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Identification of Catechol and Hydroquinone Metabolites of 4-Monochlorobiphenyl

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Polychlorinated biphenyls (PCBs) may be metabolically activated to electrophiles, which bind to proteins and nucleic acids. One activation scheme involves the formation of reactive arene oxide intermediates during cytochrome P450-catalyzed hydroxylation. We propose a second activation pathway whereby PCB catechol and hydroquinone metabolites may be oxidized to reactive semiquinones and/or quinones. By employing 4-monochlorobiphenyl (4-MCB) as a model substrate and liver microsomes from rats treated with phenobarbital and 3-methylcholanthrene, five monol and three diol metabolites were identified. The major metabolite was 4-chloro-4′-monohydroxybiphenyl, followed by, in decreasing order, 4-chloro-3′,4′-dihydroxybiphenyl, unknown B (a monol), 4-chloro-2′,3′-dihydroxybiphenyl, 4-chloro-3′-hydroxybiphenyl, 4-chloro-2′,5′-dihydroxybiphenyl, unknown A (a monol), and 4-chloro-2′-monohydroxybiphenyl. A trace of a dihydrodiol was detected by GC/MS. To elucidate the source of the diols, 4-MCB and the synthetic monol metabolites 4-chloro-2′-/-3′-/-4′-monohydroxybiphenyls were each employed as substrates in incubations with microsomes from rats treated with phenobarbital, 3-methylcholanthrene, or both inducers. The three diol metabolites were all produced from 4-MCB in incubations with microsomes from 3-methylcholanthrene-treated rats, but incubations with microsomes from phenobarbital-treated rats did not yield detectable amounts of 4-chloro-2′,3′-dihydroxybiphenyl. 4-Chloro-2′,3′-dihydroxybiphenyl was only found as a product of 4-chloro-2′-monohydroxybiphenyl. The 4-chloro-2′,5′-dihydroxybiphenyl was found in extracts of incubations with 4-chloro-2′- and -3′-monohydroxybiphenyls, while the 4-chloro-3′,4′-dihydroxybiphenyl was the only product found from 4-chloro-3′- and -4′-monohydroxybiphenyls. No other chlorinated diols were detected by GC/MS. These data suggest that the major route of biosynthesis of the diols was via a second hydroxylation step and not aromatization of dihydrodiols derived from primary arene oxides. We propose a scheme for the *in vitro* synthesis of the catechol and hydroquinone metabolites, which may be precursors for electrophilic semiquinone or quinone products with the potential for cytotoxic and genotoxic effects.

Introduction

Commercial mixtures of polychlorinated biphenyls (PCBs)[1] have been used in applications as diverse as transformer dielectrics, hydraulic fluids, pipeline lubricants, and carbonless paper (*1*). Due to their resistance to physical and biological decomposition (*2*), PCBs were widely dispersed in the global ecosystem (*3*). PCBs have been identified in surface water (*4*), lake and river sediments (*5, 6*), precipitation (*7*), vegetation (*8*), and, due to their lipophilicity, in the tissues of most aquatic and terrestrial organisms, including humans (*9*−*11*).

Studies of the PCBs are difficult since there are 209 possible structures. Coplanar isomers, i.e., those congeners possessing no *o*-chloro substituents, mainly exert their biological effects via the aryl hydrocarbon receptor (Ah receptor), for which 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD) is the prototypical receptor agonist (*12*). The higher chlorinated congeners (4−10 chlorines), except those without adjacent *meta* and *para* chlorines (*13, 14*), are more resistant to metabolic degradation. Higher chlorinated congeners may be promoters in two-stage hepatocarcinogenesis (*15*−*17*). Although largely neglected, compelling evidence suggests that the lower chlorinated PCBs (1−3 chlorines), and possibly several higher chlorinated congeners, may exert effects via Ah receptor-independent mechanisms, leading to neurotoxicity (*18*) and alterations in endocrine functions (*19*−*21*). Despite their susceptibility to metabolic transformation, many of the lower congeneric PCBs are also present in many environmental samples (*4*−*6*, *8*, *14*) and human tissues (*11*), most likely as a result of the dechlorination of higher chlorinated congeners. PCB metabolites themselves may also persist in human and animal tissues (*14*, *22*, *23*), which adds a new dimension to toxic effects (*14*, *19*, *21*) that are just beginning to be explored in detail.

Lower chlorinated PCBs are hydroxylated in reactions catalyzed by cytochromes P450 1A and 2B (*24*−*26*). Studies of the metabolism of monochlorobiphenyls (MCBs) (*24*) and dichlorobiphenyls (DCBs) (*25*) have shown that

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[1] Abbreviations: PCB, polychlorinated biphenyl; CB, chlorobiphenyl; MCB, monochlorobiphenyl; DCB, dichlorobiphenyl; 4-MCB, 4-chlorobiphenyl; OPP, *o*-phenylphenol; $NADP^+$, (oxidized) nicotinamide adenine dinucleotide phosphate; Ah receptor, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachloro-*p*-dibenzodioxin; BSTFA, bis(trimethylsilyl)-trifluoroacetamide; TMS, trimethylsilyl; PB, phenobarbital; 3-MC, 3-methylcholanthrene; DMSO, dimethyl sulfoxide; 4′-monol; 4-chloro-4′-hydroxybiphenyl; 3′-monol, 4-chloro-3′-hydroxybiphenyl; 2′-monol, 4-chloro-2′-hydroxybiphenyl; 3′,4′-diol, 4-chloro-3′,4′-dihydroxybiphenyl; 2′,3′-diol, 4-chloro-2′,3′-dihydroxybiphenyl; 2′,5′-diol, 4-chloro-2′,5′-dihydroxybiphenyl.

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the monol, and presumably the diol, metabolites arise through an intermediate arene oxide formed between adjacent, unsubstituted positions. Since many arene oxides of PCBs are unstable, i.e., the half-life and the direction of opening depend upon the stabilization of the incipient carbocation, they nonenzymatically isomerize to phenols (*27*). The evidence that arene oxides may form during the metabolism of 4-MCB and other PCBs includes (1) the occurrence of NIH shift metabolites (*26*, *28*, *29*) and (2) the identification of dihydrodiol products (*30*). Of course, the hydroxyl group may also be directly inserted into the ring, thereby precluding the formation of an arene oxide (*31*).

