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Comparative Metabolism of PCB Isomers by Three Species of Fish and the Rat

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In spite of the concern about PCB contamination of aquatic organisms, relatively little is known about metabolic fates of PCB in fish. Most of the metabolic data are based upon mammalian species, particularly the rat (YOSHIMURA and YAMAMOTO, 1973; YOSHIMURA et al., 1973; GARDNER et al., 1973; JENSEN and SUNDSTROM, 1974, HUTZINGER et al., 1972; GHIASUDDIN et al., 1976). As for metabolic studies in fish, HUTZINGER et al., (1972), found very little evidence of PCB metabolism in brook trout as compared to that of the rat. MELANCON and LECH (1976) confirmed this phenomenon, but they were able to show small amounts of conjugated metabolites in the bile of ^{14}C-tetrachlorobiphenyl treated rainbow trout. Fish are very important from the viewpoint of environmental toxicology being the vital link for human health and potential wildlife danger.

While it has been acknowledged that fish generally have relatively low metabolic capabilities of xenobiotics, as compared to mammalian species, there are instances where significant metabolic activities contributed to the species differences in susceptibility to foreign chemicals. For instance, the basis of lamprey control in the Great Lakes is the superior degradation activities of the controlling agent by fish as compared to that by lamprey.

The purpose of this study is to compare a few species of fish for their ability to metabolize PCBs and to compare the pattern of metabolism to that by rats which have been extensively studied by other scientists.

MATERIALS AND METHODS

Radiochemicals:

Two preparations of ^{14}C-2,5,2'-trichlorobiphenyl were obtained from California Bionuclear Corporation, Sun Valley, CA. Before being used, each was purified by thin-layer chromatography. The specific activities of both were 9.91 mCi/mmole, but one preparation was uniformly labeled in the monochlorinated ring and the other in the dichlorinated ring.

For counting ^{14}C, the scintillation solution consisted of 1.5 l toluene, 1.5 l methyl cellosolve, 16.5 g PPO and 0.9 g dimethyl-POPOP. Counting efficiencies calculated from an automatic external

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standard were generally between 63% and 68%.

In vivo metabolism of 2,5,2'-trichlorobiphenyl by rat:

A male Sprague-Dawley white rat about 55 days old was dosed by stomach tube with a corn oil solution of ^{14}C-2,5,2'-trichlorobiphenyl (1.77 uCi, 45 μg; approx. 0.25 μg/g). Urine was collected for one week, stored at 4° with a little toluene and along with washings of the collection apparatus extracted with diethyl ether in a continuous extraction. The aqueous layer was filtered and combined with a similar filtrate from another rat that had been fed a diet of ground Purina Laboratory Chow to which biphenyl (Pfaltz and Bauer, Inc.) had been added to make a concentration of 1%. (The collection apparatus for the biphenyl-fed rat was not washed, however.) The combined filtrate volume was about 270 ml. It was acidified with 27 ml of concentrated hydrochloric acid whereupon a precipitate formed, and then it was stored for one day at 4° in an attempt to get coprecipitation of biphenyl and trichlorobiphenyl metabolites. The acidified mixture was filtered through a Whatman extraction thimble, and the solid was rinsed with a few milliliters of water before air drying overnight. The dry solid was extracted with ether in a Soxhlet extractor for about one day to remove any free phenolic compounds still present.

The feces from the trichlorobiphenyl-dosed rat were collected daily for a week after treatment and stored at 4° with a little toluene. After the feces were partially ground, they were extracted three times by adding 50 ml each of acetone and methanol and filtering one day or three days (the third extraction) later. In the fourth extraction equal amounts of acetone and water were combined with the feces and the mixture was filtered one day later. The procedure was similar for the fifth extraction, except that one milliliter of glacial acetic acid was added to the 75 ml each of water and acetone. In the final extraction, the sixth, 2 ml of glacial acetic acid was added to the water and acetone, and the filtration was done after two days. Extracts 1-3 were combined with each other as were extracts 4-6.

The radioactivity in fecal extracts 1-3 and 4-6 were partitioned between ether and water. The residue from extracts 1-3 was dissolved in 25-30 ml each of ether and water; the layers were separated overnight in a separatory funnel. The aqueous layer was washed with ether and then the combined ether layers were washed with water. Fecal extracts 4-6 from which the acetone had evaporated were extracted twice with ether, and the combined extracts were washed with water.

