Isolation and Identification of a Polar Metabolite of Tetrachlorobiphenyl from Bile of Rainbow Trout Exposed to ^{14}C-Tetrachlorobiphenyl

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The presence of polychlorinated biphenyls (PCBs) in a wide variety of fish has been reported by VEITH and LEE (1971) and STALLING and MAYER (1972). Laboratory studies have demonstrated the uptake of dietary PCB by rainbow trout (LIEB, et al., 1974) and lake trout (SCHOETTGER, 1973), and aqueous PCB by goldfish (HATTULA and KARLOG, 1973), spot and pinfish (HANSEN, et al., 1971) and yellow perch and rainbow trout (MELANCON, 1974). Once absorbed by fish the PCBs do not appear to be readily eliminated. Although over 50% of the PCB accumulated by spot and pinfish during preexposure to 1 ppb PCB was released during 8 weeks following exposure (HANSEN, et al., 1971; HANSEN, et al., 1974) this extent of PCB elimination is not typical of fresh water fish. HATTULA and KARLOG (1973) reported that goldfish released almost 80% of accumulated PCBs during a 10-week washout period, but the amount of fat tissue in these fish showed an unexpected comparable decrease such that PCB levels in fat tissue remained constant at about 3000 ppm. LIEB, et al. (1974) examined the elimination of previously accumulated dietary PCBs by rainbow trout utilizing a PCB-free diet (16 weeks) or fast (8 weeks). As the trout receiving the PCB-free diet grew, the PCB level decreased, but the total amount of PCB per fish remained constant. The fasted fish lost weight and the PCB level increased but the total amount of PCB per fish remained constant. Another study of the elimination of previously accumulated dietary PCB was reported by SCHOETTGER (1973). In this case, lake trout showed slightly reduced PCB concentrations, but it was not reported if this was simply a growth effect.

The slow elimination of PCBs from fish may be explained by their high affinity for lipids and slow metabolism. HUTZINGER, et al. (1972) have shown that although rats and pigeons were able to hydroxylate and eliminate chlorinated biphenyls, the water in which PCB-fed trout were maintained for 4 days contained no hydroxylated metabolites. Reports of the biliary excretion of metabolites of 3-trifluoromethyl-4-nitrophenol (LECH, 1973), sulfobromophthalein (SCHMIDT and WEBER, 1973), carbaryl and DDT (LECH, et al., 1973), and Bayer 73 (STATHAM and LECH, 1975) by rainbow trout suggested that examination of trout bile following PCB exposure might be a logical source of possible PCB metabolites. For this reason, rainbow trout were exposed to aqueous ^{14}C-tetrachlorobiphenyl (TCB) with subsequent examination of bile for metabolites.

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MATERIALS AND METHODS

Rainbow trout were obtained from Kettle Moraine Springs Trout Hatchery and maintained in flowing dechlorinated tap water at 12°C for at least 1 week before use. Trout were exposed for 24 hr at 12°C to 0.5 ppm of 2,5,2',5'tetrachlorobiphenyl (Ring-UL-^{14}C) (Mallinckrodt, St. Louis, Mo.) plus unlabelled 2,5,2',5'tetrachlorobiphenyl (TCB) (Analabs, New Haven, Conn.) at a specific activity of 8,000 dpm/µg. This material, after preparative thin-layer chromatography (TLC) on silica gel (Brinkman, Westbury, N.Y.) in hexane, showed 99.8% radioactivity moving as TCB. A single peak was also found by gas chromatography using a 1/8" by 3' column of 3% OV-7 on chromosorb WHP in a Perkin-Elmer Model 270B gas chromatograph coupled to a Chicago Nuclear radioactive monitor.

At the indicated exposure times fish were sacrificed by a blow to the head and bile was collected by gallbladder puncture. The bile was pooled and an aliquot examined by TLC. The bile was treated as outlined in Figure 1. The pooled bile was applied