Our hypothesis suggesting a role for semiquinones and quinones in the activation of lower chlorobiphenyls (CBs) is based on studies of the metabolism of benzene (*32*) and *o*-phenylphenol (OPP) (*33*) and our own genotoxicity data (*34*). These concepts are developed further in the accompanying paper (*35*). The present work focuses on the identification of catechol and hydroquinone metabolites of 4-monochlorobiphenyl (4-MCB), a model substrate. We report here the identification of five monohydroxy and three dihydroxy metabolites of 4-MCB. The identification of the metabolite 4-chloro-2′,5′-dihydroxybiphenyl is particularly significant given its structural similarity to the potentially genotoxic metabolites of benzene and OPP.

Materials and Methods

4-Chlorobiphenyl was purchased from EGA-Chemie (Steinheim/Albuch, Germany) and recrystallized from methanol to 99+% purity. Anhydrous solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Other solvents and preparative TLC plates (silica, 1 mm thick, Whatman) were from Fisher Scientific (Pittsburgh, PA). $NADP^+$ (sodium salt), glucose 6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49, baker's yeast), horseradish peroxidase (EC 1.11.1.7, type VI), catalase (EC 1.11.1.6, bovine liver), and inorganic reagents were purchased from Sigma Chemical Co. (St. Louis, MO). **Caution**: *PCBs and their metabolites should be handled as hazardous compounds in accordance with NIH guidelines (36).*

Procedure for the Palladium-Catalyzed Coupling of Arylboronic Acids with Bromobenzenes and Characterization of the Synthetic Standards. The required boronic acids and all 4-MCB metabolites were synthesized according to previously described methods (*37*). All synthetic compounds were purified by crystallization or preparative TLC to 99+% purity, as determined by GC/MS. IR spectra were obtained by using a Perkin-Elmer 1600 FT-IR spectrophotometer. The ^1H NMR and ^{13}C NMR spectra were recorded on a Varian VXR-400S spectrometer using CD_2Cl_2 and acetone-d_6 (Aldrich Chemical Co.) as solvents and internal standards. Melting points were determined on a Mel-Temp apparatus and are uncorrected. All synthetic standard mono- and dihydroxybiphenyls were silylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA)/pyridine (1:1, v/v) (Sigma) prior to GC/MS using a Kratos Concept 1H instrument fitted with a fused silica capillary column (DB-5MS, 15 m × 0.25 mm id × 0.25 μm film, J &W Scientific, Folsom, CA).

4-Chloro-4′-methoxybiphenyl was synthesized from (4-methoxyphenyl)boronic acid and *p*-bromochlorobenzene in 80% yield as a white solid from diethyl ether/petroleum ether (1:4): mp 112−113 °C (lit. mp 115−116 °C, *38*); IR (KBr) 3010, 2966, 2933, 2833, 1605, 1484, 1289, 1260, 1199, 1100, 1037 cm^{-1}; ^1H NMR (400 MHz, CD_2Cl_2) δ 3.83 (s, 3H, OCH_3), 6.98, 7.39, 7.50, 7.52 (m, 2AA′BB′, 8H); MS (70 eV) *m*/*z* (relative intensity) 218 (100) [M^+], 203 (58) [$M^+ - CH_3$], 175 (52), 139 (41).

4-Chloro-3′-methoxybiphenyl was synthesized from (3-methoxyphenyl)boronic acid and *p*-bromochlorobenzene in 82% yield: colorless oil (lit. mp 32 °C, *38*); IR (neat) 3064, 3000, 2936, 2834, 1600, 1564, 1477, 1294, 1212, 1091, 1011 cm^{-1}; ^1H NMR (400 MHz, CD_2Cl_2) δ 3.87 (s, 3H, OCH_3), 6.93 (ddd, J = 8.2, 2.5, 0.9 Hz, 1H, 4-H′), 7.11 (t, J = 2.3 Hz, 1H, 2′-H), 7.19 (ddd, J = 7.6, 2.2, 0.9 Hz, 1H, 6′-H), 7.37 (t, J = 7.9 Hz, 1H, 5′-H), 7.42, 7.53 (m, AA′BB′, 4H); MS (70 eV). *m*/*z* (relative intensity) 218 (100) [M^+], 188 (28), 175 (37), 139 (35).

4-Chloro-2′-methoxybiphenyl was synthesized from (2-methoxyphenyl)boronic acid and *p*-bromochlorobenzene in 73% yield: white solid from petroleum ether; mp 49−50 °C (lit. mp 50−51 °C, *38*); IR (KBr) 3044, 2967, 2930, 2833, 1595, 1478, 1233, 1089, 1017 cm^{-1}; ^1H NMR (400 MHz, CD_2Cl_2) δ 3.80 (s, 3H, OCH_3), 7.00 (ddd, J = 7.2, 1.1, 0.4 Hz, 1H), 7.03 (m_c, $J \sim$ 7.4, 1.1 Hz, 1H), 7.29 (ddd, J = 7.4, 1.8, 0.4 Hz, 1H), 7.33 (m_c, $J \sim$ 7.3, 1.8 Hz, 1H), 7.38, 7.47 (m, AA′BB′, 4H); MS (70 eV) *m*/*z* (relative intensity) 218 (73) [M^+], 203 (12) [$M^+ - CH_3$], 168 (100) [$M^+ - CH_3Cl$], 139 (37).

