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METABOLISM OF MONOCHLOROBIPHENYLS BY HEPATIC MICROSOMAL CYTOCHROME P-450*

MARGARET W. KENNEDY, NANCY K. CARPENTIER, PAUL P. DYMERSKI, STEPHEN M. ADAMS and LAURENCE S. KAMINSKY

Division of Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A.

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Abstract—*In vitro* rat hepatic microsomal metabolism of the monochlorobiphenyls (MCBs) 2-, 3- and 4-chlorobiphenyl, has been investigated as a model for the metabolism of polychlorinated biphenyl pollutants. MCB metabolism was catalyzed by cytochrome P-450, as indicated by a dependence on NADPH and O_2, inhibition by 2-diethylaminoethyl-2,2-diphenylpropylacetate (SKF 525-A), metyrapone and CO, and the formation of type I difference spectra, on the addition of MCBs to microsomes. All MCBs yielded a 4'-monohydroxy MCB as the major metabolite, as determined by mass and nuclear magnetic resonance spectroscopy, dechlorination to 4-hydroxybiphenyl, and high-pressure liquid chromatography retention times. Minor monohydroxy and dihydroxy metabolites were also produced from the MCBs. The regioselectivity of control cytochrome P-450 for metabolism of MCBs at the 4' position was not altered by preinduction of cytochrome P-450 with 2,4,2',4'-tetrachlorobiphenyl (TCB) or cytochrome P-448 with 3,4,3',4'-TCB. 2-Chlorobiphenyl was metabolized only by control and induced cytochrome P-450; 3- and 4-chlorobiphenyl were metabolized by control and by induced cytochrome P-450 and P-448. Thus, the regioselectivity of metabolism of MCBs is independent of the chlorine position or the form of the induced cytochrome involved, but the extent of metabolism of polychlorinated biphenyls (PCBs) is determined by induction of the hepatic cytochromes P-450.

Since the first report of environmental contamination by polychlorinated biphenyls (PCBs) in 1966 [1, 2], numerous studies (e.g. Ref. [3]) have established the existence of worldwide environmental pollution by these compounds. Interpretation of the extensive toxicological studies of PCBs has been hampered by the presence of highly toxic contaminants, such as dibenzofurans, in commercial PCB mixtures [4, 5], and the difficulties associated with analysis of multicomponent PCB mixtures (209 possible compounds) [6–8]. It has also been proposed that, based on relative LD_{50} values, the toxicity of certain PCBs is attributable to hydroxylated metabolites [9], and that the mutagenicity of PCBs is a consequence of their hepatic microsomal metabolism [10, 11].

Elucidation of the mechanisms and degrees of toxicity of commercial mixtures of PCBs is thus dependent on the determination of pathways of metabolism of individual PCBs. Some PCBs, particularly those with lower numbers of chlorine substituents (between one and four), are hydroxylated by mammalian liver microsomal cytochrome P-450 [12]. The most extensive investigation of such hydroxylations has been performed on the parent compound, biphenyl, which yields primarily 4-hydroxybiphenyl with control rat liver microsomes [13]. However, induction of rat cytochrome P-450 with 3-methylcholanthrene or phenobarbital leads, in addition, to the formation of significant quantities of 2-hydroxybiphenyl or 3-hydroxybiphenyl respectively [13]. *In vitro* microsomal metabolism of purified PCBs has been investigated to a limited extent only. 4-Chlorobiphenyl yields principally 4'-chloro-4-biphenylol [11], and 2,3-dichlorobiphenyl is metabolized to 2,3-dichloro-4'-biphenylol [14], while 2,2'- and 2,4'-dichlorobiphenyl are metabolized to four and to two monohydroxylated compounds respectively [15]. *In vivo* metabolism of purified PCBs has been studied more extensively and the results have been reviewed [12]. Included in the *in vivo* studies were 4-chlorobiphenyl, dichloro-, trichloro-, tetrachloro-, pentachloro- and hexachlorobiphenyls [12].

PCBs have also been demonstrated to induce hepatic microsomal cytochrome P-450 [16]. Initially, studies indicated the induction of novel forms of cytochrome P-450 [17] but subsequent investigations, following purification of the induced cytochrome P-450, suggested that a commercial mixture of PCBs (Aroclor 1254) induces forms of cytochrome P-450 which are a mixture of those induced by phenobarbital and 3-methylcholanthrene [18]. The positions of the chloro-substituents on the biphenyl nucleus govern the extent and the forms of cytochrome P-450 which are induced [19]. Thus, PCBs, through induction of the enzymes involved, play a role in determining the manner of their own subsequent metabolism.

In an effort to determine the manner in which the metabolism of PCBs ultimately affects their toxicity, we have initiated investigations into the metabolism of individual PCBs by rat liver microsomes. In the present paper we report on the metabolism of three monochlorobiphenyls (MCBs) by control and 2,4,2',4'- or 3,4,3',4'-tetrachlorobiphenyl (TCB)-induced rat liver microsomes.

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MATERIALS AND METHODS

Monochlorinated biphenyls and biphenylols were purchased from the RFR Corp., Hope, RI, and Analabs, North Haven, CT, and were further purified via high-pressure liquid chromatography (h.p.l.c.). NADPH was obtained from the Sigma Chemical Co., St. Louis, MO. High-pressure liquid chromatography grade acetonitrile was from the Fisher Chemical Co., Pittsburgh, PA. 2-Diethylaminoethyl-2.2-diphenylpropylacetate (SKF 525-A) and metyrapone were gifts from the Smith, Kline and French Laboratories, Philadelphia, PA, and the Ciba Pharmaceutical Co., Summit, NJ, respectively. Sodium dihydrobis (2-methoxyethoxy) aluminium hydride was purchased from Pfaltz & Bauer, Stamford, CT. Water was deionized and glass-distilled. All other chemicals were of the highest grades commercially available.

High-pressure liquid chromatography. A Water Associates model 244 liquid chromatograph with a μBondapak C_{18} reversed phase column (4 mm i.d. × 30 cm) was used to purify substrates and to analyze reaction products. MCBs and metabolites were detected with a Waters Associates model 440 absorbance detector at 254 nm and the results were calculated using a Hewlett Packard 3385A automation system (recording integrator). Acetonitrile–water (75:25) was the mobile phase in analytic procedures at a flow rate of 1 ml/min. The detector responses of a series of commercially available monohydroxy MCB standards were used for estimating the responses of those products for which no standards were available, permitting their approximate quantitation.