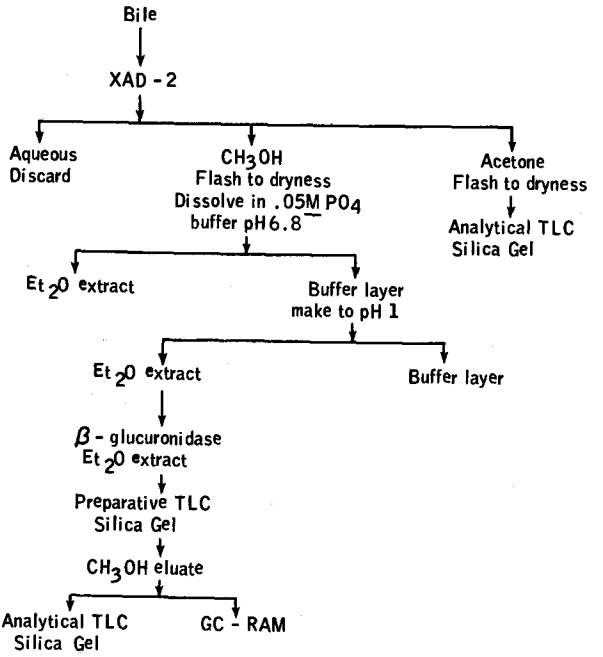

Figure 1 Fractionation scheme of bile from fish exposed to ^{14}C TCB.

to a 2 x 20 cm bed of washed Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, Pa.) in a glass column and the resin bed was washed with 50 ml of distilled water. Elution with methanol removed only part of the radioactivity from the resin. Subsequent elution with acetone removed the remainder of the radioactivity. Both the methanol and the acetone fractions were reduced in volume and examined for TCB and possible metabolites by TLC on silica gel using hexane as solvent.

Radioactivity on TLC plates was localized by scraping and counting segments of the silica gel from origin to solvent front. The samples were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillation cocktail contained 80g naphthalene, 4g PPO, and 0.05g POPOP dissolved in 600 ml toluene plus 400 ml methyl cellusolve. Silica gel plates used for preparative TLC were prewashed in methanol. Glass distilled solvents were used for TLC and all other chemicals and solvents were reagent grade.

The methanol fraction was evaporated to dryness and the residue was taken up in 0.05 M phosphate buffer pH 6.8. The buffer solution was extracted with diethyl ether at pH 6.8 and after adjustment to pH 1 with 10 N HCl. These ether extracts were examined for the presence of glucuronide conjugates. Aliquots of the ether solutions were evaporated to dryness, the residue was dissolved in 0.05 M phosphate buffer pH 6.8 and these solutions were incubated with 100 units β-glucuronidase (Sigma, St. Louis, Mo.) or β-glucuronidase plus saccharo-1,4-lactone (1×10^{-3}M) a specific inhibitor of β-glucuronidase. Incubations were terminated by the addition of 0.05 ml of 5 N HCl (control tubes received HCl before enzyme), and aliquots of the complete incubation solution were spotted on silica gel plates and developed in chloroform : methanol : ammonium hydroxide 8:4:1.

Since the acidic ether extracts from both the 24 hr and the 48 hr exposures contained most of the radioactivity, these extracts were pooled and subjected to β-glucuronidase hydrolysis followed by diethyl ether extraction at pH 6.8. The extract was reduced in volume and subjected to preparative TLC followed by analytical TLC and gas chromatography.

RESULTS AND DISCUSSION

The concentration of TCB and metabolites based on total ^{14}C in pooled bile from the 24 hr and 48 hr exposures were 5.0 ppm and 5.4 ppm respectively. Results of the TLC of crude bile radioactivity from the 24 hr exposure is shown in Figure 3A. The elution profile obtained when the crude bile was fractionated on an XAD-2 column with methanol followed by acetone is shown in Figure 2. The TLC of the methanol and acetone fractions is shown in figure 3, parts B & C. It can be seen that the TCB and more polar fractions originally present in the bile were separated by selective elution from the XAD-2 column. Because only the methanol fraction contained possible metabolites it was utilized for further study. As previously described, this fraction was separated into two fractions - neutral ether extract and acidic ether extract. The neutral ether extract contained little radioactivity, while the acidic ether extract contained a considerable amount and would be expected to obtain any glucuronides present. The data in figure 4 show the results of β-glucuronidase incubation of the acidic ether extracts from the 24 hr exposure. Similar results were obtained with the sample from the 48 hr exposure. In both cases the polar compound which remained near the origin

