gas–liquid chromatography. In the fish removed within 24 hr of treatment, endothall concentration in the flesh ranged from 0.02 to 0.1 ppm.

After 30-min exposure to water containing 2 ppm of [^{14}C]endothall, ^{14}C-labeled residues (expressed as endothall) ranging from 0.02 to 0.04 ppm were detected in the blood. After 60 min, the concentration of ^{14}C in the blood increased up to 0.08 ppm. Longer exposure up to 4 hr did not significantly increase the level of radioactivity in the blood.

The concentration of ^{14}C in the various tissues, 48 hr after the fish were fed [^{14}C]endothall through the digestive tract, is shown in Table III. The results show that the herbicide is absorbed by the intestinal tract though the fish were observed to eliminate 73% of the administered herbicide during this period. In the fish fed ^{14}C-labeled herbicide through the digestive tract, like the bath-exposed fish, the concentration of radioactivity was highest in the viscera and lowest in the flesh. The pattern of distribution of radioactivity in the various tissues in the feed-exposed fish was similar to that observed for the bath-exposed fish, with the exception that the scales had a relatively lower proportion of the total radioactivity.

These findings have demonstrated that bluegills absorb endothall directly from water as well as through the intestinal tract. Adsorption of endothall on the scales also contributes to the herbicide residues in the fish. This is supported by the observation that the proportion of ^{14}C in the scales decreased when the fish were fed endothall through the digestive tract.

Thin-layer chromatographic analysis of the methanol extract from the fish treated with the herbicide for 48 hr showed that all the ^{14}C in the alcohol extractable fraction was present in the form of unchanged endothall (R_f 0.48 and 0.75 on silica gel and cellulose plates, respectively). The ^{14}C in the extract co-chromatographed with authentic [^{14}C]endothall. In contrast to aquatic microorganisms which were found to readily degrade endothall (Sikka and Saxena, 1973), bluegills do not appear to be capable of metabolizing the herbicide.

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The Mechanism of Chlorobiphenyl Metabolism

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4-Chlorobiphenyl was administered to rabbits and the major urinary metabolites were identified as 4′-chloro-4-biphenylol and 4′-chloro-3,4-biphenyldiol. It was also shown that 4′-chloro-4-biphenylol was also converted into the diol as well as its monomethyl derivatives. The biohydroxylation pathway was further investigated using 4′-[^{2}H]-4-chlorobiphenyl as a substrate. The 4′-chloro-4-biphenylol metabolite retained 79% of the deuterium and the results are consistent with the intermediacy of an arene oxide in the first hydroxylation reaction. The diol metabolite retained ca. one-half the deuterium found in the phenol (39%) and it is therefore not formed directly from the arene oxide but by direct hydroxylation of the phenolic metabolite. The sequence of hydroxylation of chlorobiphenyl is, therefore, analogous to the stepwise hydroxylation of phenylalanine to give 3,4-dihydroxyphenylalanine and butamoxane to give 6,7-dihydroxybutamoxane.

Polychlorinated biphenyls (PCB) are industrial compounds which are now recognized as among the most widespread pollutants in the global ecosystem (Hutzinger et al., 1974b; Risebrough et al., 1968; Fishbein, 1973). Residues have been identified in both the terrestrial and aquatic environments (Koeman et al., 1969; Harvey et al., 1974; Jensen et al., 1969; Hom et al., 1974) as well as in animals (Bagley et al., 1970; Butler, 1973; Prestt et al., 1970) and humans (Biros et al., 1970; Jamieson et al., 1973). Recent work has shown that both commercial PCB mixtures and pure isomeric chlorobiphenyls are metabolized by animals (Hutzinger et al., 1972, 1974a; Safe et al., 1974, 1975a,b; Burse et al., 1974; Greb et al., 1973; Yoshimura and Yamomoto, 1973; Block and Cornish, 1959; Goto et al., 1974; Gardner et al., 1973), plants (Moza et al., 1973, 1974), and microorganisms (Wallnofer et al., 1973; Ahmed and Focht, 1973; Maas et al., 1975) to give a range of hydroxylated metabolites. The major rabbit urinary metabolites of 2,2′,5,5′-tetrachlorobiphenyl were 2,2′,5,5′-tetrachloro-3-biphenylol, 2,2′,5,5′-tetrachloro-4-biphenylol, and *trans*-3,4-dihydro-2,2′,5,5′-tetrachloro-3,4-biphenyldiol and these all can conceivably be formed from the 3,4-epoxy intermediate. NIH rearrangement (Jerina, 1974; Jerina and Daly, 1974; Daly et al., 1972) of this intermediate (i.e., *trans*-3,4-dihydro-2,2′,5,5′-tetrachloro-3,4-epoxybiphenyl) could yield the two phenolic metabolites and hydrolysis of the epoxide would yield the dihydrodiol product. The experiments described in this paper are concerned with the mechanism of PCB hydroxylation in the rabbit using 4-chlorobiphenyl, 4′-chloro-4-biphenylol, and 4′-[^{2}H]-4-chlorobiphenyl as model substrates.