2-, 3- and 4-Chlorobiphenyl were purified by h.p.l.c. using a μBondapak C_{18} reversed phase preparative column (7.8 mm i.d. × 30 cm). A solution of the MCB in 25% acetonitrile in water (approximately 100 μg/ml) was purified in 5 mg portions. A 5-min gradient was run to 60% acetonitrile at a flow rate of 4 ml/min, and fractions corresponding to the various chromatographic peaks were collected. The acetonitrile was removed with a rotatory evaporator, and the MCB residues were taken up in diethyl ether. All MCBs were shown to be homogenous by analytical h.p.l.c. following purification. The purity was confirmed by mass spectrometry.

Purified MCBs were analyzed on a Finnigan 4000 mass spectrometer/data system equipped with a solid probe. The sample (0.5 to 1.5 μg) was temperature distilled by heating to 300° at approximately 100°/min, while mass spectra were acquired every 2 sec and stored in the computer. The presence of impurities was determined from plots of the elution of the most significant ions (MSI) temperature and by comparison of the concentration curves with the total ion current (TIC). Major differences between the MSI and TIC curves suggest the presence of contaminants in the sample.

Microsomal metabolites of the MCBs were identified by h.p.l.c–mass spectrometry. The Finnigan mass spectrometer was equipped with a Finnigan liquid chromatograph interface. The interface comprises a continuous moving belt with an infra red lamp to remove solvent, a hot wire to volatilize the sample, and a two-stage differential pumping system. Because of difficulties associated with the use of the interface with an aqueous solvent, the chromatographic system was modified to incorporate a Waters Associates μPorasil column with 0.5% methanol in methylene chloride as eluting solvent at a flow rate of 0.6 ml/min.

Hydroxybiphenyls were analyzed by h.p.l.c on a Spherisorb NH_2 column using a slight modification of a published method [20]; 2-, 3- and 4-hydroxybiphenyls were well resolved in this system.

Preparation of microsomes. Male Wistar rats (200–250 g body wt) 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* for 15 min, and the supernatant fraction was chromatographed on a Sepharose CL 2B column (20 × 7 cm) by a modification [21] of the method of Tangen *et al.* [22], using the Tris buffer as eluent. The microsomes were eluted in the void volume. Protein concentration was determined by the method of Schacterle and Pollack [23]. Cytochrome P-450 concentrations were measured using the carbon monoxide difference spectral method of Omura and Sato [24].

For studies with induced microsomes, 2,4,2′,4′-TCB or 3,4,3′,4′-TCB dissolved in corn oil was administered i.p. to rats at a dose of 0.14 mmole/kg body wt (41 mg/kg) once daily for 3 days. The rats were killed 2 days after the final administration of the PCBs.

In vitro *metabolism.* MCB suspensions were prepared for use as substrates in the microsomal system by ultrasonicating the MCB in 2.5% (w/v) aqueous buffered carboxymethyl cellulose (CMC, type 7LF, Hercules Inc., Wilmington, DE) for 2 × 5 min, using a model W140 Sonifier (Heat Systems-Ultrasonics Inc., Plainview, NY). The sonifier was equipped with a microprobe and used at a setting of 6.

The mixtures were kept in an ice–water bath during sonication [25]. Appropriate amounts of the MCB suspension were diluted to 0.20 ml by the addition of 2.5% CMC, added to the microsomal suspension (2 mg protein), and the volume brought to 0.90 ml with buffer. The mixtures were incubated for 10 min at room temperature and for 1 min at 37° to allow uptake of the MCB by the microsomes. Reaction was initiated by the addition of 0.10 ml (2 mg) of NADPH in water, and the tubes were incubated in a shaking water bath at 37° (100 strokes/min) for variable times. Reaction was terminated by the rapid addition of 3 ml diethyl ether. The reaction mixtures and ether were mixed well, and the aqueous portion was frozen out in an ice–salt bath. The ether was decanted, the aqueous portion allowed to thaw, and the extraction repeated (2 × 3 ml). The ether was evaporated off and the residue was dissolved in tetrahydrofuran (200 μl). Recovery studies of 4-chlorobiphenyl and 4-chloro-

4′-biphenylol from microsomes, using this method, indicated extraction efficiences of 90.3 per cent ± 4.7 (S.E.) and 95.2 per cent ± 1.9 (S.E.) respectively (N = 10). This method of extraction did not lead to any detectable loss of metabolites by volatilization.

Products from the incubations were quantitated and identified via h.p.l.c. and h.p.l.c–mass spectrometry as described previously.

The major monohydroxylated products and some minor products from 2- and 3-MCB were reductively dechlorinated using dihydrobis (2-methoxyethoxy) aluminum hydride [26]. The dechlorinated products were extracted with ether, purified by h.p.l.c. using a μBondapak C_{18} analytical column and identified by h.p.l.c. as described previously.

Incubations designed to provide relatively large quantities of 2- and 3-MCB metabolites for n.m.r. spectroscopy used 30 mg of substrate, the concentration of MCB in the microsomal suspension being approximately 1 mM. Incubations were performed for 1 hr and NADPH was added at 15-min intervals. The metabolites were extracted with ether after acidification of the boiled reaction mixture with HCl. The ether extract was washed with water and evaporated to dryness, and the residue was taken up in 25% acetonitrile. Highly polar components of the extract were removed using Sep-Pak C_{18} cartridges (Waters Associates). Individual products were purified using h.p.l.c. on a μBondapak C_{18} preparative column as described previously. Nuclear magnetic resonance spectra were recorded at 100 MHz using a Varian XL100 instrument with deuterochloroform as a solvent and tetramethylsilane as an internal standard.

Binding to hepatic cytochrome P-450. Affinities of the MCBs for hepatic cytochrome P-450 were determined using the difference spectral method of Schenkman [27] over the range 350–500 nm. An Aminco DW-2 spectrophotometer with a cell compartment maintained at 25° was used to determine the difference spectra. One milliliter of microsomal suspension was placed in each of two optically balanced cuvettes with 1-cm light paths. To one was added 10 μl of 2.5% CMC, and to the other an MCB suspended in 10 μl of 2.5% CMC. The contents of the cuvettes were mixed well before spectra were determined.

