Metabolism of 4-Chlorobiphenyl and 4,4′-Dichlorobiphenyl in the Rat: Qualitative and Quantitative Aspects

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Qualitative and quantitative aspects of the metabolism of 4-chlorobiphenyl and 4,4′-dichlorobiphenyl have been studied in the rat. Both chlorinated biphenyls are rapidly metabolized and excreted in the bile of cannulated animals. The major metabolites of 4-chlorobiphenyl were 4-hydroxy-4′-chlorobiphenyl (65%), 3,4-dihydroxy-4′-chlorobiphenyl (22%), 3-hydroxy-4′-chlorobiphenyl (7%), and either 3-methoxy-4-hydroxy-4′-chlorobiphenyl or 3-hydroxy-4-methoxy-4′-chlorobiphenyl (2.5%). The major metabolite of 4,4′-dichlorobiphenyl involved a chlorine shift to yield 4-hydroxy-3,4′-dichlorobiphenyl (91%); 3-hydroxy-4,4′-dichlorobiphenyl (trace) was the minor metabolite of this chlorinated biphenyl. Excretion of unmetabolized chlorinated biphenyl accounted for less than 10% of the dose in either case.

Efforts in our laboratory have recently been directed toward the development of a pharmacokinetic model which will predict the disposition and excretion of polychlorinated biphenyls (PCBs) in mammals. These efforts have reemphasized the necessity of both qualitative and quantitative metabolic data for a complete understanding of the disposition of any xenobiotic. Thus, we have provided such detailed data for the metabolism of 4-chloro-(1-CB) and 4,4′-dichlorobiphenyl (2-CB), to extend and complement the previously published descriptions of the major metabolites (Safe et al., 1974, 1975a,b,c) and rates of excretion (Matthews and Anderson, 1975b) of these compounds.

We have also confirmed the structures of the metabolites and speculated on the biological mechanisms involved in their formation.

MATERIALS AND METHODS

Chemicals. Samples of uniformily ^{14}C-labeled 4-chlorobiphenyl and 4,4′-dichlorobiphenyl as well as unlabeled 4-hydroxy-4′-chlorobiphenyl, 2-, 3-, and 4-methoxy-4′-chlorobiphenyl were kindly furnished by Dr. L. A. Levy of NIEHS. All compounds were prepared by published synthetic methods (Oswald et al., 1974) and their structural identity and purity were confirmed by mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy, and melting points. The labeled compounds were analyzed for radiochemical purity by means of a gas chromatograph equipped with a proportional counter detector. The specific activities were 0.931 mCi/mmol for the 4-chlorobiphenyl and 3.96 mCi/mmol for the 4,4′-dichlorobiphenyl. Unlabeled 4-chlorobiphenyl and 4,4′-dichlorobiphenyl were obtained from Aldrich Chemical Corp. (Milwaukee, Wis.). Reagents for silylation procedures were obtained from Pierce Chemical Co. (Rockford, Ill.). Methyl iodide and anhydrous K_2CO_3 were reagent grade chemicals obtained from Fisher Scientific Co. (Raleigh, N.C.). Reference samples of 3-hydroxy-4,4′-dichlorobiphenyl and 4-hydroxy-3,4′-dichlorobiphenyl were obtained from RFR Corp. (Hope, R.I.) and analyzed for identity and purity by GC–MS and ^1H and ^{13}C NMR spectrometry.

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Biological Methods. Adult male Sprague-Dawley rats (250–300 g) were dosed with 30 mg/kg of the radiolabeled chlorobiphenyl which had been diluted with the respective unlabeled compound. Corn oil (Mazola Pure Corn Oil) was used as the vehicle for daily dosing, which was repeated for 6 days in the case of the 4,4-dichlorobiphenyl and 7 days with the 4-chlorobiphenyl. Metabolite collection, isolation, and radioactivity measurements were similar to methods recently described (Chen et al., 1976). The metabolites were purified by multiple thin-layer chromatography using silica gel G plates and benzene saturated with formic acid–ether (10:2), then hexane–methanol (10:1) as the solvent system. Estimates of the rate of metabolism were made from bile excretion experiments utilizing methods which have been described previously (Matthews and Anderson, 1975a).

