POLYCHLORINATED BIPHENYLS: HYDROXYLATED URINARY METABOLITES OF 2,5,2',5'-TETRACHLOROBIPHENYL IDENTIFIED IN RABBITS

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Summary:

Rabbits fed 2,5,2',5'-tetrachlorobiphenyl excreted three hydroxylated tetrachlorobiphenyl metabolites in urine. Two were identified as 3-hydroxy-2,5,2',5'-tetrachlorobiphenyl and 4-hydroxy-2,5,2',5'-tetrachlorobiphenyl by chromatographic and spectrometric comparison with synthetically prepared compounds. The third was shown by infrared and mass spectra, and the infrared spectrum of its dehydration product, to be trans-3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl. Metabolism of 2,5,2',5'-tetrachlorobiphenyl probably occurs via an arene oxide intermediate.

Introduction:

2,5,2',5'-Tetrachlorobiphenyl is one component of the class of man-made environmental pollutants known as polychlorinated biphenyls (1-3). The existence of metabolic products of polychlorinated biphenyls in animals had not been reported until Hutzinger and co-workers published evidence of hydroxylated products of a dichlorobiphenyl and a tetrachlorobiphenyl in rat and pigeon excreta (4). They reported that in rats and pigeons 2,5,2',5'-tetrachlorobiphenyl was metabolized to a compound containing four chlorine atoms and exhibiting a molecular ion of 306 e.m.u. in the mass spectrum. They postulated that the metabolite was a hydroxy-tetrachlorobiphenyl, but structural proof was not produced. Previously other workers had reported that hydroxylated urinary metabolites of various chlorinated aromatic chemicals were formed in other animals (5-7). More recently Yoshimura and Yamamoto found a phenolic polychlorinated biphenyl metabolite of 3,4,3',4'-tetrachlorobiphenyl in rat feces (8). The absence of this metabolite in the urine of the rat could be an indication of microbial degradation of this polychlorinated biphenyl in the lower rat gut. Our work was undertaken in the hope that determination

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of the substitution positions of chlorine and hydroxy groups on the biphenyl nucleus of urinary metabolites might provide clues to the mechanism of metabolic conversion of polychlorinated biphenyls in animals.

Materials and Methods:

2,5,2',5'-Tetrachlorobiphenyl was prepared by Webb's modification of the Gomberg-Bachmann coupling reaction (2, 9). The final product was more than 99% pure based on gas chromatographic analysis (10).

Three male New Zealand white rabbits weighing 2.0 to 2.5 kg were each given a corn oil solution of 10 mg 2,5,2',5'-tetrachlorobiphenyl by stomach tube. Three control rabbits received only corn oil. All rabbits were provided with food and water ad libitum throughout the study. Urine was collected and analyzed daily for one week.

Ether extracts of urine were analyzed by electron capture gas chromatography (10). Chromatograms of extracts of urine of tetrachlorobiphenyl-fed rabbits exhibited three peaks that were absent in chromatograms of the control rabbits' urine extracts. The samples were subsequently put on 11 x 250 mm columns of silica gel that had been deactivated by the addition of 3% water. Metabolites were eluted in 10 ml cuts with 200 ml portions of petroleum ether and of petroleum ether containing 1, 3, 12, and 20% ethyl acetate, respectively. Metabolites I and II, designated by their elution order, appeared in the 3% ethyl acetate-petroleum ether fraction and metabolite III eluted in the 20% ethyl acetate-petroleum ether fraction. Fraction collection was monitored by electron capture gas chromatography.

Metabolites I and II were further purified by two-dimensional thin layer chromatography on 0.5 mm thick, 20 x 20 cm silica gel layers containing fluorescent indicator. Toluene and methylene chloride were the developing solvents. Metabolite III was purified by two-dimensional thin layer chromatography with ethyl ether and methylene chloride as the developing solvents (R_f's = 0.9 and 0.1, respectively). Metabolites obtained after ether extraction of the silica gel adsorbent were pure enough for infrared spectroscopic analysis.