4-Chloro-3′,4′-dimethoxybiphenyl was synthesized from (3,4-dimethoxyphenyl)boronic acid and *p*-bromochlorobenzene in 78% yield: white solid from diethyl ether/petroleum ether, 1:3; mp 81−82 °C; IR (KBr) 3061, 3005, 2930, 2825, 1596, 1520, 1487, 1252, 1144, 1023 cm^{-1} ; ^1H NMR (400 MHz, CD_2Cl_2) δ 3.87, 3.89 (2s, 6H, OCH_3), 6.94 (d, J = 8.4 Hz, 1H, 5′-H), 7.08 (d, J = 2.0 Hz, 1H, 2′-H), 7.12 (dd, J = 8.2, 2.2 Hz, 1H, 6′-H), 7.39, 7.52 (m, AA′BB′, 4H); MS (70 eV) *m*/*z* (relative intensity) 248 (100) [M^+], 233 (42) [$M^+ - CH_3$], 205 (35).

4-Chloro-2′,3′-dimethoxybiphenyl was synthesized from (2,3-dimethoxyphenyl)boronic acid and *p*-bromochlorobenzene in 60% yield: white solid from diethyl ether/petroleum ether, 1:3; mp 42−43 °C; IR (KBr) 3067, 2933, 2822, 1578, 1467, 1261, 1083, 1006 cm^{-1}; ^1H NMR (400 MHz, CD_2Cl_2) δ 3.59, 3.89 (2s, 6H, OCH_3), 6.91 (dd, J = 7.7, 1.6 Hz, 1H), 6.95 (dd, J = 8.2, 1.6 Hz, 1H), 7.11 (dd, J = 8.2, 7.7 Hz, 1H, 5′-H), 7.39, 7.50 (m, AA′BB′, 4-H); MS (70 eV) *m*/*z* (relative intensity) 248 (79) [M^+], 233 (42) [$M^+ - CH_3$], 198 (100) [$M^+ - CH_3Cl$].

Demethylation Reaction. The methoxy compounds were demethylated with boron tribromide as previously described (*37*).

4-Chloro-4′-hydroxybiphenyl (4-monol) was obtained from 4-chloro-4′-methoxybiphenyl in 92% yield: white solid from chloroform/petroleum ether, 1:3; mp 143−144 °C (lit. mp 147−147.5 °C, *39*); IR (KBr) 3386, 3033, 1605, 1485, 1260, 1105 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 6.94, 7.43, 7.51, 7.60 (m, 2AA′BB′, 8H), 8.47 (s, 1H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.70, 128.70, 128.81, 129.58, 131.81, 132.75, 140.65, 158.36; MS (70 eV) *m*/*z* (relative intensity) 276 (100) [M^+], 261 (95) [$M^+ - CH_3$], 73 (58).

4-Chloro-3′-hydroxybiphenyl (3-monol) was obtained from 4-chloro-3′-methoxybiphenyl in 78% yield: white solid from chloroform/petroleum ether, 1:10; mp 78−79 °C (lit. mp 86 °C, *40*); IR (KBr) 3286, 3056, 1592, 1474, 1289, 1202, 1094 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 6.85 (ddd, J = 8.1, 2.2, 1.1 Hz, 1H, 6′-H), 7.10 (m_c, 2H, 2′-, 4′-H), 7.28 (t, J = 8.1 Hz, 1H, 5′-H), 7.45, 7.62 (m, AA′BB′, 4H), 8.52 (s, 1H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 114.51, 115.54, 118.88, 129.28, 129.64, 130.87, 133.70, 140.66, 141.97, 158.77; MS (70 eV) *m*/*z* (relative intensity) 276 (62) [M^+], 261 (100) [$M^+ - CH_3$].

4-Chloro-2′-hydroxybiphenyl (2-monol) was obtained from 4-chloro-2′-methoxybiphenyl in 82% yield: white solid from chloroform/petroleum ether, 1:10; mp 42−43° C (lit., 51−52° C, *41*); IR (KBr) 3310, 3043, 1589, 1476, 1347, 1201, 1090 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 6.92 (m_c, $J \sim$ 7.5, 1.2 Hz, 1H), 6.98 (ddd, J = 8.1, 1.2, 0.4 Hz, 1H), 7.19 (m_c, $J \sim$ 8.1, 1.7 Hz, 1H), 7.29 (ddd, J = 7.6, 1.7, 0.4 Hz, 1H), 7.41, 7.60 (m, AA′BB′, 4H), 8.47 (s, 1H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.98, 120.93, 127.89, 128.80, 129.75, 131.23, 131.76, 132.86, 138.48, 154.95; MS (70 eV) *m*/*z* (relative intensity) 276 (47) [M^+], 261 (38) [$M^+ - CH_3$], 93 (100).

4-Chloro-3′,4′-dihydroxybiphenyl (3′,4′-diol) was obtained from 4-chloro-3′,4′-dimethoxybiphenyl in 74% yield: white solid from chloroform/petroleum ether, 1:3; mp 121−122 °C; IR (KBr) 3487, 3308, 1598, 1520, 1488, 1303, 1202, 1113 cm^{-1}; ^1H NMR (400 MHz, acetone-d_6) δ 6.90 (d, J = 8.2 Hz, 1H, 5′-H), 6.99 (dd, J = 8.1, 2.2 Hz, 1H, 6′-H), 7.12 (d, J = 2.1 Hz, 1H, 2′-H),

7.38, 7.57 (m, AA′BB′, 4H), 8.06 (bs, 2H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 114.72, 116.65, 119.32, 128.76, 129.53, 132.61, 132.75, 140.77, 146.12, 146.41; MS (70 eV) m/z (relative intensity) 364 (22) [M^+], 73 (100).

4-Chloro-2′,3′-dihydroxybiphenyl (2′,3′-diol) was obtained from 4-chloro-2′,3′-dimethoxybiphenyl in 50% yield: white solid from chloroform/petroleum ether, 1:3; mp 112−113 °C; IR (KBr) 3500, 3422, 1617, 1589, 1471, 1320, 1211, 1072 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.78 (t, J = 7.8 Hz, 1H, 5′-H), 6.87 (dd, J = 7.8, 1.7 Hz, 1H), 6.92 (dd, J = 7.8, 1.7 Hz, 1H), 7.41, 7.61 (m, AA′BB′, 4H), 8.29 (s, 2H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 115.36, 120.58, 122.06, 128.06, 128.85, 131.69, 132.93, 138.38, 143.53, 146.00; MS (70 eV) m/z (relative intensity) 364 (42) [M^+], 349 (15) [$M^+ - CH_3$], 276 (13), 73 (100).