Identification Methods. GLC analysis was performed on a Hewlett-Packard Model 5700A gas chromatograph equipped with dual-flame ionization detectors. The columns used were: (1) $^1/_8$ in. × 6 ft stainless steel containing 10% OV-101 on 100/120 mesh Chromosorb W, and (2) $^1/_8$ in. × 2 m stainless steel containing 10% OV-225 on 100/120 mesh Chromosorb W-HP with a helium flow rate of 25 mL/min. The injection port and column were maintained at 250 °C. Electron impact (EI) mass spectra were obtained through the use of a Finnigan Model 9500 gas chromatograph interfaced by means of a glass jet separator to a Finnigan Model 1015D mass spectrometer. The ionizing electron energy was a nominal 70 eV. Molecular weights were confirmed by chemical ionization mass spectrometry. Chemical ionization (CI) mass spectra were obtained on a similar GC–MS system, except that no separator was used. For the CI GC–MS data, isobutane was the carrier as well as the reagent gas. The source pressure was maintained at 0.8 Torr, as measured by the thermocouple gauge supplied by the manufacturer. A System Industries 150 interactive data system was used for data acquisition and analysis. Samples were chromatographically introduced into the mass spectrometer by means of a 2 mm i.d. glass column 1.5 M long containing either 3% OV-101 or 3% OV-225 on 100/120 mesh Chromosorb W-HP.

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-100-12 spectrometer with Fourier transform and a 16K 620/L computer. Infrared spectra were obtained on a Perkin-Elmer 621 grating infrared spectrophotometer.

Trimethylsilyl (Me_3Si) ethers were prepared by placing a 0.1-mL aliquot of each sample in a reaction vial, evaporating the solvent with dry N_2, and adding approximately 0.1 mL of silylating reagent (hexamethyl-

Table I. Relative Intensities of Important Ions from the Mass Spectra of the 4-Chlorobiphenyl Metabolites

m/e	M_1	M_2	M_3	M_4
366			2.0	
364			6.1	
306				10.1
304				30
293				2.5
291				6.5
278	31.2	27		31.5
276	87.8	66	1.9	76.5
263	35.3	35.5		2.0
261	100	100		5.5

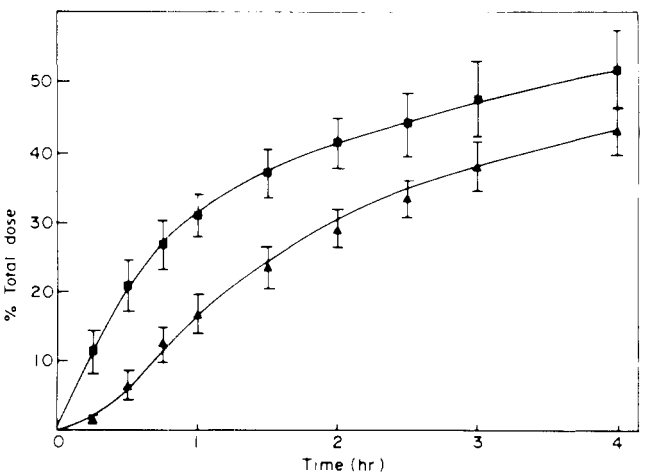

Figure 1. Cumulative excretion of total chlorinated biphenyl-derived radioactivity in bile. Data obtained by bile duct cannulation followed by IV administration of 0.6 mg/kg of 4-chlorobiphenyl (■) or 4,4′-dichlorobiphenyl (▲) into the femoral vein. Each point represents the mean ± standard deviation obtained with at least three animals.

disilazane–trimethyldichlorosilane–pyridine (3:1:9). The reaction mixture was allowed to stand at room temperature for at least 30 min.

