In Vitro Metabolism of Permethrin Isomers by Carp and Rainbow Trout Liver Microsomes

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Carp liver microsomal esterases hydrolyze *trans*-permethrin much more extensively than *cis*-permethrin and the same relationship holds for rainbow trout liver microsomes, although they appear to be less active. There is a strong preference with both isomers and microsomal mixed-function oxidases of both species for hydroxylation at the 4′ position of the alcohol moiety as opposed to other sites. The methyl group trans to the carboxy is usually hydroxylated more extensively than the *cis*-methyl group, the greatest specificity being with carp microsomes acting on *cis*-permethrin. The bile of rainbow trout exposed in vivo to ^{14}C-alcohol-labeled *trans*-permethrin contains little or no permethrin but instead consists mainly of conjugates cleaved by β-glucuronidase but not by aryl sulfatase.

Permethrin is a synthetic pyrethroid with high insecticidal potency and low mammalian toxicity (Elliott et al., 1973). The low toxicity of permethrin to mammals may be due in part to its rapid metabolism (Miyamoto, 1976; Abernathy and Casida, 1973) by ester hydrolysis and hydroxylations at certain preferred sites on the molecule (Gaughan et al., 1977; Elliott et al., 1976). *trans*-Permethrin is more susceptible than *cis*-permethrin to esterase attack by mouse liver microsomes (Soderlund and Casida, 1977a). This difference in hydrolysis rate of the two isomers may be related to the greater persistence and toxicity of *cis*-permethrin in rats (Gaughan et al., 1977).

Permethrin is extremely toxic to fish (Miyamoto, 1976; Zitko et al., 1977). The LC_{50} of the technical grade compound is 18 μg/L in rainbow trout (Glickman and Lech, 1978). Knowledge of the metabolism of permethrin in fish may contribute to understanding the mechanisms of selective toxicity of permethrin and other pyrethroids and to selection of suitable pyrethroid insecticide synergists. The present investigation is concerned with the in vitro metabolism of *cis*- and *trans*-permethrin in carp and rainbow trout liver microsomal fractions, the role of microsomal esterase and oxidase systems in permethrin metabolism in fish (Shono and Casida, 1978), and the biliary metabolites of permethrin in rainbow trout.

MATERIALS AND METHODS

Chemicals. (1*RS*)-*trans*-Permethrin (*t*-per), (1*RS*)-*cis*-permethrin (*c*-per), and ^{14}C-acid and ^{14}C-alcohol preparations of these isomers (Figure 1) were generously provided by FMC Corporation (Agricultural Chemical Division, Middleport, NY). The site of the label in the ^{14}C-acid preparation was at the carbonyl carbon of the acid moiety and the site in the ^{14}C-alcohol sample was at the methylene carbon on the benzyl ring. Unlabeled standards for permethrin metabolites were synthesized by the methods of Unai and Casida (1977). These compounds included several hydroxy esters, i.e., 4′-hydroxy-3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (4′-HO-per), 3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2-*trans*-hydroxymethyl-2-*cis*-methylcyclopropanecarboxylate (*t*-HO-per), 3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2-*cis*-hydroxymethyl-2-*trans*-methylcyclopropanecarboxylate (*c*-HO-per), and 4′-hydroxy-3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2-*trans*-hydroxymethyl-2-*cis*-methylcyclopropanecarboxylate (4′-HO, *t*-HO-per). Standards of alcohol moiety products included 3-phenoxybenzyl alcohol (PBalc), 3-phenoxybenzaldehyde (PBald), 3-phenoxybenzoic acid (PBacid), and 4′-hydroxy-3-phenoxybenzyl alcohol (4′-HO-PBalc). Acid moiety metabolite standards used were 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (Cl_2CA), 3-(2,2-dichlorovinyl)-2-*cis*-hydroxymethyl-2-*trans*-methylcyclopropanecarboxylic acid (*c*-HO-Cl_2CA), 3-(2,2-dichlorovinyl)-2-*trans*-hydroxymethyl-2-*cis*-methylcyclopropanecarboxylic acid (*t*-HO-Cl_2CA), and the lactone of *c*-HO-Cl_2CA, *c*-HO-Cl_2CA-lactone.