4-Chloro-2′,5′-dihydroxybiphenyl (2′,5′-diol) was synthesized from the quinone (*42*) which was reduced with sodium dithionite: white solid from water; mp 116−117 °C (lit. mp 117−118.5 °C, *43*); IR (KBr) 3279, 1597, 1486, 1449, 1353, 1249, 1183, 1092 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ 6.69 (dd, J = 8.5, 3.0 Hz, 1H, 4′-H), 6.79 (d, J = 2.9 Hz, 1H, 6′-H), 6.82 (d, J = 8.5 Hz, 1H, 3′-H), 7.40, 7.58 (m, AA′BB′, 4H), 7.85 (s, 2H, OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 116.37, 117.46, 117.94, 128.81, 131.73, 132.87, 138.65, 147.93, 151.65; MS (70 eV) m/z (relative intensity) 364 (100) [M^+], 349 (18) [$M^+ - CH_3$], 314 (33) [$M^+ - CH_3Cl$], 73 (70).

Microsomal Preparation. Male Sprague−Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN), provided Rodent Chow (Purina, St. Louis, MO) and tap water *ad libitum*, and maintained on a 12 h light−dark cycle in a controlled environment at a temperature of 22 °C. After a 7 day quarantine period, the rats were injected ip on three consecutive days with either 400 μmol of phenobarbital (PB, saline) per kilogram body weight or 100 μmol of 3-methylcholanthrene (3-MC, corn oil) per kilogram body weight. A group also received simultaneous injections of phenobarbital and 3-methylcholanthrene (PB/3-MC) as described. Control animals received vehicle only.

On the fourth day, each rat (200−220 g) was sacrificed by asphyxiation with carbon dioxide, and the liver was perfused *in situ* with ice-cold 0.25 M sucrose/0.1 mM EDTA (pH 7.3) and then excised. Whole liver homogenate was prepared by mincing and then grinding with an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH) in 3 or 4 vol of ice-cold sucrose/EDTA. The homogenate was centrifuged at 10000*g* for 20 min (Sorvall, rotor SS-34). The supernatant was then centrifuged (Beckman, L7 Ultracentrifuge, rotor SW41) at 100000*g* for 1 h. The supernatant (cytosol) was decanted and the microsomal pellet was resuspended in sucrose/EDTA. Protein was determined by the method of Lowry et al. (*44*) using bovine serum albumin as standard. The microsomal suspensions were frozen in aliquots at −80 °C until use.

Metabolism Studies. The final incubation medium consisted of 25 mM Tris-HCl buffer (pH 8.0) (*45*), 2 mM $MgCl_2$, 2 mg of microsomal protein/mL, and a NADPH-regenerating system of 5 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 0.75 unit/mL glucose-6-phosphate dehydrogenase. After a 5 min preincubation, substrate, dissolved in dimethyl sulfoxide (DMSO), was added to a final concentration of 1 mM (0.5%, DMSO). All incubations were at 37 °C in a shaking water bath. Reactions were stopped by adding HCl to 0.36 M. NaCl was added and the metabolites were extracted with diethyl ether. The combined extracts were dried over anhydrous $MgSO_4$, which was then washed with 1 × 1 mL ether. The extract and wash were evaporated to a residue, which was stored in the dark at room temperature until analysis.

For metabolite identification via GC/MS, a 15 mL reaction mixture was prepared with microsomes from rats treated with PB/3-MC. The reaction was stopped after 45 min. For the time course experiment, a 25 mL reaction mixture was prepared with microsomes from rats treated with PB/3-MC. At the appropriate time, a 2 mL aliquot was taken and the reaction was stopped and prepared as described earlier. For the metabolism of 4-MCB and 4-chloro-2′-/-3′-/-4′-monohydroxybiphenyl to the diols, the final reaction volume was 2 mL and the reaction was stopped after 20 min.

GC/MS Identification of Metabolites. The dry metabolite residues were dissolved in ethyl acetate and then derivatized with BSTFA/pyridine (1:1, molar basis). Standards were prepared as described in the following for use as a reference. GC/MS was done with a Varian 3400 GC fitted with a methyl silicone capillary column (15 m × 0.25 mm id × 0.32 μm film thickness) connected in series with a Finnigan INCOS 50 mass spectrometer. The GC temperature program was 60 °C for 1 min, increased to 280 °C at 1 °C/min, and then held at 280 °C for 5 min.

Quantitation of 4-MCB and Metabolites. Stock solutions of the mono- and dihydroxy metabolites were prepared in ethyl acetate. A 400 μM mixed metabolite standard was prepared from the individual stock solutions, and additional mixed standards ranging from 300 to 12.5 μM were prepared by dilution with ethyl acetate. To prevent autoxidation of the diol metabolites, the solvent was evaporated from 250 μL aliquots of each mixed metabolite standard, and the residue was stored at room temperature in a glass vial sealed with a Teflon-lined cap. Standards of 4-MCB were prepared in a similar manner and ranged from 0.5 to 8.0 mM.

A solution of 400 μM 2,2′-biphenyldiol was prepared with dry ethyl acetate. The 2,2′-biphenyldiol served as an internal standard, and the solution was stored at 4 °C over $MgSO_4$ in a sealed glass container wrapped with aluminum foil. On the day of determination, residues of both samples and standards were dissolved in 230 μL of 400 μM 2,2′-biphenyldiol and then 20 μL of BSTFA/pyridine (1:1, molar basis) was added. When kept at room temperature and protected from water, the trimethylsilyl (TMS)-derivatized metabolites were stable for at least 4 days.