RESULTS

Metabolites of 4-Chlorobiphenyl. Four metabolites were isolated from the excreta of rats dosed with 4-chlorobiphenyl. Preliminary analysis by GC–MS showed these to be two isomeric chlorobiphenylols, a chlorobiphenyldiol, and a methoxychlorobiphenylol (Table I). These were each analyzed in detail. These same metabolites were isolated from the bile of cannulated animals which had been treated intravenously with 4-chlorobiphenyl-^{14}C. Identification of the metabolites from bile was by cochromatography with metabolites previously isolated from the excreta. Less than 10% of the radiolabeled material extracted from bile after acid hydrolysis cochromatographed with the parent compound. The metabolism and excretion of 4-chlorobiphenyl in the bile was rapid; the initial half-life being only 3.6 h, Figure 1.

Monohydroxy Metabolites. The major metabolite of 4-chlorobiphenyl accounted for 65% of the radioactivity excreted and has previously been identified as 4-hydroxy-4′-chlorobiphenyl (Safe et al., 1974). The reported structure of this metabolite was confirmed by gas chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy.

A second monohydroxy metabolite accounted for only 7% of the total radioactivity, so its identification was based on more circumstantial evidence. The mass spectral data were obtained by use of GC–MS, with the metabolite analyzed as the trimethylsilyl ether. The prominent peaks at m/e 276, 278, and 261, 263, with the proper relative abundances for one chlorine, show the metabolite to be a hydroxychlorobiphenyl. This metabolite was available in neither sufficient quantity nor purity to permit analysis by nuclear magnetic resonance or infrared spectroscopy. Thus, identification was by comparative gas chromatographic behavior with that of standard compounds. The metabolite sample was converted to the methyl ether derivative and its GC behavior compared to that of the available methoxy-4′-chlorobiphenyl standards. It was found to coelute with 3-methoxy-4′-chlorobiphenyl on OV-101 at 220 °C and on OV-225 at 190 °C, while it was clearly resolved from the isomeric 2- or 4-methoxy-4′-chlorobiphenyl.

Table II. Calculated ^{13}C Chemical Shifts for the Isomeric 4-Chlorobiphenyldiols Compared to the Observed ^{13}C NMR Spectrum[a]

3′-5′	2′-6′	2′-5′	2′-4′	3′-4′	2′-3′	Obsd
157.2	155.7	148.5	155.7	143.1	143.1	143.9
157.2	155.7	147.0	155.6	141.5	141.6	143.5
144.4	139.7	139.7	139.7	139.7	139.7	139.0
139.7	133.5	133.5	133.5	135.7	133.5	133.2
133.9	130.1	130.3	130.3	133.5	130.3	132.7
129.3	129.3	129.3	130.2	129.3	129.3	128.7
129.3	129.3	129.3	129.3	129.3	129.3	128.7
128.7	128.7	128.7	129.3	128.7	128.7	127.8
128.7	128.7	128.7	128.7	128.7	128.7	127.8
107.4	116.2	117.6	128.7	121.5	123.0	119.5
107.4	108.9	116.1	108.9	117.6	121.5	115.7
101.9	108.9	116.0	103.5	116.1	116.0	114.0

[a] All chemical shifts relative to Me_3Si.

Dihydroxy Metabolite. The mass spectrum of the trimethylsilyl derivative of this metabolite shows molecular ions at m/e 364, 366 and indicates the presence of one chlorine. This information is consistent with expectations for a dihydroxychlorobiphenyl metabolite. ^1H and ^{13}C nuclear magnetic resonance spectra were obtained. The ^1H NMR spectrum shows an AA′BB′ absorption pattern, indicating that the chlorine substituted ring is intact, as well as a complex absorption due to the protons on the hydroxylated ring. Table II lists the predicted chemical shifts for each of the carbons in the remaining possible isomers. These are calculated by treating the hydroxylated ring as a substituted benzene and assuming substituent effects on chemical shifts to be additive according to the method of Levy and Nelson (1972). Thus, all possibilities except 2,3-dihydroxy-4′-chlorobiphenyl and 3,4-dihydroxy-4′-chlorobiphenyl can be eliminated from further consideration. As has been well documented (Conley, 1966), infrared spectroscopy can be useful in determining the substitution pattern on an aromatic ring. Thus, an IR spectrum of the sample was obtained, after it was converted to the diacetate to reduce hydrogen bonding effects. The aromatic C–H out of plane bending vibrations show peaks at 813 cm^{-1} with a shoulder at 820 and 882 cm^{-1} while there was no peak in the regions 705–745 or 760–780 cm^{-1}, indicating a 1,2,4-substitution pattern on the trisubstituted aromatic ring. Thus, the structure was assumed to be 3,4-dihydroxy-4′-dichlorobiphenyl. This metabolite accounted for 22% of the radioactivity excreted.