In vivo metabolism of 2,5,2'-trichlorobiphenyl by goldfish:

A goldfish (6.3 g) in a glass jar containing 5 liters of water was fed ^{14}C-2,5,2'-trichlorobiphenyl (1.56 μg, 133,000 dpm) that had been applied to a brine shrimp pellet at a ratio of 0.25 μg/g. After one week at 15-20°C, the water in the jar was divided into three portions and extracted three times with ether by using each portion of ether to extract each portion of water. The fish carcass

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was coarsely ground and extracted by standing with acetone-ethanol for two days.

In vitro degradation of 2,5,2'-trichlorobiphenyl-^{14}C:

Male Sprague-Dawley rats weighing approximately 200 to 350 g were killed by a blow just behind the head. Bullheads (Ictalurus sp.) were obtained from Lake Kegonsa (Dane County, Wisconsin) through the Wisconsin Department of Natural Resources and were maintained in the laboratory at 4° for over 6 weeks without feeding. Rainbow trout (about 20-23 cm long, 125 g) were supplied by the Nevin Fish Hatchery, Madison, Wisconsin. Goldfish (about 60 g each) were of the Comet variety and were obtained from a local pet store. The fish were put into ice water to anaesthetize them before decapitation and removal of the livers.

The liver was homogenized with 0.2 M pH 7.4 (pH measured at 25°) potassium phosphate buffer to make a 30% (w/v) homogenate. After centrifuging at 10,000xg at 4° for 20 minutes, the supernatant portion was the enzyme solution added to tubes for incubation.

The incubation solution, unless stated otherwise in the text, consisted of 3 ml 0.2 M pH 7.4 potassium phosphate buffer, 1 ml NADPH (2 mg/ml) in buffer, 1 ml of enzyme solution and 0.01 ml 2,5,2'-trichlorobiphenyl-^{14}C (22,100 dpm, 1 nanomole in 10 µl dimethylsulfoxide). The incubations were done at 37° in a water bath shaker. After incubation each tube was extracted first with 7 ml diethyl ether and two additional times with 5 ml ether each time.

The extracts were combined, evaporated and redissolved in ether before taking an aliquot to measure the recovery of ^{14}C. The remaining extract was used for thin-layer chromatography (Silica gel G, 0.25 mm, unactivated; Skellysolve B-acetone, 6:1) and autoradiography to determine the amount of degradation. For each extract three areas of silica gel (Rf = 0.0, 0.2, and 0.55) indicated by the radioautograpms were scraped from the plate into counting vials that contained 10 ml each of counting solution.

In an experiment to follow the progress of the reaction in incubating tubes containing 1 ml enzyme, 2 mg NADPH in 2 ml buffer and 1 nanomole 2,5,2'-trichlorobiphenyl in 10 µl of dimethylsulfoxide, the degradation was stopped after various lengths of time by adding 2 M HCl. Extraction and analysis were as described above except the scintillation solution contained ethyl acetate in place of methyl cellosolve.

To test the effect of substrate concentration on the rate of formation of products, additional non-radioactive 2,5,2'-trichlorobiphenyl (Analabs, New Haven, Connecticut; lot 001) was added as necessary to produce 0.08 (no unlabeled substrate), 0.2 and 4 ppm in the incubation solution. The incubation lasted 130 minutes. Extraction and analysis were as described above except that the developing solvent for chromatography was Skellysolve B-ethyl acetate-acetic acid (40:10:1); this solvent produces a different pattern of

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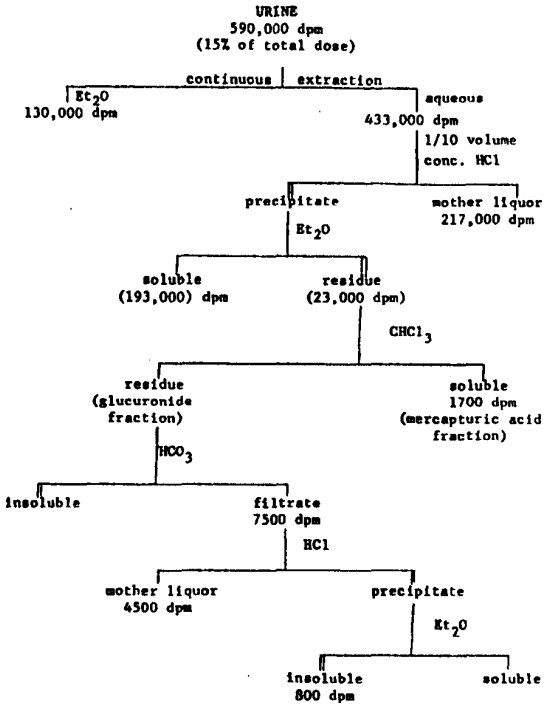


Figure 1. Distribution of radioactivity in urine. Extraction procedure based on WEST et al. (1956)

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spots to be scraped.