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The three isomeric hydroxy-2,5,2',5'-tetrachlorobiphenyls were synthesized by coupling 2,5-dichloroanisole with 2,5-dichlorobenzene via the 2,5-dichlorodiazonium chloride (2, 9) followed by cleavage of the methyl ethers with pyridine hydrochloride (11). The hydroxy isomers were separated into three distinct bands by thin layer chromatography on 0.5 mm thick, silica gel plates containing fluorescent indicator, with toluene as developing solvent. NMR spectra of individual isomers were obtained on the Varian HA-100 spectrometer. NMR coupling constants, J, of the two protons on the hydroxylated ring were 0.25 Hz, 2.4 Hz, and 8.6 Hz for the 4-, 3-, and 6-hydroxy-2,5,2',5'-tetrachlorobiphenyls, respectively. Coupling constants for the ortho, meta, and para protons in 2,5-dichlorophenol were 8.55 Hz, 2.38 Hz, and 0.24 Hz, respectively. Kofler hot stage melting point ranges of tetrachlorobiphenyl compounds crystallized from ethyl ether were 94-96°C for the 3-hydroxy isomer, 100-102°C for the 4-hydroxy isomer, and 109.5-111.5°C for the 6-hydroxy isomer.

Results:

Metabolites I and II were identified as the 3- and the 4-hydroxy-2,5,2',5'-tetrachlorobiphenyl, respectively, by comparison of their spectral and chromatographic characteristics with those of the synthetic materials. Table I compares the chromatographic data and Figure 1 the IR spectra. All hydroxy-tetrachlorobiphenyl isomers, whether synthetic or metabolic, had identical mass spectra: typically, a molecular ion at 306 a.m.u. and a chlorine cluster at the molecular ion, indicating four chlorine atoms per molecule. Although synthetic 6-hydroxy-2,5,2',5'-tetrachlorobiphenyl could be extracted from rabbit urine with ether, it was not found as a metabolite.

The mass spectral fragmentation pattern of metabolite III suggested that it was a dihydro-diol tetrachlorobiphenyl. It displayed a molecular ion at 324 a.m.u. (306 a.m.u. + H_2O) and indicated four chlorine atoms per molecule. When a methylene chloride solution of metabolite III was treated with concentrated sulfuric acid, metabolite III was dehydrated to 4-hydroxy-

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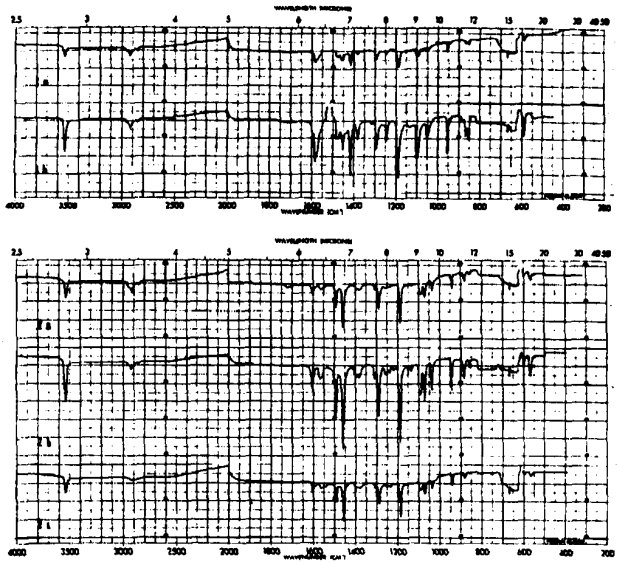

Figure 1:

Infrared spectra of metabolite I(1a), 3-hydroxy-2,5,2',5'-tetrachlorobiphenyl (1b), metabolite II (2a), 4-hydroxy-2,5,2',5'-tetrachlorobiphenyl (2b), and one of the dehydration products of metabolite III (2c). Spectra were obtained in CCl_4 solutions in 0.1 mm NaCl ultramicro liquid cells (Wilks Scientific Corp., South Norwalk, Conn.). Major bands seen in solution spectra were also evident in spectra of hydroxy-tetrachlorobiphenyls in KBr discs.

2,5,2',5'-tetrachlorobiphenyl and some 3-hydroxy-2,5,2',5'-tetrachlorobiphenyl isomer. Chromatographic and spectral data for the dehydration products are shown in Table I and Figure 1, respectively.

The infrared spectrum of metabolite III in micro-KBr disc (Figure 2)

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Table 1. Chromatographic data of synthetic and metabolic hydroxy-2,5,2',5'-tetrachlorobiphenyls (TCB).

Product	GLC R_A*	TLC R_f†
3-Hydroxy-2,5,2',5'-TCB‡	1.43	0.59
Metabolite I	1.43	0.59
Dehydration products of metabolite III	1.46, 1.54	0.58, 0.51
4-Hydroxy-2,5,2',5'-TCB	1.54	0.51
Metabolite II	1.54	0.51
6-Hydroxy-2,5,2',5'-TCB	1.36	0.78

*Retention time relative to the pesticide, aldrin, on 6 ft x 1/4 in. 10% DC-200 column at 200°C. Nitrogen flow was 120 ml/min.