Methoxybiphenyl Metabolite. Only trace amounts of this metabolite were isolated, 2.5% of the total dose, thus positive identification was not possible. This metabolite was analyzed by GC–MS as the trimethylsilyl ether. The resulting mass spectrum showed molecular ions at m/e 306 and 308 (one Cl), with intense peaks at m/e 276 and 278 corresponding to the loss of CH_2O from the molecular ion. The sample was treated with CH_3I/K_2CO_3. The resulting derivative showed molecular ions at m/e 320

and 322 (one Cl) and coeluted with the similar derivative of the dihydroxy metabolite, when gas chromatographed on OV-101 (220 °C) or OV-225 (190 °C). Thus the oxygen functions were assumed to be in the same positions and this metabolite was either 3-methoxy-4-hydroxy-4′-chlorobiphenyl or 3-hydroxy-4-methoxy-4′-chlorobiphenyl. This methylated catechol is probably the product of a reaction mediated by a nonspecific catechol-*O*-methyltransferase (Molirold and Axelrod, 1971).

Metabolites of 4,4′-Dichlorobiphenyl. The major metabolite of 4,4′-dichlorobiphenyl was examined by GC–MS. Mass spectra of both underivatized material and the trimethylsilyl ether were acquired. The underivatized material showed a molecular ions at *m/e* 238, 240, and 242, with the approximate 9:6:1 ratio indicating two chlorines. This was confirmed by examination of the mass spectrum of the trimethylsilyl ether, which exhibits molecular ions at *m/e* 310, 312, and 314 (two Cl), with fragment ions at *m/e* 295, 297, and 299 (M – CH_3) and *m/e* 259, 261, and 263 (M – CH_3 – HCl). Thus this metabolite is a hydroxydichlorobiphenyl and was further analyzed by ^1H NMR spectrometry.

The ^1H NMR spectrum of the major metabolite consisted of an AA′BB′ pattern centered at $\delta H = 7.42$ ppm with major peaks at 7.55, 7.52, 7.44, 7.40, 7.34, and 7.32 ppm for the 4-chloro ring (Wilson, 1975). The ABX pattern for the hydroxychloro ring is obscured by the AA′BB′ absorption except for peaks at $\delta H = 7.41$ ppm and 7.02 ppm. The hydroxyl proton absorbs at $\delta H = 3.6$ ppm. A reference sample of 4-hydroxyl-3,4′-dichlorobiphenyl gave an identical spectrum under the same conditions. This spectrum is also very similar to that previously attributed to the metabolite of 4,4′-dichlorobiphenyl in the rat (Safe et al., 1974). A reference sample of 3-hydroxy-4,4′-dichlorobiphenyl gave a spectrum with an AA′BB′ pattern centered at $\delta H = 7.45$ ppm with major peaks at 7.56, 7.47, 7.43, and 7.34 ppm. The nonobscured ABX peaks were observed at 7.24, 7.22, 7.11, 7.09, 7.04, and 7.02 ppm. Thus, the ^1H NMR data suggest 4-hydroxy-3,4′-dichlorobiphenyl to be the major metabolite. This identification was confirmed by cochromatography of the metabolite and 4-hydroxyl-3,4′-dichlorobiphenyl on 3% OV-101 and 3% OV-225 at 180 °C.