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MATERIALS AND METHODS

Chlorobiphenyl Substrates. 4′-Chloro-4-biphenylol was synthesized as described (Savoy and Abernethy, 1942).

Table I. Identification and Recovery of the Rabbit Urinary Metabolites of 4-Chlorobiphenyl and 4′-Chloro-4-biphenylol

Compd (M^+)[a]	Substrate[c] 4-Chlorobiphenyl Free, mg	4-Chlorobiphenyl Bound, mg	4′-Chloro-4-biphenylol Free, mg	4′-Chloro-4-biphenylol Bound, mg	NMR data for the metabolites, δ, ppm
4′-Chloro-4-biphenylol (*m/e* 204)	80.4	15.0	72.6	12.9	7.55 (d, J = 8.2 Hz), 7.48 (d, J = 8.2 Hz), 7.42 (d, J = 8.2 Hz), 7.18 (d, J = 8.2 Hz)
4′-Chloro-3-methoxy-4-biphenylol[b] 4′-Chloro-4-methoxy-3-biphenylol (*m/e* 234)			13.2	3.0	8.0–7.0 (m), 3.94 (s), 3.91 (s)
4′-Chloro-3,4-biphenyldiol (*m/e* 220)	10.5	1.5	45.6	7.5	7.43 (d, J = 8.2 Hz), 7.37 (d, J = 8.2 Hz), 7.08 (d, J = 1.9 Hz), 7.01 (q, J = 8.2, 1.9 Hz), 6.93 (d, J = 8.2 Hz)

[a] M^+ = molecular ion. [b] The two methoxy isomers could not be separated by TLC and were analyzed by gas–liquid chromatography (GLC) as described (Safe et al., 1975b); the relative percentages of the methoxy isomers were determined by the GLC peak areas and the values given in the table were calculated from the yield of diol obtained after demethylation of the mixture. [c] The figures given are based on milligrams of compound recovered from the urine of a single rabbit; each rabbit (average wt, 5 kg) was given 300 mg of substrate by intraperitoneal injection in a vegetable oil solution.

4-Chloro-4′-iodobiphenyl (1.0 g, prepared from the corresponding 4-amino-4′-chlorobiphenyl) was dissolved in dry tetrahydrofuran (25 ml) and lithium aluminum deuteride (0.20 g) was carefully added. The mixture was then refluxed for 8 hr, cooled, and quenched by the addition of deuterium oxide (5 ml). The products were isolated with ether extraction and purified by preparative thin-layer chromatography (TLC). The major product was 4′-[^{2}H]-4-chlorobiphenyl (0.280 g, 85% purity by mass spectrometry) and some 4,4′-[$^2H_2$]biphenyl (0.05 g) was also isolated. Unlabeled 4-chlorobiphenyl was obtained from commercial sources (Eastman) and used directly as a metabolic substrate.

Animal Feeding Experiments. The chlorobiphenyl substrate (300 mg) was dissolved in vegetable oil (5–10 ml) and administered intraperitoneally to a male rabbit (ca. 5 kg) which was housed in a metabolic cage. Urine and feces were collected for 10 days after administration of the xenobiotic.

Extraction and Analysis. The urine samples were acidified to pH 4–5 with the addition of glacial acetic acid and extracted with ether to give the free metabolite extract. The urine samples were then carefully acidified with concentrated sulfuric acid to give a 2 N sulfuric acid solution which was then heated on a steam bath for 2 hr. The solution was cooled and then extracted with ether to give the bound metabolite extract.

The 4-chlorobiphenyl and 4′-[^{2}H]-4-chlorobiphenyl metabolites were isolated by preparative TLC as described (Safe et al., 1975b) and the 4′-chloro-4-biphenylol metabolites were also obtained by TLC (Safe et al., 1975a). The crude hydroxy fractions were purified via their acetate derivatives and the corresponding hydroxylated metabolites were obtained by subsequent basic hydrolyses of the acetates. Demethylations were carried out by treatment of the metabolite with boron tribromide (0.1 ml) in methylene chloride (2 ml) for 9 hr at 25°. The solution was diluted with water (15 ml) and methylene chloride (20 ml). The demethylation product was then obtained by evaporation of the dried methylene chloride extract.

Spectroscopic Methods. Mass spectra were obtained on a Varian MAT CH-7 spectrometer and nuclear magnetic resonance (NMR) spectra were recorded on a Varian HR220 spectrometer using deuteriochloroform as solvent.