Partition coefficients. The partition of MCBs between an aqueous buffer and a microsomal phospholipid preparation was determined as follows: MCB (12–100 μg) in 25 μl of an acetone solution was added to 2 ml of microsomes (1–4 mg protein/ml) in a centrifuge tube. The tubes were mixed and allowed to stand for 10–15 min at room temperature, and then centrifuged for 10 min at 200 *g* to sediment undissolved MCB. This supernatant fraction (0.5 ml) was pipetted into a 10-ml volumetric flask containing 5 ml methanol, and the flask was brought to volume with methanol (Flask No. 1). CMC (2.5%, 0.3 ml) was added to the remaining microsomal suspension and the tube was centrifuged for 10 min at 200 *g*. At this gravitational force, the microsomes sediment in the presence of CMC. The supernatant fraction (0.6 ml) was pipetted into a 10-ml volumetric flask which was brought to volume with methanol (Flask No. 2). The flasks were placed in the refrigerator overnight and the clear methanolic solution was analyzed via h.p.l.c. for the content of MCB.

μg MCB in Flask No. 1 = total μg MCB present;

μg MCB in Flask No. 2 = μg MCB in aqueous solution;

Flask No. 1 − Flask No. 2 = μg MCB in microsomal solution.

Partition coefficients were calculated according to the method of Parry *et al.* [28], using the phospholipid : protein ratio for microsomes determined by Borgese *et al.* [29].

Calculation of kinetic constants. Rates of MCB metabolism were calculated in nmoles product/min/nmole cytochrome P-450; Lineweaver–Burk, $(S)/V$ vs (S), and direct linear plots were used to calculate apparent K_m and apparent V_{max} values. The direct linear plot [30] was particularly valuable in revealing the existence of the two enzyme–one substrate systems. The values presented for the kinetic constants are averages calculated using two or more of the methods, which were in good agreement generally.

Molecular orbital calculations. Extended Hückel molecular orbital calculations were performed for biphenyl and the MCBs [31–33]. Biphenyl and the 3- and 4-chlorobiphenyls were assumed to be in the planar configuration, and for 2-chlorobiphenyl the two phenyl rings were assumed to be perpendicular to one another for the purposes of the calculations. The chlorine parameters used in the calculations were: $\xi(3S) = 2.033$, V.S.I.E.(3S) = −30.0 eV; $\xi(3P) = 2.033$, V.S.I.E.(3P) = −15.0 eV; and $\xi(3d) = 2.03$, V.S.I.E.(3d) = −9.0 eV. The net atomic charges were calculated by Mulliken population analysis.

RESULTS

MCB binding to microsomes. Difference spectra arising from the interactions of 2-, 3- or 4-MCB with microsomal cytochrome P-450 from control, 3,4,3′,4′-TCB- or 2,4,2′,4′-TCB-induced microsomes are shown in Figs. 1, 2 and 3. In agreement with the results of Goldstein *et al.* [19], 2,4,2′,4′-TCB induced microsomes had a reduced-CO difference spectral maximum at 450 nm, while 3,4,3′,4′-TCB induced microsomes had a spectral maximum at 448 nm. All MCBs yielded type I binding spectra with control and induced microsomes with absorbance peaks at approximately 390 nm and troughs at approximately 420 nm. However, the extent of the absorbance differences (Δ absorbance$_{390\text{–}420\,nm}$) for the MCBs was differentially affected by the state of induction of the microsomes. 2-Chlorobiphenyl interacted to the greatest extent with 2,4,2′,4′-TCB-induced cytochrome P-450 and showed virtually no binding to 3,4,3′,4′-TCB-induced cytochrome P-448 (Fig. 1). 3-Chlorobiphenyl exhibited enhanced binding with both forms of induced cytochromes relative to controls but to a greater extent with 2,4,2′,4′-TCB-induced cytochrome P-450 (Fig. 2). With 4-chlorobiphenyl the major binding was to the 3,4,3′,4′-TCB-induced cytochrome P-448 (Fig. 3), but 2,4,2′,4′-TCB induction also produced some increase in binding relative to controls.

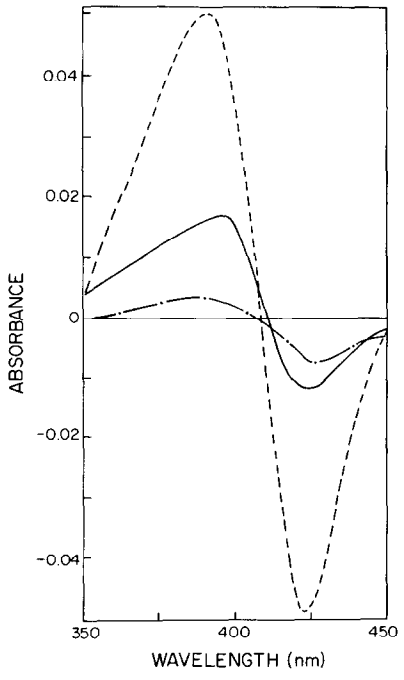

Fig. 1. Difference spectra of 2-chlorobiphenyl with liver microsomes from control rats (0.89 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (——), 2,4,2′,4′-tetrachlorobiphenyl-induced rats (1.75 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (- - -), and 3,4,3′,4′-tetrachlorobiphenyl-induced rats (2.20 nmoles cytochrome P-448/mg protein, 2 mg protein/ml) (—·—). 2-Chlorobiphenyl was added in an ultrasonicated suspension of carboxymethyl cellulose (40 μM). An equivalent volume of carboxymethyl cellulose solution was added to the reference cuvette. Spectra were recorded at 25° in 0.02 M Tris–HCl buffer, pH 7.4.