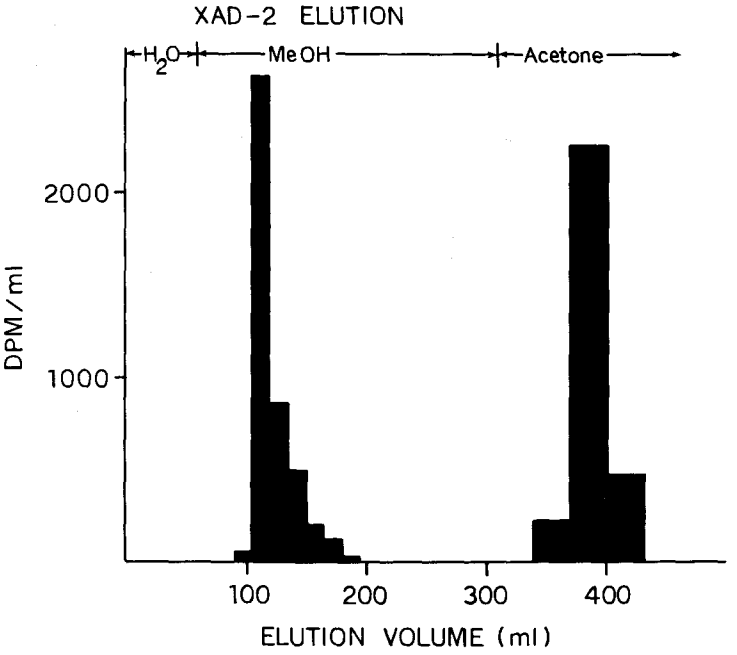

Figure 2 Elution profile of bile ^{14}C from trout exposed to ^{14}C TCB.

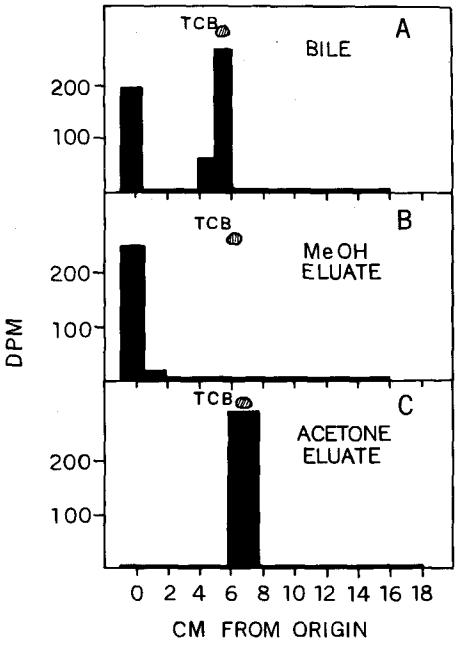

Figure 3 Thin-layer chromatograms of (a) pooled trout bile; (b) methanol eluate from XAD-2 column; and (c) acetone eluate from XAD-2 column. Solvent: hexane. The dark spots indicate the mobility of TCB standard.

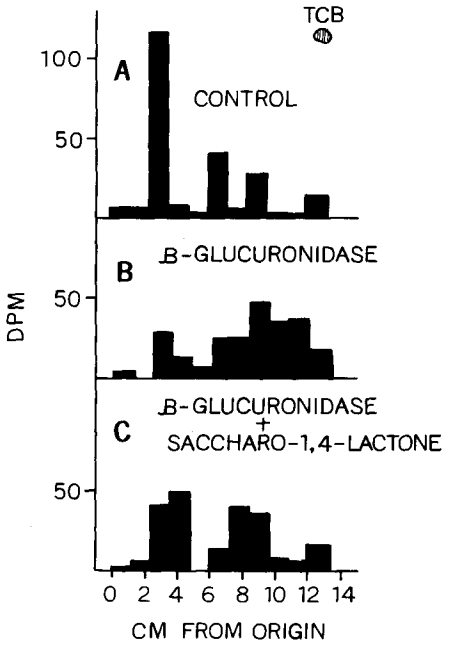

Figure 4 Thin-layer radiochromatograms of acidic ether extracts from methanol fractions of bile following 48 hr exposure to ^{14}C TCB; (a) control; (b) after hydrolysis by β-glucuronidase; (c) after hydrolysis by β-glucuronidase in presence of saccharo-1,4-lactone. Solvent:chloroform:methanol:ammonium hydroxide 8:4:1. Dark spot indicates mobility of TCB standard.

disappeared following β-glucuronidase incubation and was replaced by a compound with a mobility almost as great as that of TCB. It can be seen that the presence of saccharo-1,4-lactone almost completely blocked this change in mobility.