A Hewlett-Packard 5890A GC fitted with an HP-1 dimethyl silicone gum column (30 m × 0.25 mm id × 1 μm film thickness) was used to separate and quantify the metabolites. The injector and flame ionization detector temperatures were 250 and 300 °C, respectively. The carrier gas, helium, was maintained at 10 psi head pressure. The temperature program was from 140 to 245 °C at 5 °C/min and then held at 245 °C for 6 min. Peaks were recorded with an HP-3392A integrator. The relative response factors of 4-MCB and each metabolite were calculated as the ratio of the area of the compound to the area of the internal standard, 2,2′-biphenyldiol. Standard curves for 4-MCB and each metabolite were constructed by using the relative response factor. A correlation coefficient ≥0.98 was obtained for each fitted, linear standard curve.

Results

Identification of Monohydroxy and Dihydroxy Metabolites of 4-MCB. The first objective was to determine the spectra of metabolites formed from 4-MCB by using GC/MS (Figure 1). The chlorinated metabolites were readily identified by the natural isotope abundance ratio of 3:1 for M^+:$[M + 2]^+$ for a monochlorinated biphenyl. On the basis of prior reports (*28*, *45*−*48*), we expected to find 4-chloro-4′-hydroxybiphenyl and 4-chloro-3′,4′-dihydroxybiphenyl, which were synthesized to aid in their identification. Surprisingly, five monol and three diol metabolites of 4-MCB were found in the ether extracts of microsomes from PB/3-MC-treated rats (Figure 1). These results led to the synthesis of additional mono- and dihydroxy metabolites, as indicated in the Materials and Methods.

Three monol metabolites (M^+ = 276) and three dihydroxybiphenyl metabolites (M^+ = 364) were identified (Figure 1) by comparison with GC/MS chromatograms of a mixture of the standards. Two monol metabolites (M^+ = 276) were found that did not match any standard. These were designated unknown A and unknown B (Figure 1). Since all possible monohydroxylated metabo-

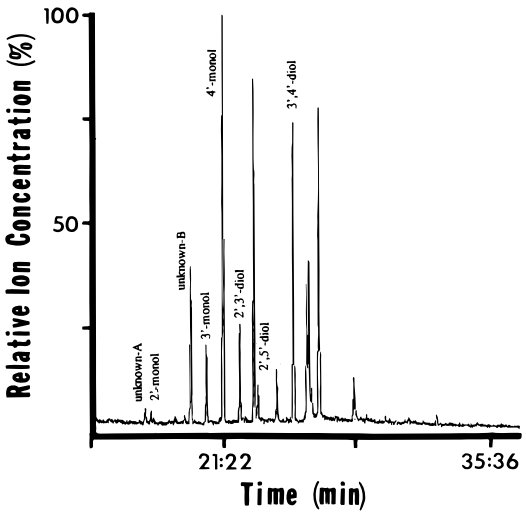


Figure 1. GC/MS chromatogram of the diethyl ether-extractable monol and diol metabolites of 4-monochlorobiphenyl. The following metabolites were identified: 2′-monol (4-chloro-2′-hydroxybiphenyl), 3′-monol (4-chloro-3′-hydroxybiphenyl), 4′-monol (4-chloro-4′-hydroxybiphenyl), 2′,3′-diol (4-chloro-2′,3′-dihydroxybiphenyl), 2′,5′-diol (4-chloro-2′,5′-dihydroxybiphenyl), and 3′,4′-diol (4-chloro-3′,4′-dihydroxybiphenyl). 4-Chlorobiphenyl (1 mM) was incubated with a NADPH-regenerating system and microsomes from rats treated with phenobarbital and 3-methylcholanthrene. After 45 min at 37 °C, the reaction was stopped with HCl and the metabolites were extracted with diethyl ether (3 × 10 mL). The ether was dried over anhydrous $MgSO_4$, decanted, and then evaporated. The residue was dissolved in ethyl acetate, and the trimethylsilyl derivatives were prepared for GC/MS analysis. The GC was fitted with a 1:100 split injector port and a methyl silicone capillary column (15 m × 0.25 i. × 0.32 μm film) connected in series with the MS. The temperature program was as follows: Held at 60 °C for 1 min, ramped to 280 °C at 1 °C/min, and then held at 280 °C for 5 min. The unlabeled peaks indicate the positions of fatty acids/cholesterol esters.

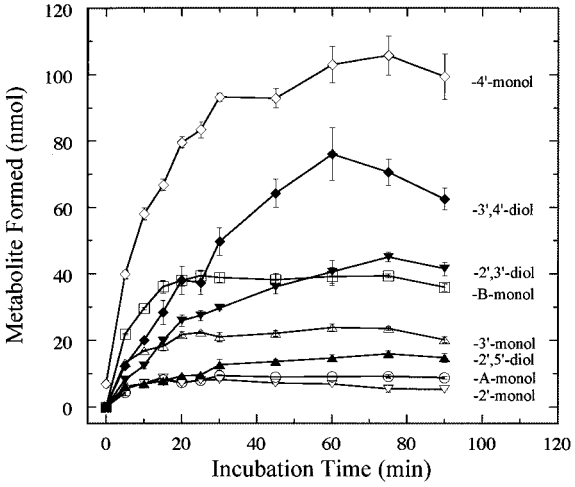


Figure 2. Time course of the appearance of monol and diol metabolites of 4-monochlorobiphenyl: 4-chloro-2′-hydroxybiphenyl (▽), 4-chloro-3′-hydroxybiphenyl (△), 4-chloro-4′-hydroxybiphenyl (◇), 4-chloro-A-hydroxybiphenyl (○), 4-chloro-B-hydroxybiphenyl (□), 4-chloro-2′,5′-dihydroxybiphenyl (▲), 4-chloro-3′,4′-dihydroxybiphenyl (◆), 4-chloro-2′,3′-dihydroxybiphenyl (▼). A 25 mL reaction mixture consisting of 1 mM 4-chlorobiphenyl, liver microsomes from phenobarbital- and 3-methylcholanthrene-treated rats, and a NADPH-regenerating system were incubated at 37 °C. At the appropriate time, a 2 mL aliquot was removed and the reaction was stopped with HCl. The metabolites were extracted with diethyl ether (3 × 2 mL), dried over anhydrous $MgSO_4$, and decanted, and then the solvent was evaporated. On the day of GC analysis, the residue was dissolved in 2 mL of ethyl acetate containing 400 μM 2,2′-dihydroxybiphenyl as an internal standard. A 230 μL aliquot was derivatized with 20 μL of BSTFA/pyridine (1:1, molar basis). A dimethyl silicone column (30 m × 0.25 mm id × 1 μm film) was used to separate the metabolites. The temperature program was 140 to 245 °C at 5 °C/min and then held at 245 °C for 6 min. The metabolites were identified by comparison with synthetic standards prepared in a similar manner. Quantitation was done by the internal standards ratio method. All values represent the means ± standard deviation of three determinations.