A comparison by TLC of radioactive compounds from *in vivo* and *in vitro* metabolism was made by developing with benzene-ethyl acetate (12:1) on a Silica gel C layer.

RESULTS AND DISCUSSION

About 15% of the radioactivity in the rat's dose was accounted for in the first seven day's urine (Fig. 1), and 57% was extracted from the feces (Fig. 2). Of the urinary ^{14}C, 55% (i.e. 8.25% of total excretion) was extracted with ether at neutral and acidic pH's. In addition, some radioactivity in the acidic mother liquor may be ether soluble since this solution was not extracted with ether. An even higher fraction, 85% of the fecal ^{14}C (i.e 48.5% of total excretion) was extracted into ether. Of the total ^{14}C recovered from one week's excreta 78% was partitioned into ether and is presumably low in conjugated metabolites. Together with the evidence that there is very little mercapturic acid (unlike biphenyl metabolism) one is forced to conclude that excretion of the trichlorobiphenyl is mostly carried out without extensive conjugation processes in rats.

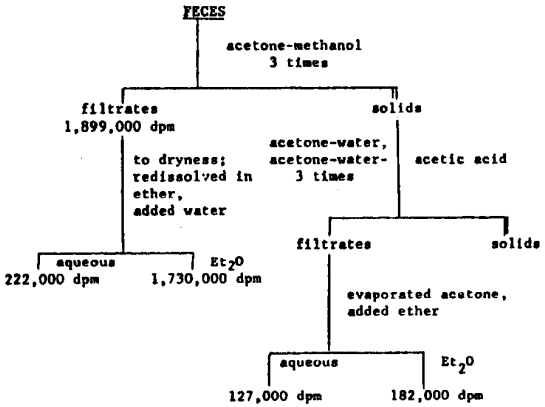


Figure 2. Distribution of radioactivity in feces.

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TABLE 1

Effect of time on in vitro degradation of ^{14}C-2,5,2'-trichlorobiphenyl by rat liver: the data expressed in % of total radioactivity recovered* in each Rf zone in each thin-layer chromatogram**.

Incubation time	Metabolic products Rf=0	Rf=0.2	PCBs remaining Rf=0.6
Min.	%	%	%
0	0.5	1.1	98.4
10	6.3	4.6	89.0
20	7.8	6.8	85.4
40	8.0	6.3	85.7
60	7.4	6.9	85.6
130	7.9	7.8	84.3

* Total radioactivities recovered varied from 70,000 to 122,000 cpm per chromatogram.

** Silica gel G, mobile phase hexane-acetone (6:1).

Of the dose to the goldfish altogether 78% of the ^{14}C was recovered; 48.2% was found in the carcass and 29.8% was found in the ambient water. Less than one-third of the ^{14}C in the aquarium water could be extracted by ether; this is about 9% of the originally added dose.

Data in Table 1 show that in the ether extract of in vitro rat experiments the ratio of metabolites to substrate was reached and maintained after the first 20 minutes of incubation. When the concentration of substrate was increased, there was an increase in the moles of metabolites in the extract but this translates to a smaller ratio of metabolites to trichlorobiphenyl (Table 2). As a result an incubation condition close to the optimum enzyme activity (1 nanomole per tube, 120 min., 37°C) was adopted to conduct the following comparative in vitro study.

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,5,2'-trichloro-
tal radio-
r chromato-

PCBs remaining Rf=0.6
%
98.4
89.0
85.4
85.7
85.6
84.3

0 to 122,000

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TABLE 2

Effect of substrate concentration upon in vitro degradation of ^{14}C-2,5,2'-trichlorobiphenyl by rat liver: the data are expressed in % of total radioactivity recovered in each spot in each thin-layer chromatogram* found.

Compound (Rf on TLC)	Amount of added PCBs per tube (nanomoles) 1	2.5	50
	%	%	%
Metabolite 1 (0)	1.7	1.2	0.7
Metabolite 2 (0.06)	1.3	0.9	1.0
Metabolite 3 (0.15)	3.5	2.0	1.9
Metabolite 4 (0.4-0.45)	15.8	9.7	4.4
PCB (0.67)	77.6	86.2	92.0

* Silica gel G, mobile phase hexane-ethyl acetate-acetic acid (40:10:1).

The in vitro experiments show considerable variation in the amount of metabolism among species (Table 3). The most notable result, however, is that 6-9% of the 2,5,2'-trichlorobiphenyl incubated with goldfish liver enzymes was recovered as more polar metabolites. Rat liver enzymes produced more metabolites than goldfish enzymes, but from incubations with trout liver enzymes no significant amount of metabolites was recovered. In the case of bullheads, the amount of metabolism was less than 0.5%, which is only slightly above the cpm from heat inactivated control samples. The results from bullhead #2 are more suggestive of metabolism than those from bullhead #1.