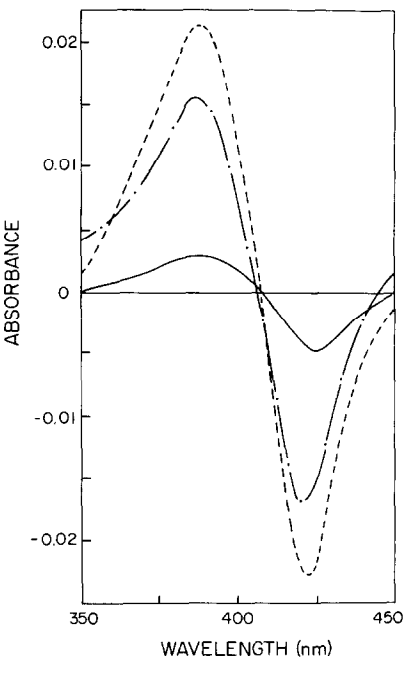

Fig. 2. Difference spectra of 3-chlorobiphenyl with liver microsomes from control rats (0.87 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (——), 2,4,2′,4′-tetrachlorobiphenyl-induced rats (1.60 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (- - -), and 3,4,3′4′-tetrachlorobiphenyl-induced rats (2.13 nmoles cytochrome P-448/mg protein, 2 mg protein/ml) (—·—). 3-Chlorobiphenyl was added in an ultrasonicated suspension of carboxymethyl cellulose (50 μM). Other conditions were as in Fig. 1.

Partition coefficients of MCBs. The partition coefficients of the MCBs for the microsomal suspension-aqueous buffer system are presented in Table 1. The partition coefficients for the MCBs are not significantly different from one another when determined at a concentration of 2 mg of microsomal protein/ml. For all three MCBs the partition coefficients decreased with increasing microsomal protein concentration.

In vitro *microsomal metabolism of MCBs.* Solutions of 4-chlorobiphenyl in methanol, ethanol, dimethylsulfoxide (DMSO), acetone, propylene glycol or acetonitrile (0.025 ml), or an ultrasonicated suspension in CMC (0.20 ml), each containing the same quantity of 4-chlorobiphenyl, were added to microsomes (0.90 ml). The final protein concentration was 2 mg/ml and the final substrate concentration, 2.65 mM. The reaction was initiated by the addition of 2 mg NADPH in *N*-tris-(hydroxymethyl)-2-aminoethane sulfonic acid (TES) buffer (0.10 ml). Metabolites were quantitated by h.p.l.c.

Ethanol and DMSO diminished the rates of product formation by 70 per cent, and acetone and methanol caused approximately 10 per cent stimulation relative to experiments with CMC as substrate vehicle. Acetonitrile and propylene glycol were

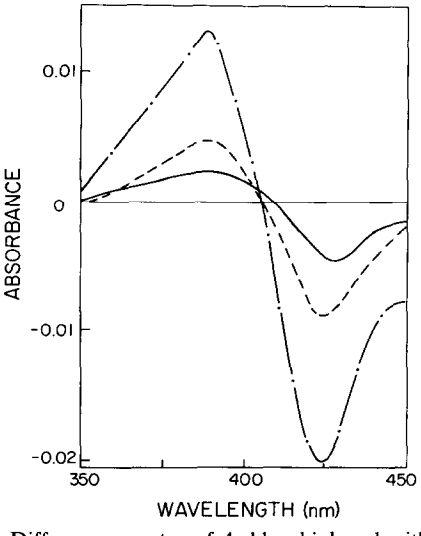

Fig. 3. Difference spectra of 4-chlorobiphenyl with liver microsomes from control rats (0.91 nmole cytochrome P-450/mg protein, 2 mg protein/ml) (——), 2,4,2′,4′-tetrachlorobiphenyl-induced rats (1.23 nmoles cytochrome P-450/mg protein, 2 mg protein/ml) (— —), and 3,4,3′,4′-tetrachlorobiphenyl-induced rats (2.28 nmoles cytochrome P-448/mg protein, 2 mg protein/ml) (—·—). 4-Chlorobiphenyl was added in an ultrasonicated carboxymethyl cellulose suspension (23 μM). Other conditions were as in Fig. 1.

Table 1. Partition coefficients of 2-, 3- and 4-chlorobiphenyl between microsomal suspensions and aqueous buffer*

MCB	Partition coefficients $\times 10^{-3}$		
	1 mg protein/ml	2 mg protein/ml	4 mg protein/ml
2-Cl	9.30 ± 1.50† (N = 4)	5.70 (N = 2)	3.00
3-Cl	9.70 ± 1.90 (N = 4)	5.80 ± 0.50 (N = 5)	4.10
4-Cl	7.50 ± 0.10 (N = 3)	5.30 ± 0.70 (N = 3)	2.80

* Microsomes were isolated from control rat liver and studied at concentrations of 1, 2 and 4 mg protein/ml.
† Values are means ± S.D.

approximately equivalent to CMC. CMC was chosen as the substrate vehicle for all subsequent studies in view of these results and of reports by Hook *et al.* [25] that a CMC suspension of biphenyl was without effect on its metabolism or on a number of phases of microsomal electron transport. Acetone has also been reported to behave as an uncompetitive inhibitor of cytochrome P-450 [25], while methanol and acetonitrile inhibit ethoxy coumarin deethylation [34]. Furthermore, in contrast to the organic solvents, addition of CMC to microsomal suspensions in concentrations equivalent to those used in this study did not produce any difference spectrum in the range of 350–500 nm.

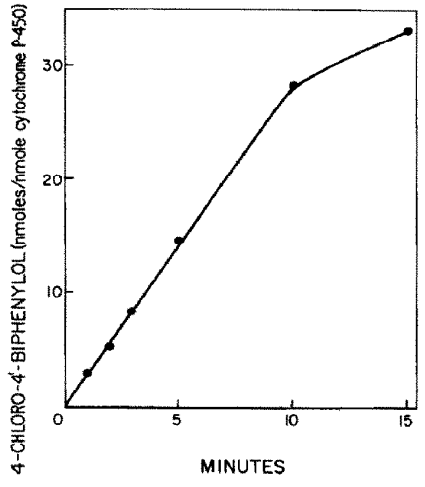

Fig. 4. Effect of time on the microsomal metabolism of 4-chlorobiphenyl to 4'-chloro-4-biphenylol. Microsomes were isolated from control rats (0.86 nmole cytochrome P-450/mg protein, 2 mg protein/ml). 4-Chlorobiphenyl was added to the microsomes in an ultrasonicated suspension of carboxymethyl cellulose (1.0 mM). Reactions were performed at 37° and pH 7.4.