lites with a hydroxy group in the nonchlorinated ring were identified, the unknowns were probably 4-chloro-2-monohydroxybiphenyl and 4-chloro-3-monohydroxybiphenyl. A third unknown, in trace amounts, had $M^+ = 366$, which corresponded to that expected of 4-chlorobiphenyl dihydrodiol (not shown). With the exception of the three unknowns, the sample and reference GC/MS chromatograms had almost identical retention times and mass fragmentation patterns.

Time Course for the Formation of Monol and Diol Metabolites. The major metabolite of 4-MCB was 4-chloro-4′-hydroxybiphenyl, followed by 4-chloro-3′,4′-dihydroxybiphenyl (Figure 2). About 50% of the maximum amounts of 4-chloro-2′-/-3′-/-4′-hydroxybiphenyls was achieved in less than 10 min. The levels of 4-chloro-3′-hydroxybiphenyl and 4-chloro-4′-hydroxybiphenyl plateaued at about 30 min while the 4-chloro-2′-hydroxybiphenyl peaked at 30 min and then declined over the next 60 min to the level at 5 min. The levels of 4-chloro-2′,5′-/-2′,3′-/-3′,4′-dihydroxybiphenyls reached 50% of their maximum levels in about 15 min; therefore, there was at least a 5 min lag in the production of dihydroxybiphenyl metabolites compared to the monohydroxybiphenyl metabolites. The levels of all three dihydroxybiphenyl metabolites plateaued around 45 min (Figure 2). The unknown monols were quantified using the standard curves for 4-chloro-2′-hydroxybiphenyl and 4-chloro-3′-hydroxybiphenyl for unknown A and unknown B, respectively. Unknown B was the second most abundant monohydroxy product of 4-MCB metabolism (Figure 2).

The amount of 4-MCB recovered from each time point was found to decline during the course of the incubation from 2 μmol at time zero to about 1.4 μmol at 90 min (not shown), with the following linear curve fit $(1.8-5.3) \times 10^3$, $r = 0.9$. In contrast, the sum of the 4-MCB and the hydroxylated metabolites remained relatively constant at about 1.9 μmol for the first 60 min and then decreased to about 1.7 μmol at 75 and 90 min (not shown). On the basis of these data, extraction recoveries were estimated to be 85−95%, depending upon incubation time. The data were not corrected further. However, it was demonstrated that the catechol metabolites are susceptible to autoxidation and that the quinone of 4-chloro-2′,5′-dihyroxybiphenyl binds to amino acids and proteins (not shown). Thus, it was likely that a small percentage of the diol metabolites was not extractable, and as demonstrated the total recovery decreased with time (not shown).

The identities of the metabolites were confirmed by GC/MS. The only metabolite found in the 0 min sample was a trace of the 4′-monol (not shown). Extracts from all other time points contained the described metabolites (see above), including trace amounts of the dihydrodiol, which was below the detection limit of the GC analysis and therefore not quantifiable.

Formation of Dihydroxybiphenyl Metabolites from the Monohydroxybiphenyl Metabolites of 4-MCB: Effects of Inducers. Two micromoles each of

Table 1. Formation of Diol Metabolites from 4-Chlorobiphenyl and Three 4-Chloromonohydroxybiphenyls Incubated for 20 min with Rat Liver Microsomes from PB-, 3-MC-, and PB/3-MC-Treated Rats

inducer treatment parent compound	amount of diol metabolite formed[a]		
	2′,3′-diol	2′,5′-diol	3′,4′-diol
phenobarbital (PB)			
4-chlorobiphenyl	nd[b]	6.51 ± 1.0	13.9 ± 1.0
4-chloro-2′-biphenylol	8.67 ± 1.3	53.2 ± 1.3	nd
4-chloro-3′-biphenylol	nd	14.8 ± 1.1	36.1 ± 6.4
4-chloro-4′-biphenylol	nd	nd	14.9 ± 0.8
3-methylcholanthrene (3-MC)			
4-chlorobiphenyl	19.1 ± 0.6	10.6 ± 0.2	22.9 ± 2.3
4-chloro-2′-biphenylol	12.0 ± 1.5	99.4 ± 1.8	nd
4-chloro-3′-biphenylol	nd	16.0 ± 1.1	28.0 ± 4.9
4-chloro-4′-biphenylol	nd	nd	69.3 ± 6.1
PB/3-MC			
4-chlorobiphenyl	18.9 ± 1.6	10.6 ± 1.3	34.5 ± 5.1
4-chloro-2'-biphenylol	12.9 ± 1.1	128 ± 2.6	nd
4-chloro-3′-biphenylol	nd	31.4 ± 0.6	51.1 ± 6.4
4-chloro-4′-biphenylol	nd	nd	91.6 ± 13

[a] nmol of metabolite; mean ± standard deviation ($n = 4$). [b] nd, none detected.

4-MCB, 4-chloro-2′-hydroxybiphenyl, 4-chloro-3′-hydroxybiphenyl, and 4-chloro-4′-hydroxybiphenyl was incubated for 20 min with microsomes from PB-, 3-MC-, and PB/3-MC-treated rats (Table 1). The major dihydroxybiphenyl metabolite of 4-MCB in each microsomal group was the 3′,4′-diol. The 2′,3′-diol was the second most abundant metabolite with the exception of the PB-induced microsomal group, which had no detectable 2′,3′-diol. The 2′,5′-diol was the least abundant dihydroxy metabolite of 4-MCB.