Preparation of Microsomes. Rainbow trout, *Salmo gairdneri*, were obtained from Kettle Moraine Springs Hatchery (Adelle, WI). Carp, *Cyprinus carpio*, were supplied by the U.S. Department of the Interior, Fish and Wildlife Service, La Crosse, WI. All fish were maintained in flowing charcoal filtered, tap water at 12 °C for at least 1 week prior to use.

Rainbow trout (75–120 g) and carp (950–1350 g) were sacrificed by a blow to the head. The livers were excised, rinsed in cold 0.15 M KCl, and homogenized in four volumes of 0.25 M sucrose using a Potter-Elvehjem-type glass–Teflon homogenizer (Elcombe and Lech, 1978). Each liver homogenate was centrifuged at 8500*g* × 20 min, and the supernatant was decanted and centrifuged at 165000*g* × 60 min. The microsome pellet obtained was resuspended in 0.15 M KCl and centrifuged again at 165000*g* × 60 min. The washed pellet was suspended in 0.25 M sucrose to a concentration equivalent to 1 g wet weight of liver per milliliter of suspension.

All microsomes were used on the same day they were prepared. Estimations of protein concentrations were by the method of Lowry et al. (1951).

Incubation Conditions. Each incubation mixture consisted of the following: 1 mL of liver microsomes (14.5–14.8 mg of protein) equivalent to 1 g fresh liver weight; NADP (0 or 1.25 μmol), glucose-6-phosphate (10 μmol), and glucose-6-phosphate dehydrogenase (2.5 units; Sigma Chemical Co., St. Louis, MO) to regenerate NADPH; and Tris-HCl buffer (66 mM, pH 7.4) to 2.5 mL in 25-mL Erlenmeyer flasks. With rainbow trout liver microsomes, 0.1 μmol (39 μg) of [^{14}C]permethrin (1 μCi) in 10 μL of dimethylformamide was added to each flask while approximately 0.05 μmol (18 μg) of [^{14}C]permethrin

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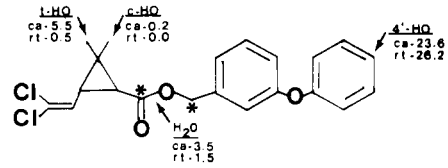
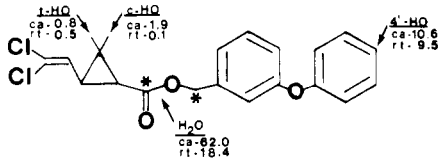
Table I. Metabolism of ^{14}C-Alcohol Preparations of *cis*- and *trans*-Permethrin by Rainbow Trout and Carp Liver Microsome-NADPH Systems

	% of extracted radiocarbon			
	cis-permethrin		*trans*-permethrin	
compd	carp	trout	carp	trout
permethrin	61.2 (95.9)[a]	72.3 (89.3)	31.0 (37.2)	74.0 (82.4)
4′-HO-per	24.7	23.4	6.0	7.1
t-HO-per	3.0	0.5	0.6	0.4
c-HO-per	0	0	0.6	0.1
4′-HO, *t*-HO-per	3.7	0	0	0
PBalc	3.2 (3.6)	0.7 (3.4)	50.6 (60.5)	13.4 (16.1)
PBald	0	0	0	0.2
PBacid	0.3	0	0.5 (0.5)	0
4′-HO-PBalc	1.2	1.5	7.4	3.3
unknown	2.7 (0.5)	1.6 (7.3)	3.3 (1.8)	1.5 (1.5)

[a] Values in parentheses are the results in the absence of NADPH. Where not stated, the values were 0.



1RS,cis-permethrin



1RS,trans-permethrin

Figure 1. Structures of (1*RS*)-*cis*- and (1*RS*)-*trans*-permethrin showing labeling positions in alcohol moieties (55.9 mCi/mmol) and acid moieties (58.2 mCi/mmol) and sites and extent of metabolism. The numbers are percentage values for carp (ca) and rainbow trout (rt) microsome–NADPH systems. Hydrolysis is an average of acid- and alcohol-labeled preparations. Hydroxylation sites are average of acid-labeled preparations (esters plus appropriate cleavage products) and alcohol-labeled preparations (esters plus appropriate cleavage products). Unidentified metabolites are not included.