RESULTS

A summary of the major rabbit urinary metabolites of 4-chlorobiphenyl and 4′-chloro-4-biphenylol is given in Table I. The former substrate was converted into two metabolites, 4′-chloro-4-biphenylol and 4′-chloro-3,4-biphenyldiol; 4′-chloro-4-biphenylol was metabolized to give 4′-chloro-3,4-biphenyldiol as the major product and smaller amounts of a mixture of 4′-chloro-3-methoxy-4-biphenylol and 4′-chloro-4-methoxy-3-biphenylol. The latter two compounds could not be separated by TLC; however, the NMR spectrum of this fraction gave two methoxyl signals at δ 3.94 and 3.91 ppm. Treatment of the mixture with boron tribromide in methylene chloride gave 4′-chloro-3,4-biphenyldiol as the sole product. These metabolism results with 4-chlorobiphenyl and 4′-chloro-4-biphenylol were similar to those obtained from the metabolism of these substrates in the rat (Safe et al., 1974; Safe et al., 1975a).

Administration of 4′-[^{2}H]-4-chlorobiphenyl also gave 4′-chloro-4-biphenylol and 4′-chloro-3,4-biphenyldiol as metabolites and these were examined by mass spectrometry. The phenol gave molecular ion species at *m/e* 205 and 204 and 79% of the deuterium was retained in this metabolite. Examination of the NMR spectrum showed that the signal at 7.18 ppm was diminished in its expected intensity and confirmed the shift of the 4-^{2}H atom to the 3 position. The mass spectrum of the 4′-chloro-3,4-biphenyldiol isolated from this experiment gave molecular ion peaks at *m/e* 221 and 220 and 39% of the deuterium was retained in this metabolite. The mass spectral data were obtained using the metabolites isolated from the free urine extract. Treatment of the deuterated chlorobiphenyls with 2 N sulfuric acid results in some H–^{2}H exchange and for this reason the mass spectral data for the metabolites isolated from the bound fraction were not used for calculation of the ^{2}H retention.

DISCUSSION

The results obtained for the metabolism of 4-chlorobiphenyl and 4′-chloro-4-biphenylol in the rabbit were similar to the experiments already described for rats (Safe et al., 1974, 1975a). In addition, the metabolism of a series of chlorobiphenyls including 2,3-dichlorobiphenyl, 2,4,6-trichlorobiphenyl, 2,3,5,6-tetrachlorobiphenyl, and 2,3,4,5,6-pentachlorobiphenyl also gave the corresponding phenols,

catechols, and methoxyphenols as the major metabolites (Goto et al., 1974). It is conceivable that the formation of these products could occur via an arene oxide which on rearrangement would give the phenol and on hydrolysis followed by dehydrogenation would give the catechol (Daly et al., 1972). The methoxyphenols could then be formed by methylation of the catechols. The conversion of 4′-chloro-4-biphenylol into the corresponding diol presents a second alternative pathway for the formation of this metabolite. Analysis of the two urinary metabolites of 4′-[^{2}H]-4-chlorobiphenyl clearly resolves this problem. The mass spectrum of 4′-chloro-4-biphenylol shows that 79% of the deuterium is retained in the metabolite and this is consistent with an arene oxide intermediate in which the hydrogen has migrated from the site of hydroxylation to a neighboring carbon atom (the NIH shift). This result is supported by the NMR spectrum of the metabolite in which the signal at 7.18 ppm is of diminished intensity due to the presence of deuterium. The NIH shift has also previously been reported for the oxidation of biphenyl to give biphenylol (Daly et al., 1968) and 4-fluorobiphenyl to give 4′-fluoro-4-biphenylol (Daly et al., 1969).

The mass spectrum of the 4′-chloro-3,4-biphenyldiol metabolite gave molecular ions at *m/e* 220 and 221 and 39% of the deuterium from the original 4′-[^{2}H]-4-chlorobiphenyl substrate was retained in the metabolite. This result supports the formation of the diol by two independent hydroxylation reactions in which the first step occurs via an arene oxide followed by a second biological hydroxylation reaction. The monooxygenase enzymes responsible for the insertion of the second hydroxyl group thus remove one-half of the deuterium present at the 3 position in 4′-chloro-4-biphenylol. This sequence of events is analogous to the stepwise hydroxylation of phenylalanine to give tyrosine which is in turn hydroxylated yielding 3,4-dihydroxyphenylalanine (Guroff et al., 1966). Similarly, it has also been shown that the oxidation of butamoxane to give 6,7-dihydroxybutamoxane proceeds via two consecutive hydroxylation reactions rather than by the more direct route through an arene oxide intermediate (Murphy et al., 1974).

The arene oxides of several polycyclic aromatic hydrocarbons are known to be more active as carcinogens than either the parent hydrocarbon, the corresponding phenols, or dihydrodiols (Grover et al., 1971). The results reported in this paper show that a lower chlorinated PCB isomer, 4-chlorobiphenyl, is also metabolized via an arene oxide intermediate. Since the biological properties of PCB and their metabolites are not fully understood this is, therefore, an area of environmental concern. The possible toxicological properties of PCBs and their metabolites are currently under investigation in our laboratory.

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