A range of substrate concentrations of 0.2–2.0 mM was chosen to span the K_m value determined in a preliminary study of 4-chlorobiphenyl metabolism, and this was used for all subsequent studies. The initial rates of product formation from the microsomal metabolism of 4-chlorobiphenyl were linear for 10 min and deviated slightly from linearity thereafter (Fig. 4). To ensure linearity for all the substrates, all metabolic studies were run for 5 min.

The h.p.l.c. u.v. spectral responses at 254 nm of a series of MCBs and monohydroxy MCBs are presented in Table 2. All the MCBs exhibited a single peak after purification, and the purity was confirmed by mass spectrometry. The responses are relative to that of 4-chlorobiphenyl arbitrarily set at 1.000. These results provide a number of relationships which can be used for predicting the responses of other metabolites: (1) the response of a biphenyl with a 4-chloro substituent > 3-chloro- > 2-chloro; (2) a 2-hydroxyl substituent diminished the response of an MCB markedly and virtually independently of whether the hydroxyl and chlorine substituents were on the same or different phenyl rings (c.f. 3-chloro-2-biphenylol and 3'-chloro-2-biphenylol with 3-chlorobiphenyl); and (3) a 4-hydroxyl substituent (on

Table 2. High-pressure liquid chromatographic u.v. spectral responses for monochlorobiphenyls and monochlorobiphenylols at 254 nm

Compound	Spectral response (relative to 4-chlorobiphenyl = 1.000)
4-Chlorobiphenyl	1.000
2-Chlorobiphenyl	0.271
3-Chlorobiphenyl	0.824
2-Chloro-4-biphenylol	0.653
2-Chloro-5-biphenylol	0.333
3-Chloro-2-biphenylol	0.314
3'-Chloro-2-biphenylol	0.365
3-Chloro-4-biphenylol	0.683
4'-Chloro-4-biphenylol	0.638

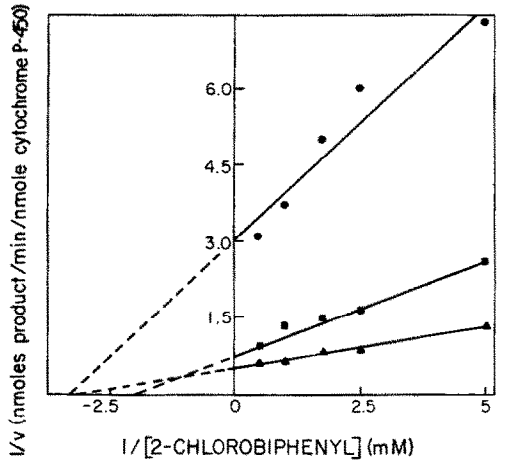

Fig. 5. Effect of 2-chlorobiphenyl concentration on its rate of microsomal metabolism. Microsomes from control (0.91 nmole cytochrome P-450/mg protein) (■), 2,4,2′,4′-tetrachlorobiphenyl-induced (1.49 nmoles cytochrome P-450/mg protein) (▲), or 3,4,3′,4′-tetrachlorobiphenyl-induced (2.03 nmoles cytochrome P-448/mg protein) (●) rat livers were incubated (2 mg protein/ml) with varying concentrations of 2-chlorobiphenyl at 37° and pH 7.4. The reciprocals of the rates of formation of the major monohydroxylated metabolite are plotted against the reciprocals of the substrate concentrations.

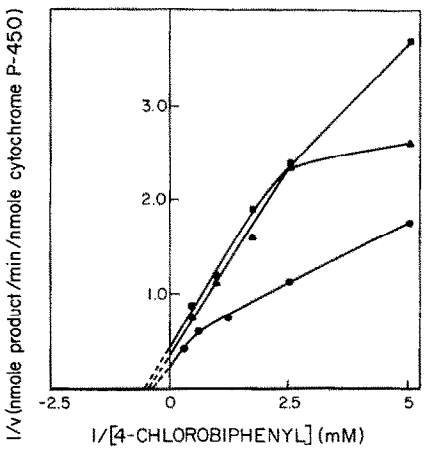

Fig. 7. Effect of 4-chlorobiphenyl concentration on its rate of microsomal metabolism. Microsomes from control (0.89 nmole cytochrome P-450/mg protein) (■), 2,4,2′,4′-tetrachlorobiphenyl-induced (1.59 nmoles cytochrome P-450) (▲), or 3,4,3′,4′-tetrachlorobiphenyl-induced (2.03 nmoles cytochrome P-448/mg protein) (●) rat livers were incubated (2 mg protein/ml) with varying concentrations of 4-chlorobiphenyl at 37° and pH 7.4. The reciprocals of the rates of formation of the major monohydroxylated metabolite are plotted against the reciprocals of the substrate concentrations.

either ring) enhanced the response of 2-chlorobiphenyl and diminished the responses of 3- and 4-chlorobiphenyl to the extent that they all became approximately equivalent (c.f. 2-chloro-4-biphenylol, 3-chloro-4-biphenylol and 4′-chloro-4-biphenylol). Similar relationships, based on relative retention times, were also apparent and will be discussed later.

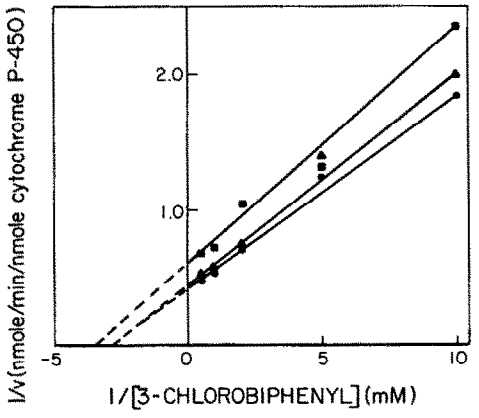

Fig. 6. Effect of 3-chlorobiphenyl concentration on its rate of microsomal metabolism. Microsomes from control (0.91 nmole cytochrome P-450/mg protein) (■), 2,4,2′,4′-tetrachlorobiphenyl-induced (1.49 nmoles cytochrome P-450/mg protein) (▲), or 3,4,3′,4′-tetrachlorobiphenyl-induced (2.03 nmoles cytochrome P-448/mg protein),(●) rat livers were incubated (2 mg protein/ml) with varying concentrations of 3-chlorobiphenyl at 37° and pH 7.4. The reciprocals of the rates of formation of the major monohydroxylated metabolite are plotted against the reciprocals of the substrate concentrations.

