferentially metabolized by microsomal cytochromes P-450 from BNF-induced rats. Particularly with DCBs having both chloro substituents on the same phenyl ring a planar configuration favors monohydroxylation by BNF-induced systems, and this configuration also favors a differentiation in the regioselectivities of BNF- and PB-induced systems.

Although the absence of standards of all monohydroxylated metabolites prevents an accurate assessment of the relative rates of metabolism of the various DCBs, estimates can be made based on the detector responses of the substrates. With microsomes from control rats the DCBs with both chloro substituents on one ring are more rapidly metabolized than the DCBs with one chloro substituent on each ring. The partition coefficients of the DCBs between microsomes and aqueous buffer are not significantly different, which indicates that solubility differences are not responsible for differences in the rates of metabolism.

The formation of dihydrodiols from the substrate DCBs and the inhibition of formation by the epoxide hydrase inhibitor 1,2-epoxy-3,3,3-trichloropropane is indirect proof of the formation of intermediate arene oxides. Recently direct evidence for the formation of an arene oxide from 2,5,2′,5′-tetrachlorobiphenyl has been reported [41]. Further support for arene oxide intermediates is provided by the NIH shift [42] observed and quantitated with 4,4′-DCB. Previously, an NIH shift with deuterium-labeled 4-chlorobiphenyl was reported following *in vitro* microsomal metabolism [10]. An NIH shift was also observed following 4,4-dihalobiphenyl metabolism *in vivo* [43].The greatest rates of dihydrodiol formation were with the BNF-induced system, but no relationship of rates with chloro substituent positions is discernible. The enhanced rates of dihydrodiol formation with BNF-induced systems could be a consequence of (1) the induction of epoxide hydratase by BNF, (2) enhanced rates of epoxide formation from the parent DCBs by the BNF-induced system, or (3) the greater proximity of cytochromes P-450 and epoxide hydratase to each other in the microsomal membrane of BNF-induced rats. Although we could find no published reports on the inducibility of epoxide hydratase by BNF, the inducibility of the enzyme by 3-methylcholanthrene, a mixed function oxidase inducer with very similar properties to BNF, has been investigated extensively. Although it has been reported that epoxide hydratase and the rate-limiting component of the mixed function oxidases are under common biosynthetic control [44], and that 3-methylcholanthrene does induce enhanced epoxide hydratase activity with a diverse group of arene and alkene oxides (average 1.5-fold increase) [45], the preponderance of data clearly indicates that there is no induction [46–48]. Since monohydroxylation and dihydrodiol formation are alternative products of the intermediate epoxide (Scheme 1), enhanced rates of for-



Scheme 1

mation of one alternative as a consequence of BNF-induced increases in the common intermediate should also result in increases in the other alternative. Although this is in some cases true, e.g. with 3,4-DCB both monohydroxy and dihydrodiol metabolites are increased with BNF induction, in the majority of cases, and particularly with an ortho chloro substituent where the dihydrodiol is increased by BNF induction, the monohydroxy metabolite is decreased. It thus appears possible that BNF facilitates dihydrodiol formation by increasing the proximity of coupling of the cytochrome with the hydratase. This possibility has been invoked previously to explain the metabolism of naphthalene in a reconstituted partially purified mixed function oxidase system [49].

4-Chlorobiphenyl has been demonstrated to be a mutagen in the Ames test through hepatic microsomal mixed function oxidase catalyzed metabolism to an epoxide intermediate [50]. The DCBs would be expected to be similarly mutagenic, and in view of the relatively greater efficiency of the epoxide hydratase in the BNF-induced system, the greatest mutagenicity should arise from metabolism catalyzed by the PB-induced system. Since PCBs such as 2,4,2′,4′-tetrachlorobiphenyl induce cytochrome P-450 which catalyzes DCB metabolism in a virtually identical manner to that of PB-induced cytochromes, it is possible that PCBs are capable of facilitating their own mutagenicity and thus carcinogenicity by inductive processes. There is some evidence with 2,2′-DCB, however, that while PB induction increases the metabolically related reversible binding of metabolites to microsomal protein, the effect is primarily related to secondary metabolism, and that arene oxides are responsible for no greater than 25 per cent of the effect [13].

In conclusion, we have demonstrated that the DCBs are metabolized by hepatic microsomal cytochromes P-450 to yield monohydroxy and dihydrodiol metabolites and unstable metabolites of intermediate polarity. For DCBs with both chloro substituents on the same phenyl ring, those with planar configurations have differing regioselectivities for hydroxylation with different forms of cytochrome P-450 and greater affinity for BNF-induced cytochromes P-450, while DCBs in non-planar configurations are susceptible to hydroxylation with the same regioselectivities (4′-hydroxylation) by different forms of cytochrome P-450, and have greater affinity for PB-induced cytochromes P-450. The sites of hydroxylation of DCBs with one chloro substituent on each ring are under the electronic influence of the chloro substituents. Rates of formation of dihydrodiols are increased in the BNF-induced system.

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