The mass spectrum of the minor metabolite of 4,4′-dichlorobiphenyl also shows molecular ions at 310, 312, and 314 (9:6:1) when examined as the trimethylsilyl ether and is nearly identical with that of the major metabolite. Since insufficient material was available for an NMR spectrum, identification is based upon GC–MS evidence. The methyl ether of the minor metabolite was found to coelute with the methyl ether of 3-hydroxyl-4,4′-dichlorobiphenyl on the 3% OV-101 and 3% OV-225 columns and gave a mass spectrum identical with that of the reference material. Thus, this metabolite is assumed to be 3-hydroxyl-4,4′-dichlorbiphenyl.

As with 4-chlorobiphenyl, the metabolites of 4,4′-dichlorobiphenyl were identified in the bile of cannulated animals by cochromatography and less than 10% of the radiolabeled material extracted from bile after acid hydrolysis or excreted in urine and feces cochromatographed with the parent compound. The metabolism and excretion of 4,4′-dichlorobiphenyl was somewhat less rapid than that of 4-chlorobiphenyl, but an initial half-life of 6.1 h for excretion in the bile implies that metabolism and excretion were still relatively rapid, Figure 1.

DISCUSSION

The metabolites of 4-chlorobiphenyl which we have identified can best be explained in terms of an arene oxide intermediate involving the 3′ and 4′ carbons (Daly et al., 1972). The hydroxy metabolites probably arise from the rearrangement of the arene oxide to either a 3′- or 4′-keto functional group, followed by enolization to the aromatic hydroxide. Since the 4′-keto group would be stabilized more by the other benzene ring than would the 3′-keto group, it predominates as a metabolite of 4-chlorobiphenyl. The dihydroxy metabolite probably arises from the action of an epoxide hydrase on the arene oxide to form a dihydrodiol, which is then dehydrogenated by a cytoplasmic dehydrogenase (Ayengar et al., 1959) to form the catechol. The final metabolite is explained by further metabolism of the catechol by a catechol-*O*-methyltransferase. Catechol-*O*-methyltransferase is found in the cytoplasm of liver and other tissues, and plays an important role in the inactivation of catechols, such as noradrenaline (Molirold and Axelrod, 1971). As anticipated for lipophilic xenobiotics such as these chlorinated biphenyls, there was little excretion prior to metabolism. The observation that each of the metabolites was excreted in bile of cannulated animals which received an intravenous dose proves that these metabolites were formed in the animal and not in the gut as a result of microbial metabolism. The rate of excretion indicates that metabolism was rapid and that the initial half-life in the intact animal should be relatively short. The term "initial half-life" is used to differentiate the first from subsequent half-lives which are likely to be significantly longer as a result of the accumulation of these lipophilic compounds in the more lipophilic and poorly perfused tissues such as adipose tissue and skin.

It is also of interest to note that cumulative excretion of these chlorobiphenyls in bile in 4 h (Figure 1) exceeds their cumulative excretion in feces in 48 h (Matthews and Anderson, 1975b). This observation, and the observation that in the intact animal a significant portion of a dose of these chlorobiphenyls is excreted in the urine (Matthews and Anderson, 1975b), implies that much of the material excreted in bile is adsorbed from the gut and subsequently excreted in the urine. This speculation is supported by our unpublished observation that the gut contents of animals treated intravenously and sacrificed between 15 min and 4 h following treatment often contained more radioactivity than was excreted in the feces in 24 h. The time for passage through the gut of these rats is approximately 10 to 12 h. These results also imply that the metabolites of 4-chlorobiphenyl are more readily absorbed than those of 4,4′-dichlorobiphenyl, a phenomenon which may be related to the degree of chlorination, but more study will be required to fully elucidate the processes involved.