Following β-glucuronidase hydrolysis of the pooled acidic ether extracts the ether extractable material was purified by TLC. Great difficulty was experienced in recovering ^{14}C from the silica gel of the TLC plate. There appeared to be more than one radioactive peak in agreement with figure 4. However, only one of these was recovered in sufficient quantity for analysis by the gas chromatograph-radioactive monitor. The TLC data in figure 5A show that this material was not the original TCB, while the data in figure 5B show that this material has the same R_f as the material released by β-glucuronidase hydrolysis shown in figure 4. The results of gas chromatography shown in Table 1 indicate that the elution temperature of the trimethylsilyl derivative of the β-glucuronidase released compound matched that of the TMS derivative of authentic 4-hydroxy-TCB.

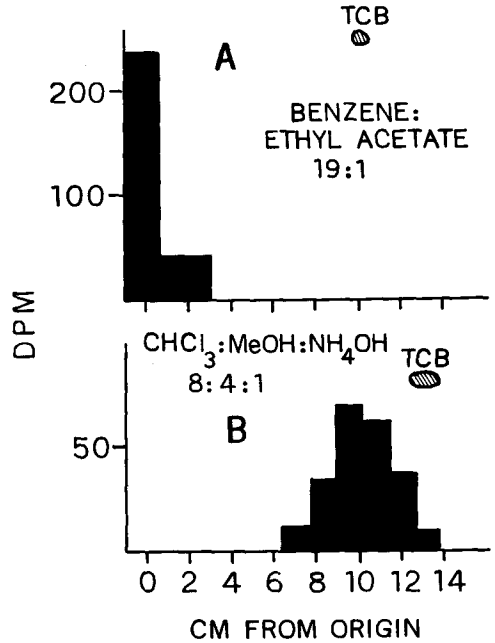

Figure 5 Thin-layer radiochromatograms of TCB metabolite using (a) solvent:benzene:ethyl acetate: 19:1 and (b) solvent: chloroform:methanol:ammonium hydroxide 8:4:1. The dark spots indicate the mobility of TCB standard.

TABLE 1

Gas chromatography of standard TCB, and the trimethylsilyl ethers of HO-TCB and TCB metabolite using a 3% OV-7 on chromosorb WHP column with a 100°-240° program.

Compound	Elution Temperature
TCB	179°
TMS HO-TCB	203°
TMS TCB Metabolite	203°

Studies concerned with exposure of fish to PCBs have indicated a high recovery of unmodified starting material (LIEB, et al., 1974, HATTULA and KARLOG, 1973) and such observations have been related to an absence of PCB metabolism.

In 1965 CREAVEN, et al. reported the ability of trout homogenates to hydroxylate biphenyl, though at a much lower rate than similar preparations from higher vertebrates. Since that time there have been numerous reports of both *in vivo* and *in vitro* metabolism of xenobiotic compounds by a number of fish species.

Among these are the reports of PRITCHARD et al. (1973) and SANBORN et al. (1975) on the ability of flounder and sunfish, respectively, to metabolize small amounts of chlorinated compounds. PRITCHARD described the limited ability of flounder to metabolize and eliminate injected ^{14}C DDT as DDE and more polar metabolites. Only 2% of the total contained in fractions other than urine was metabolized while greater than 75% of the urine ^{14}C (2% of injected dose) appeared as metabolites. HAMELINK et al. (1971) have proposed a theory of biocentration of pollutants in fish in which high levels of lipid soluble pollutants are taken up directly from the water and accumulated according to their lipid-water partitioning. SANBORN et al. studied the uptake and elimination of ^{14}C labelled trichlorobiphenyl, tetrachlorobiphenyl and pentachlorobiphenyl in addition to DDT and DDE. When extracts from PCB exposed fish were examined by TLC it was found that although over 80% of the radioactivity from trichlorobiphenyl exposed fish was more polar than the starting material the values were only 1.16% and 0.69% for tetrachlorobiphenyl and pentachlorobiphenyl, respectively. The extent of elimination and metabolism of these PCBs appeared to be inversely related to the degree of chlorination.

The present report demonstrated the appearance, in rainbow trout bile, of ^{14}C-labelled conjugated metabolites following exposure to ^{14}C-labelled TCB. After glucuronidase hydrolysis, the TMS ether of one of the compounds matched the TMS ether of hydroxy TCB during gas chromatography.

Although the extent of TCB metabolism by rainbow trout and also by sunfish appears to be slight, even this low level of metabolism could be important in view of the high concentration of PCBs found in various fish species and the possibility of significant amounts of HO-PCBs being released into the environment from the large fish biomass.

Work is continuing on the appearance of PCB metabolites in fish tissue to determine whether such metabolites arise from metabolism by fish per se or by microorganisms in the water or intestinal tract.

ACKNOWLEDGMENTS

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