Incubation with 4-chloro-2′-hydroxybiphenyl yielded the 2′,5′-diol as the major metabolite, which was 6-, 8-, and 10-fold greater than the 2′,3′-diol for the PB-, 3-MC-, and PB/3-MC-induced microsomes, respectively (Table 1). As expected, the 3′,4′-diol was not a product of 4-chloro-2′-hydroxybiphenyl. 4-Chloro-3′-hydroxybiphenyl was metabolized to 2′,5′-diol and 3′,4′-diol in all three of the microsomal incubations. The 3′,4′-diol was about 1.6−2.5 times greater than the 2′,5′-diol. The 2′,3′-diol was not detected as a product of 4-chloro-3′-hydroxybiphenyl. The only diethyl ether-extractable diol found, as expected, from the 4-chloro-4′-hydroxybiphenyl incubation was the 3′,4′-diol (Table 1).

Discussion

The findings of previous investigators with benzene (*32*) and *o*-phenylphenol (*33*) implied that 4-MCB, as well as many other PCBs, will experience multistep hydroxylation reactions catalyzed by cytochrome P450 to yield catechol and hydroquinone metabolites. These metabolites may subsequently autoxidize or alternatively lose one or two electrons via an enzyme-mediated mechanism, thereby producing semiquinone and/or quinone products. Semiquinones and quinones characteristically react with nucleophilic targets within protein, RNA, or DNA to produce covalently bound adducts (*49*). Taken together, the analogy with benzene and OPP, as well as the metabolism of PCBs to dihydroxy metabolites (*30*), suggested the involvement of lower halogenated PCBs in cytotoxic and/or genotoxic events.

Biosynthesis of Monol Metabolites of 4-MCB. The initial goal of the current study was to confirm the previously reported production of 4-chloro-4′-hydroxybiphenyl and 4-chloro-3′,4′-dihydroxybiphenyl as metabolites of 4-MCB (*28*, *45*−*48*). However, GC/MS analysis of the diethyl ether extracts of the initial microsomal incubation of 4-MCB (Figure 1) revealed five monol, three diol, and one dihydrodiol metabolites. By assuming no NIH shift involving the chlorine, only five monol metabolites were possible, so that all monol metabolites of 4-MCB were found.

Safe et al. (*28*) determined that 79% of the 4-chloro-4′-hydroxybiphenyl isolated from the urine of a rabbit treated with 4-chloro-4′-deuteriobiphenyl had the deuterium in the 3′-position. This was direct evidence for an intermediate arene oxide, the 3′,4′-epoxide, which preferentially isomerized to yield the 4′-monol and presumably little or no 3′-monol.

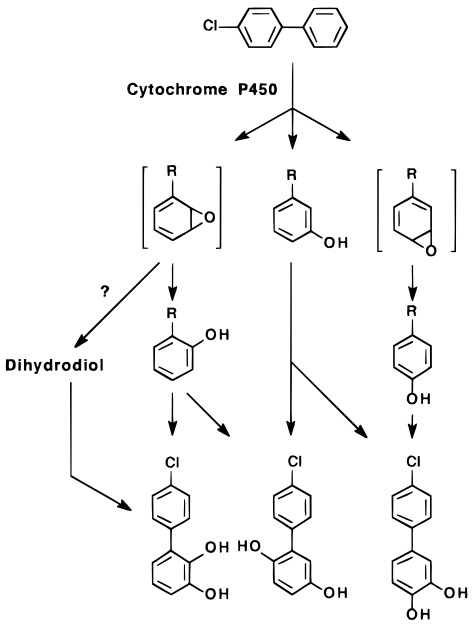
To our knowledge, this is the first reported identification of 4-chloro-3′-hydroxybiphenyl and 4-chloro-2′-hydroxybiphenyl (Figures 1 and 2). Quantitatively there were 2−3 times more 3′-monol than −2′-monol (Figure 2). Both metabolites could have been derived from a 2′,3′-arene oxide. Conceptually, resonance stabilization of the intermediate carbocation during opening of the epoxide suggests that the 2′-monol would be the preferred rearrangement product of a 2′,3′-arene oxide. The most reasonable explanation for the origin of the 3′-monol is to propose a direct insertion mechanism, which seems to be typical for *meta*-hydroxylated metabolites (*31*) and implied by the results of Safe et al. (*28*).

The two monol metabolites (unknown A and unknown B) not matching our standards must be 4-chloro-2-hydroxybiphenyl and 4-chloro-3-hydroxybiphenyl (Figures 1 and 2). Quantitatively, unknown B was the third most abundant metabolite, while unknown A was the least abundant metabolite found in the time course experiment (Figure 2).

Biosynthesis of Dihydroxy Metabolites of 4-MCB. Although arene oxides are generally considered the toxic intermediates of PCB metabolism (*50*), we have hypothesized the involvement of catechol and hydroquinone products as mediators of genotoxicity. The results reported herein confirm those of Safe et al. (*28*) and others (*29*) that 4-chloro-3′,4′-dihydroxybiphenyl is the second major metabolite of 4-MCB. The 3′,4′-diol presumably arises as a secondary hydroxylation product of the 4′-monol via direct insertion of the 3′-hydroxy group (*28*). Evidence for two additional diol metabolites, the 2′,3′- and 2′,5′-diols (Figures 1 and 2), was obtained by matching their GC/MS characteristics with those of synthetic standards. This is the first report for the microsome-mediated production of 2′,3′-diol and 2′,5′-diol as metabolites of 4-MCB (Scheme 1).