The metabolites of 4,4′-dichlorobiphenyl can also best be explained by the existence of an arene oxide intermediate. The position of this arene oxide, i.e., meta–para, is consistent with that which would account for the reported metabolites of PCBs which have unchlorinated meta and para positions (Gardner et al., 1973; Safe et al., 1975a; Chen et al., 1976). A meta–para arene oxide intermediate has also been proposed in the metabolism of one PCB which is chlorinated in the para position but does not have any adjacent unsubstituted carbon atoms (Hutzinger et al., 1974; Sundstrom et al., 1976). Safe et al. (1976) have reported 4-hydroxy-3,4′-dichlorobiphenyl to be a minor metabolite of 4,4′-dichlorobiphenyl in the rabbit. It was anticipated, as has been reported by Safe et al. (1974, 1975b), that the metabolism of 4,4′-dichlorobiphenyl by the rat would proceed via oxidation of the ortho–meta positions since they are not chlorinated and should be more available to enzymatic attack. If

oxidation had occurred at the ortho–meta positions, 3-hydroxy-4,4′-dichlorobiphenyl should have been the major rather than the minor metabolite of this PCB. As it is, the rate of 4,4′-dichlorobiphenyl metabolism and the predominance of the 4-hydroxy metabolite imply that the formation of an arene oxide between a chlorinated and an unchlorinated carbon atom proceeds quite readily so long as other substitution of the molecule does not restrict the approach of the enzyme.

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Esterase and Oxidase Activity of House Fly Microsomes Against Juvenile Hormone Analogues Containing Branched Chain Ester Groups and Its Induction by Phenobarbital

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Branched-chain esters of 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid and 3,7,11-trimethyl-2,4-dodecadienoic acid were compared as substrates for microsomal esterases prepared from the insecticide-susceptible CSMA strain of house flies. In vitro hydrolysis of the seven esters was very slight compared to that measured previously with straight-chain esters of the same group of compounds. The analogues were readily metabolized by microsomal oxidases of the CSMA strain and by those of an insecticide-resistant strain (Rutgers). Phenobarbital in the diet of adult house flies caused increased oxidative metabolism of the analogues, up to 16-fold in the CSMA strain and up to fourfold in the Rutgers strain. In bioassays conducted during the pupal stage, when microsomal oxidase activity is low compared to esterase activity, there was considerable difference in response to the seven analogues. Methoprene, the isopropyl ester of the 11-methoxy acid, was highly effective at 0.1 μg/pupa while some other analogues were ineffective at 10 μg/pupa. Since there was only a twofold difference among the analogues, in the rate of hydrolysis by the esterases, it is concluded that other factors determine the biological activity of these compounds.

In house fly bioassays of the morphogenic activity of juvenile hormone analogues of the alkyl-3,7,11-trimethyl-2,4-dodecadienoates, Henrick et al. (1976) found that of the four most toxic analogues, three were branched-chain esters such as isopropyl (methoprene ZR-515) and *sec*-butyl (ZR-644). In our study of the metabolism of six analogues of the same general type by housefly esterases, we found that methoprene was most resistant to these enzymes (Yu and Terriere, 1975). This result correlated well with morphogenic activity in bioassays performed at the same time. Similar results were obtained with blow flies and flesh flies, only methoprene being highly active in bioassays and highly resistant to esterase action (Terriere and Yu, 1977). These observations suggest that house fly esterases may be unable to accomodate compounds in which the ester function is a branched-chain alkyl and that resistance to esterase attack in vivo may be an important requirement for biological activity.

Compounds of the type mentioned above, including methoprene, are also converted to various oxidation products in the house fly (Quistad et al., 1975) and are metabolized by the microsomal oxidases of the house fly (Yu and Terriere, 1975, 1977) and of the blow fly and flesh fly (Terriere and Yu, 1977). The suitability of the various analogues as substrates for these oxidases seems to vary considerably but this may be only apparent since, under the conditions of assay for the oxidases, the esterases are also present and active. Furthermore, the oxidases also attack the products of the esterase, the JHA acids (Yu and Terriere, 1977), thus complicating the interpretation of assay results. Another aspect of these oxidative reactions is that phenobarbital, a well-known inducer of microsomal oxidases in the house fly (Yu and Terriere, 1973), has a

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