†Silica gel plates developed with toluene. R_f of 2,5-dichlorophenol in toluene was 0.55.

‡Tetrachlorobiphenyl.

indicated the presence of a conjugated diene (1648 cm^{-1}, with rather high intensity). The C-O stretching bands for axial and equatorial secondary alcohols were present at 1088 cm^{-1} and 1043 cm^{-1} (12). The prominent phenolic C-O stretching band at approximately 1185 cm^{-1} (12) evident in the infrared spectra of 3- and 4-hydroxy tetrachlorobiphenyls (Figure 1), was absent in the spectrum of metabolite III, indicating that metabolite III was not a phenol. Metabolite III was therefore identified as a 3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl, since this structure was uniquely consistent with all of the above data.

Infrared spectral data were also used to establish the conformation of the hydroxy groups at positions 3 and 4. If metabolite III were a trans-dihydro-diol, an equilibrium between the diaxial and diequatorial forms would

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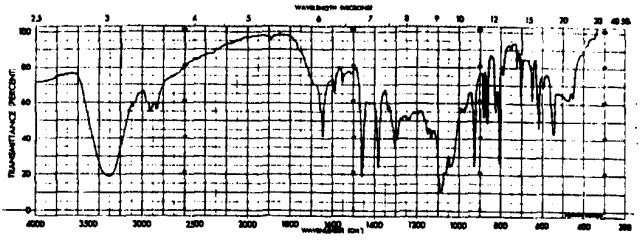

Figure 2:

Infrared spectrum of metabolite III in micro-KBr disc (14).

exist in non-poler solutions. Increasing the solvent polarity would disrupt this equilibrium. Little or no change would be noted on changing the solvent polarity if the vicinal hydroxy groups were in the cis conformation. The equilibrium was monitored in the C-O stretching region of the infrared at 1100 to 1000 cm^{-1} (12). Figure 3 shows a large increase in the axial C-O stretching band at 1032 cm^{-1} and a decrease in the equatorial C-O stretching band at 1088 cm^{-1} when the solvent polarity was increased from that of CS_2 to that of 10% dioxane-CCl_4. The trans conformation of metabolite III is thus established, and metabolite III may be further characterized as trans-3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl.

In vivo formation in animals of trans vicinal diol metabolites from aromatic compounds has been cited as evidence for arene oxide intermediates (13). Having identified the trans-3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl metabolite in this study, we suspect that the arene oxide, 2,5,2',5'-tetrachlorobiphenyl-3,4-oxide, is its immediate precursor through hydration. Isomeric rearrangement of the arene oxide intermediate or dehydration of the dihydrodiol would explain the formation of the 3- and 4-hydroxy metabolites.

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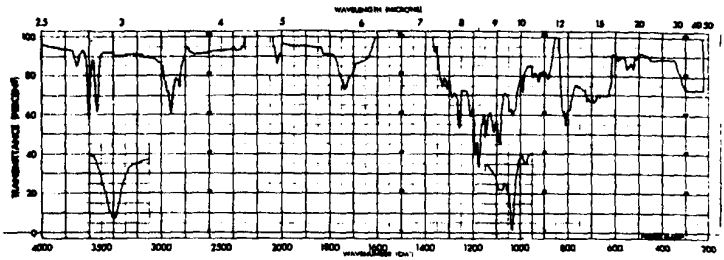

Figure 3:

Infrared spectra of metabolite III in CS_2 (top) and in 10% dioxane-CCl_4 (lower inserts). The diaxial conformation of vicinal trans hydroxy groups predominates in the more polar 10% dioxane-CCl_4 solvent as evidenced by the increase in the C-O stretching band of axial hydroxy groups at approximately 1030 cm^{-1} and a corresponding decrease in the band for the equatorial hydroxy groups at approximately 1090 cm^{-1}. Bands at approximately 1200 cm^{-1} are due to O-H in-plane stretching vibrations in dilute solution (12). Spectra were obtained in 0.1 mm KBr ultramicro liquid cell (Wilks Scientific Corp.) in 10% dioxane-CCl_4 or 3 mm KBr micro-liquid cell (Perkin-Elmer Corp., Norwalk, Conn.) in CS_2.

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