Figures 5–7 show the rates of formation of the major products of the metabolism of 2-, 3- and 4-chlorobiphenyl by control and 2,4,2′,4′-TCB- or 3,4,3′,4′-TCB-induced rat liver microsomes as a function of substrate concentration. Rates are based on the detector responses for the metabolites being equivalent to that of 4′-chloro-4-biphenylol (see previous discussion on detector responses). Lineweaver–Burk plots of the data for the 2- and 3-chlorobiphenyls were linear (Figs. 5 and 6) and yielded the apparent V_{max} and K_m values reported in Table 3. With 4-chlorobiphenyl, the Lineweaver–Burk plots were biphasic (Fig. 7), and this was confirmed in experiments where a wider range of substrate concentrations (0.05–2.0 mM) was used. Apparent V_{max} and K_m values for each of the phases of 4-chlorobiphenyl metabolism were calculated by the method of successive approximations [35, 36] (Table 3).

The rate of formation of the major metabolite of 2-chlorobiphenyl was increased by induction of cytochrome P-450 and decreased by induction of cytochrome P-448 (based on mg of microsomal protein). With 3-chlorobiphenyl, rates were enhanced following induction of both forms of cytochrome but to a greater extent by cytochrome P-448.

The mass spectral scans of the h.p.l.c. eluent containing the products of metabolism were limited to greater than *m/e* 100 due to background from solvent impurities. Selective ion monitoring of the chlorine clusters was performed at *m/e* 188, 189 and 190 for identification of substrates, 204, 205 and 206 for identification of monohydroxylated MCBs, and 220, 221 and 222 for identification of dihydroxylated MCBs. The natural isotope abundance ratio of 3:1

Table 3. Apparent V_{max} and K_m values for the metabolism of 2-, 3- and 4-chlorobiphenyl by control, 2,4,2′,4′-tetrachlorobiphenyl (TCB)- or 3,4,3′,4′,-tetrachlorobiphenyl-induced rat liver microsomes

Substrate	Inducer	K_m (mM)	V_{max} (nmoles product/min/ nmole cytochrome P-450)	V_{max} (nmoles product/min/ mg microsomal protein)
2-Cl	Control (N = 4)	0.62 ± 0.18*	1.23 ± 0.16	1.06 ± 0.18
	2,4,2′,4′-TCB (N = 3)	0.25 ± 0.06	1.09 ± 0.23	1.74 ± 0.36
	3,4,3′,4′-TCB (N = 3)	0.55 ± 0.28	0.26 ± 0.11	0.52 ± 0.23
3-Cl	Control (N = 4)	0.29 ± 0.07	1.51 ± 0.48	1.26 ± 0.49
	2,4,2′,4′-TCB (N = 3)	0.27 ± 0.10	1.40 ± 0.33	2.26 ± 0.34
	3,4,3′,4′-TCB (N = 3)	0.34 ± 0.03	2.02 ±0.27	4.01 ± 0.58
4-Cl	Control (N = 3)	6.2 ± 1.6, 0.17 ± 0.01	7.95 ± 1.77, 0.78 ± 0.03	7.66 ± 0.88, 0.76 ± 0.06

* Values are means ± S.D.

for $M^+:(M+2)^+$ was an additional criterion imposed on the selected ion monitoring reconstructed ion currents. The results indicated that 4-chlorobiphenyl metabolism produced a single monohydroxy MCB with trace quantities of a dihydroxy MCB, 2-chlorobiphenyl metabolism gave rise to a major and a minor monohydroxy MCB and traces of two dihydroxy MCBs, and 3-chlorobiphenyl metabolism gave rise to two monohydroxy MCBs in the ratio of 10:1 and trace quantities of two dihydroxy MCBs. Identification of dihydroxy MCBs was based only on elemental composition and molecular weight since standards were not available.

The h.p.l.c. retention time of the monohydroxylated MCB metabolite from 4-chlorobiphenyl was identical to that of the 4′-chloro-4-biphenylol standard, and could not be separated from the standard when it was added to the microsomal extract, under a variety of solvent conditions. 4′-Chloro-4-biphenylol was thus assumed to be the major metabolite of 4-chlorobiphenyl. 4-Chlorobiphenyl products from control, 2,4,2′,4′- or 3,4,3′,4′-TCB-induced microsomes all exhibited identical retention times under a variety of solvent conditions on the h.p.l.c. For 3-chlorobiphenyl the major monohydroxylated metabolite had a retention time similar to that of 3-chloro-4-biphenylol. This retention was different from that of 3-chloro-2-biphenylol and 3′-chloro-2-biphenylol, the latter two of which were indistinguishable by h.p.l.c. Dechlorination of the major monohydroxylated metabolite yielded 4-hydroxybiphenyl, while the minor metabolite yielded 3-hydroxybiphenyl. The n.m.r. spectrum of the major monohydroxy compound exhibited signals at 6.9 (d,2H) and 7.2 (d,2H) p.p.m. with coupling constants of approximately 8.0 Hz. This is typical of the pattern for an AA′BB′ system which is consistent with hydroxylation at the 4-position of the unsubstituted phenyl ring. The protons from the Cl-substituted ring gave rise to a multiplet at 7.3–7.6 p.p.m. The spectrum of the metabolite differed from that of 3-chloro-4-biphenylol. Based on all of these results the major product was 3′-chloro-4-biphenylol. The same major metabolite was obtained from 3-chlorobiphenyl using control or induced microsomes. For 2-chlorobiphenyl the retention time of the major metabolite differed from that of the 2-chloro-4-biphenylol and 2-chloro-5-biphenylol standards. Dechlorination of the major metabolite yielded 4-hydroxybiphenyl, while the minor monohydroxy metabolite yielded 3-hydroxybiphenyl. The major metabolite was thus 2-chloro-4′-biphenylol and this was confirmed by the n.m.r. spectrum. The spectrum exhibited one-half of the AA′BB′ pattern with a doublet at 6.88 p.p.m. (2H), while a multiplet at 7.26–7.40 p.p.m. accounted for the remainder of the protons. This spectrum is thus consistent with 4-hydroxylation of the unsubstituted phenyl ring. The