TABLE 3

In vitro metabolism of 2,5,2'-trichlorobiphenyl by livers of three species of fish and the rat. The data are expressed in percentages of the originally added 2,5,2'-trichlorobiphenyl.

Source of the enzyme	Metabolites of 2,5,2'-trichlorobiphenyl % of ^{14}C scraped from TLC plates* Very polar metabolites (Rf = 0.0) 0 min.	120 min.	Less polar metabolites (Rf = 0.2) 0 min.	120 min.
Rainbow trout-1	0.04	0.073	0.068	0.072
Rainbow trout-2	0.13	0.15	0.24	0.19
Bullhead-1	0.16	0.16	0.09	0.21
Bullhead-2	0.12	0.32	0.11	0.33
Goldfish-1	0.06	2.38	0.28	3.89
Goldfish-2	0.19	6.58	0.27	2.50
Rat-1	0.16	4.84	0.47	7.93
Rat-2	0.40	5.70	2.43	29.0

*Remaining radioactivity was at the 2,5,2'-trichlorobiphenyl position

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In vitro and in vivo metabolites show some chromatographic similarities. The autoradiograph (Fig. 3) shows spots at Rf = 0.45 and Rf = 0.55 from extracts of in vitro experiments with both rat and goldfish livers and also from the ether soluble portion of the extract of rat feces. The spot at Rf = 0.45 was also present in the ether extract of water from the in vivo goldfish experiment and in the ether soluble compounds from rat urine, but these sources did not show a definite spot at Rf = 0.55. The fish carcass contained some starting compound and some ^{14}C at Rf = 0 but no detectable ^{14}C at intermediate Rf's. There were at least five other metabolites from the ether soluble portions of rat urine and feces, but they were not present in the in vitro experiments in amount detected in this procedure.

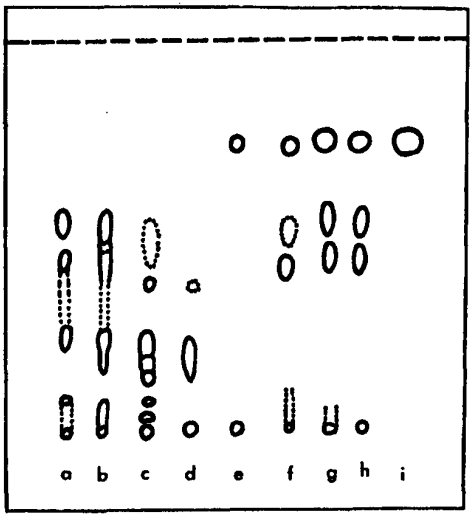


Figure 3. Thin-layer chromatography of in vivo and in vitro metabolites of 2,5,2'-trichlorobiphenyl. Absorbent, Silica gel G. Developing solvent, benzene-ethyl acetate, 12:1, v:v. a) ether soluble compounds from rat feces; b) same as (a), larger amount; c) ether soluble compounds from rat urine; d) same as (c), lesser amount; e) extract of goldfish carcass; f) ether extract of water in goldfish aquarium; g) extract of in vitro rat experiment; h) extract of in vitro goldfish experiment; i) ^{14}C-2,5,2'-trichlorobiphenyl.

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The rat in vivo metabolism data obtained here closely agree with those reported by GHIASUDDIN et al., (1976) on the same TLC system. Based upon mass spectroscopic data they conclude that the initial metabolic step of trichlorobiphenyl is the ring hydroxylation to form monohydroxy derivative. It is interesting to note here that the in vitro metabolic pattern of the goldfish is almost identical to that of the rat (Fig. 2 g,h), indicating the similarity of the metabolic route. The large difference was instead in the in vivo patterns where the rat produced very extensive metabolic pattern involving production of more polar metabolites as opposed to the case with the goldfish which showed a similar pattern as the in vitro data involving the formation of monohydroxy derivatives. As for the very polar metabolic products which stay at the origin of the TLC, it is possible that they are conjugated products, such as the ones described by MELANCON and LECH (1976) in rainbow trout, since their solvent system is roughly comparable to ours. In this connection, it is interesting to note that the radioactive material in the ambient water (Fig. 2 f) which represents the original ^{14}C-PCB and the excreted metabolic products contained large amounts of polar metabolites. In view of their in vivo and in vitro metabolic activities and their availability, goldfish should be regarded as a good material for future fish metabolism studies.

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