The origin of the catechol and *p*-hydroquinone metabolites was investigated by incubating 4-MCB, 4-chloro-2′-hydroxybiphenyl, 4-chloro-3′-hydroxybiphenyl, and 4-chloro-4′-hydroxybiphenyl with microsomes from PB-, 3-MC-, and PB/3-MC-treated rats (Table 1). By starting with the 2′-monol substrate, only the 2′,3′- and 2′,5′-diols were recovered from the diethyl ether extracts. Interestingly, about 7−10 times more 2′,5′-diol was recovered in the organic extracts than 2′,3′-diol (Table 1). Thus, *para* hydroxylation of 2′-monol was preferred over *ortho* hydroxylation (Scheme 1). No evidence was found for any other diol metabolites of 2′-monol by GC/MS. These experiments were not designed to examine the mechanism of secondary hydroxylation, i.e., isomerization of an arene oxide versus direct insertion; however, the lack of evidence for the 2′,4′-diol clearly suggests that the 2′-hydroxy substituent greatly influences the formation of

Scheme 1. Proposed Metabolism of 4-Chlorobiphenyl to Dihydroxy Metabolites[a]



[a] Primary hydroxylation of 4-chlorobiphenyl is catalyzed by cytochrome P450 to yield 2′,3′-arene oxide, 3′-monol, and 3′,4′-arene oxide. The intermediate arene oxides nonenzymatically isomerize to the 2′-monol and4′-monol, respectively. The monols subsequently undergo a second hydroxylation step, catalyzed by cytochrome P450. Three arene oxides of the 2′-monol are possible, one of which rearranges to give 2′,3′-diol and the other two, assuming that both are formed, isomerize to 2′,5′-diol. Two arene oxides of 3′-monol are conceivable: one rearranges to produce 2′,5′-diol and the other 3′,4′-diol. The major secondary metabolism product is 4-chloro-3′,4′-dihydroxybiphenyl, which probably arises from the direct insertion of a hydroxy group into the 3′-position of 4′-monol. A 2′,3′-dihydrodiol is proposed to account for the "large" amount of 2′,3′-diol that was observed (see text for a full explanation).

the diols. This proposal is supported by the fact that, in the absence of the 2′-hydroxy group, i.e., 4-MCB, the preferred site of hydroxylation is the 4′-position.

Incubation of 4-chloro-3′-hydroxybiphenyl gave the 3′,4′-diol and the 2′,5′-diol, although the former was 1.5−2 times more abundant than the latter (Table 1). No evidence was found for the 3′,5′-diol metabolite. The 2′,3′-diol metabolite also was not a detectable product of 4-chloro-3′-hydroxybiphenyl, probably due to steric hindrance of the 2′-position. Again, *ortho* and *para* hydroxylation relative to the 3′-hydroxy substituent was evident. Given that the 4′-position is the major site of hydroxylation of 4-MCB (Figure 1), we expected considerably more 3′,4′-diol than 2′,5′-diol (Table 1). Obviously the 3′-hydroxy group influences the secondary hydroxylation such that *para* hydroxylation (6′-position) becomes favorable, whereas with 2′-monol as the initial substrate no 2′,6′-diol was detectable.

It is clear that 4-chloro-4′-hydroxybiphenyl was not the only source for the 3′,4′-diol (Figures 1 and 2), since secondary hydroxylation of 4-chloro-3′-hydroxybiphenyl contributes to the pool of 4-chloro-3′,4′-dihydroxybiphenyl (Table 1). As noted earlier, it has been previously shown that the 3′-hydroxylation of 4-chloro-4′-monohydroxybiphenyl is via a direct insertion mechanism (*28*). No hints were found by GC/MS for the formation of 2′,4′-diol, the only alternative same ring diol product of 4′-monol metabolism.

Where Does the 4-Chloro-2′,3′-dihydroxybiphenyl Come From? When using 4-MCB as the initial substrate, the rank order of diol formation in the time course experiment (Figure 2) and that in the secondary metabolism experiment (Table 1) were identical. Both results showed that twice as much 2′,3′-diol as 2′,5′-diol was extractable from incubations with 4-MCB. In the secondary metabolism experiment (Table 1) using 4-chloro-2′-hydroxybiphenyl as initial substrate, the 2′,5′-diol was found at 6−10-fold higher yields than the 2′,3′-diol. Furthermore, no 2′,3′-diol was recovered from incubations with 4-chloro-3′-monohydroxybiphenyl, while the 2′,5′-diol was found as a product of both 4-chloro-2′-monohydroxybiphenyl and 4-chloro-3′-monohydroxybiphenyl. Thus, a contradiction existed because, with 4-MCB (Figure 2 and Table 1), more 2′,3′-diol than 2′,5′-diol was found, in contrast to using monohydroxybiphenyls as substrate (Table 1) where more 2′,5′-diol than 2′,3′-diol was found.

The simplest explanation of this anomaly was to propose a second source for the "extra" 2′,3′-diol, namely, a dihydrodiol. We did, in fact, find a diethyl ether-extractable dihydrodiol as a minor metabolite by using MS. Unfortunately, the amount of the dihydrodiol was below the detection limit of the GC and therefore was not quantifiable. Perhaps a 2′,3′-arene oxide was hydrated by epoxide hydrolase to yield the dihydrodiol (Scheme 1), which subsequently aromatized during the incubation, despite the absence of dihydrodiol dehydrogenase, or during the extraction procedure.

Conclusions. We found three previously unreported monohydroxy metabolites of 4-MCB (2′-monol, 3′-monol, and 2-monol), as well as two diol metabolites (2′,3′- and 2′,5′-dihydroxy). The production of the diol products can be explained by secondary hydroxylation of the monol metabolites and by the formation of at least one dihydrodiol that aromatizes to the 2′,3′-diol.

In the following paper (*35*), we are reporting separately that 4-MCB forms adducts with deoxyguanosine 3′-monophosphate. One of the major adducts was interpreted as due to an arene oxide, while two other major adducts were formed only upon the addition of an enzymatic oxidation system (horseradish peroxidase and hydrogen peroxide) (*35*). Preliminary studies suggest that the semiquinone and/or quinone product(s) of 4-chloro-2′,5′-dihydroxybiphenyl binds to protein and DNA as well as participates in redox cycling, which suggests patterns of cytotoxicity and genotoxicity exhibited by benzene (*32*) and *o*-phenylphenol (*33*). The data in the present paper and in the following paper strongly support the involvement of two classes of electrophiles, the arene oxides and the hydroquinone metabolites, arising during PCB metabolism.

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