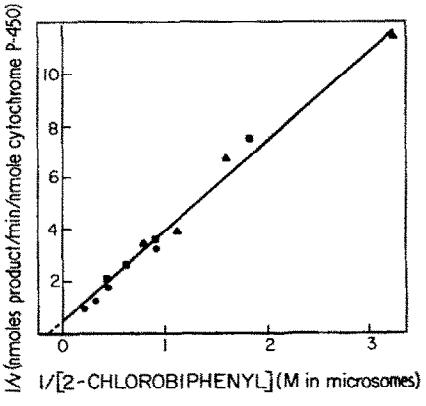

Fig. 8. Effect of microsomal protein concentration on the rates of microsomal metabolism of 2-chlorobiphenyl. Control rat liver microsomes (0.94 nmole cytochrome P-450/mg microsomal protein) were incubated at 1 mg protein/ml (●), 2 mg protein/ml (■), 3.5 mg protein/ml (▲), with varying concentrations of 2-chlorobiphenyl added in a carboxymethyl cellulose suspension. The inverse of the rate of formation of the major monohydroxylated metabolite is plotted against the inverse of the concentration of the substrate in the microsomes. Concentrations were calculated based on partition coefficients reported previously.

same major metabolite was obtained with control or induced microsomes. The n.m.r. spectrum of the metabolite was different from that of 2-chloro-4-biphenylol.

The effect of microsomal protein concentration on the rates of metabolism of 2-chlorobiphenyl is shown in Fig. 8. At a constant substrate concentration, the rate of product formation (per nmole of cytochrome P-450) decreased with increasing microsomal protein concentration. However, when the substrate concentrations within the microsomes were calculated, taking into account the effect of microsomal protein concentration on the partition coefficients of the MCBs (Table 1), and replotted (Fig. 8), the effect of protein concentration was eliminated.

Effects of inhibitors. In Fig. 9 the effects of a number of inhibitors of cytochrome P-450 on the rates of microsomal metabolism of 4-chlorobiphenyl are shown. At saturating substrate concentrations, metyrapone (2 mM) inhibited the rate of metabolite formation by 46 per cent, SKF 525-A (2 mM) by 87 per cent, displacement of air by bubbling for 10 min with nitrogen by 83 per cent, and bubbling with a CO/O_2 (80/20) mixture for 10 min by 94 per cent, while replacement of NADPH by an equivalent concentration of NADH only yielded 7 per cent of the activity.

Molecular orbital calculations. The net charge on each carbon atom of the MCBs and biphenyl, as determined from the molecular orbital calculations,

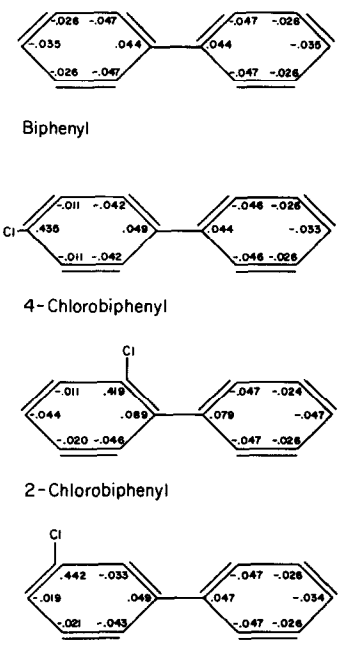

Fig. 10. Net atomic charges on the carbon atoms of biphenyl and 2-, 3- and 4-chlorobiphenyl. Net charges were calculated by Mulliken population analysis and extended Hückel molecular orbital calculations.

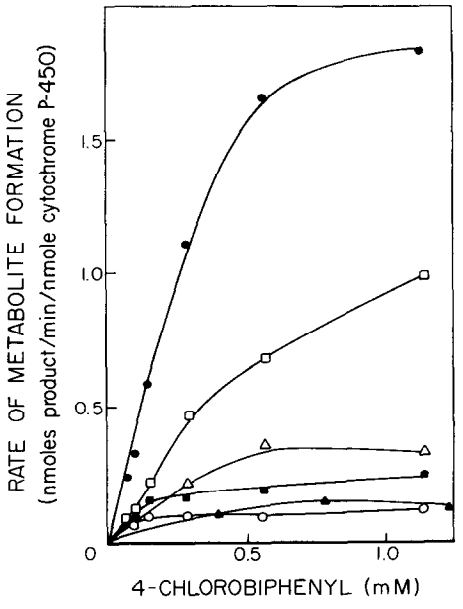

Fig. 9. Effects of inhibitors on the microsomal metabolism of 4-chlorobiphenyl. Control rat liver microsomes (0.89 nmole cytochrome P-450/mg protein, 2 mg protein/ml) were incubated with varying amounts of an ultrasonicated suspension of 4-chlorobiphenyl (0.07–1.13 mM) and NADPH (2.4 mM) at 37° and pH 7.4, no inhibitors added (●); metyrapone (2 mM) added (□); nitrogen was bubbled through the reaction mixture for 10 min prior to initiation (△); SKF 525-A (2 mM) added (■); NADH (2.7 mM) replaced the NADPH (▲); CO/O_2 (80/20) was bubbled through the reaction mixture for 10 min prior to initiation (○). Product was quantitated by h.p.l.c.

is presented in Fig. 10. Chlorine substitution at the 4-, 3- or 2-positions of biphenyl was virtually without effect on the net charge of the carbon atoms of the unsubstituted phenyl ring except in the case of the 4'-carbon of 2-chlorobiphenyl, which had a slightly more negative charge. The net charges on the 4- and 4'-carbons of 2-chlorobiphenyl were virtually equivalent.

DISCUSSION

The dependence of the rat hepatic microsomal metabolism of MCBs on NADPH and O_2, and the inhibition of this metabolism by CO [24, 37], SKF 525A [38] and metyrapone [39] strongly implicate cytochrome P-450 in the metabolism. A role for cytochrome P-450 in the catalysis of MCB metabolism is further supported by the type I difference spectra observed on the addition of MCBs to microsomal suspensions [27].

For the 2- and 3-chlorobiphenyls the combination of mass and n.m.r. spectrometry, dechlorination and h.p.l.c. retention times of the major metabolites unambiguously identify the metabolites as 2'-chloro-4-biphenylol and 3'-chloro-4-biphenylol, respectively. In the case of 4-chlorobiphenyl, the identity of the h.p.l.c. retention time of its major metabolite with that of 4-chloro-4'-biphenylol, together with the various reports that this is the major metabolite of 4-chlorobiphenyl *in vivo* in rats [40] and *in vitro* with rat liver microsomes [10, 12], serve to identify unambiguously this metabolite. It is thus apparent that chlorine substituents at the 2-, 3- or 4-positions of biphenyl do not alter the regioselectivity of control

rat liver cytochrome P-450 for hydroxylation at the 4-carbon of biphenyl. This unaltered regioselectivity is consistent with the results of molecular orbital calculations, which indicate that monochloro substituents at the 2-, 3- or 4-positions of biphenyl do not alter significantly the net charge on the carbon atoms of the unsubstituted phenyl rings relative to values for biphenyl. The 4- and 4′-carbons of 2-chlorobiphenyl, however, have similar net charges but, since no 2-chloro-4-biphenylol was detected as a metabolite, it is possible that steric effects of the 2-chloro-substituent prevented formation of this metabolite. It has been reported recently that 4- and 2-bromobiphenyl also yield primarily 4′-monohydroxylated products from the *in vivo* metabolism of rabbits [41]. In the same study, however, it was reported that the major metabolite of 3-bromobiphenyl was either 3-bromo-4-biphenylol or 5-bromo-2-biphenylol. It is not clear whether the difference between our results on the position of hydroxylation of 3-chlorobiphenyl and those reported for 3-bromobiphenyl result from the difference in the halogen substituent or from the difference in the type of experimental animal.

In contrast to the failure of 2-, 3- or 4-monochloro substituents to affect the regioselectivity of biphenyl metabolism by control cytochrome P-450, these substituents apparently overcame the regioselectivity toward biphenyl metabolism exhibited by induced forms of cytochrome P-450. We have reported previously that control rat liver microsomal metabolism of biphenyl (by Wistar rats from the same colony as that used in the present study) yielded 4- and 2-hydroxybiphenyl in the ratio of 14:1 and that this ratio was altered to 2.6:1 by prior induction of the microsomal cytochrome P-448 with 3-methylcholanthrene [42]. A similar change in regioselectivity of biphenyl metabolism following the induction of cytochrome P-448 was observed with Long–Evans [43] and Sprague–Dawley rats [13]. It was also demonstrated that phenobarbital induction of the microsomal cytochrome P-450 resulted in the formation of significant quantities of 3-hydroxybiphenyl from biphenyl (ratio of 4-/3-hydroxybiphenyl, 7:1) [13]. In the present studies, however, no such changes in regioselectivity were noted following induction by 2,4,2′,4′-TCB or 3,4,3′,4′-TCB and only the same major metabolites were detected under all conditions of induction for each of the MCBs. These results are consistent with previous studies using 4-chlorobiphenyl [44]. These TCBs have been demonstrated to induce forms of cytochrome P-450 and forms of cytochrome P-448, respectively [19]. Furthermore, in studies of induction by Aroclors 1254 and 1260 (commercial mixtures of PCBs), it was demonstrated that these mixtures induced forms of cytochrome P-450 and P-448 which were indistinguishable from those induced by phenobarbital and 3-methylcholanthrene [45, 46]. The results of the studies of MCB binding and MCB metabolic rates indicate that, except possibly for the metabolism of 2-chlorobiphenyl by cytochrome P-448, all three of the MCBs are metabolized by all of the variously induced microsomes. Thus, the effects of the 2-, 3- and 4-monochloro substituents on biphenyl in overcoming the regioselectivities of induced forms of cytochrome P-450 are not a consequence of a failure to catalyze metabolism.

Kinetic studies on the microsomal metabolism of the MCBs are complicated by the multicomponent, heterogeneous nature of the system [47]. However, following an extensive review of the literature [47] it was concluded that microsomal cytochrome P-450 metabolism is amenable to the determination of rate constants. The kinetic constants determined from microsomal metabolism should not, however, be considered as absolute values but rather as indicators of changes in enzyme activity [47] and are thus designated 'apparent' rate constants. The identity of the partition coefficients of the three MCBs for the system aqueous buffer–microsomal suspension indicates that the determined rate constants are probably not controlled by differential partition of the substrates into the microsomal environment of cytochrome P-450. In constrast, the observed dependence of the kinetic constants for 2-chlorobiphenyl metabolism on the microsomal protein, and thus lipid, concentration is apparently a consequence of the alteration in partition coefficient as a function of protein concentration. Thus, redetermination of the kinetic constants based on substrate concentrations within the microsomes eliminated the effect of microsomal protein concentration on the rate constants.

For 2-chlorobiphenyl, the results of the binding studies are essentially in agreement with the rate constants determined from the rates of formation of 2-chloro-4′-biphenylol. The apparent V_{max} value with 3,4,3′,4′-TCB-induced cytochrome P-448 is significantly lower than those with 2,4,2′,4′-TCB-induced cytochrome P-450 and control cytochrome P-450, which corresponds with the spectrally determined extents of binding of 2-chlorobiphenyl to the control and induced cytochromes. The apparent V_{max} values for 3-chlorobiphenyl metabolism (based on values per mg of microsomal protein) indicate enhanced rates of metabolism with both types of induced cytochromes, with the cytochrome P-448 producing the fastest rates. The binding studies also indicated enhanced binding to both types of induced cytochromes, although the 2,4,2′,4′-TCB-induced cytochrome P-450 produced the greater extent of binding. For 4-chlorobiphenyl metabolism all Lineweaver–Burk plots were biphasic. With control microsomes the faster phase yielded a rate of product formation which was significantly greater than the corresponding rates with 2- or 3-chlorobiphenyl.

In summary, we have demonstrated that rat liver microsomal cytochrome P-450 metabolism of MCBs yields 4′-hydroxylated MCBs as the major product irrespective of the state of induction of the cytochrome. The rates of metabolism of the MCBs were, however, differentially affected by the state of induction of the microsomal cytochrome P-450.

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