(0.5 μCi) was added to the flasks containing carp liver microsomes. Both *cis*- and *trans*-permethrin labeled in either the acid or alcohol moiety were incubated with and without NADPH. An incubation mixture containing boiled microsomes served as the control.

The flasks were incubated for 1 h at 30 °C in a Dubnoff shaking water bath whereupon the incubation was terminated by transferring the contents of the individual flasks to 15-mL screw-top culture tubes containing 0.5 g of $(NH_4)_2SO_4$. The incubations were then acidified to pH 1.8 with 1 N HCl and extracted three times with 4-mL aliquots of cold ethyl ether. In the case of the rainbow trout, the protein precipitates were further extracted by sonicating the residue in 2 mL of methanol for 10 s. The ether extracts were dried over Na_2SO_4 (0.5 g). The extraction efficiency was approximately 90% under the stated conditions.

Thin-Layer Chromatography (TLC) and Metabolite Identification. Two-dimensional TLC on 20 × 20 cm silica gel plates was used to analyze the ether and methanol extracts from incubations of the permethrin isomers with rainbow trout liver microsomes and ether extracts from those with carp microsomes. Detection and quantitation of the metabolites were by autoradiography and liquid scintillation counting (LSC) of appropriate zones of the TLC plate as previously described (Ueda et al., 1975). The following two-dimensional TLC solvent systems were used to identify individual metabolites by cochromatography: BE (benzene/ethyl acetate, 6:1) × CE (carbon tetrachloride/ether, 3:1) for 4′-HO-per, *t*-HO-per, *c*-HO-per, PBalc, PBald, and *c*-HO-Cl_2CA-lactone; BFE (benzene saturated with formic acid/ether, 10:3) twice × BEM (benzene/ethyl acetate/methanol, 15:5:1) for PBacid, 4′-HO-PBalc, Cl_2CA, *t*-HO-Cl_2CA and *c*-HO-Cl_2CA; BEM × BE for 4′-HO,*t*-HO-*c*-per. For R_f values in these solvent systems, see Gaughan et al. (1977) and Unai and Casida (1977).

In Vivo Metabolism. Rainbow trout exposed to a sublethal concentration (5 μg/L) of pure *trans*-[^{14}C-alcohol]permethrin for 2–24 h were dissected, and bile was removed by gall bladder puncture. Pooled bile samples were acidified to pH 1.8 with 1 N HCl and extracted five times with equal volumes of ethyl ether, giving extraction efficiencies of 90–99% for ^{14}C. The ether extracts were dried over Na_2SO_4.

To examine chromatographic properties of the biliary products, an aliquot of the ether extract (approximately 150 000 dpm) was subjected to two-dimensional TLC on a methanol-washed silica gel plate along with standards of *trans*-permethrin, PBalc, and PBacid. The plate was run with Bu/AcA/H_2O (butanol/glacial acetic acid/water, 6:1:1) and then in the second dimension with BFE twice. UV light was used to detect the standards prior to autoradiography. A portion of the bile–ether extract (25 000 dpm following ether evaporation) was incubated in 100 μL of $NaHPO_4$ buffer (pH 6.9) with and without 100 Sigma units of β-glucuronidase at 37 °C for 1 h. A comparable aliquot was treated with 1 unit of aryl sulfatase in acetate buffer (pH 5) at 37 °C for 24 h. Reactions were terminated by the addition of 100 μL of acetone to the incubates. The incubates were then directly applied to 5 × 20 cm silica gel TLC plates and the plates were developed in BFE twice along with standards of *trans*-permethrin, PBalc, and PBacid. The plates were scraped in 1 × 2 cm segments and the silica gel subjected to LSC.

RESULTS

In Vitro Metabolism by Carp and Rainbow Trout. *Liver Microsomes.* The data in Tables I and II clearly show that carp and rainbow trout liver microsomes metabolize permethrin by oxidation (cis isomer) or hydrolysis and oxidation (trans isomer). Hydrolysis seems to be the predominant route of metabolism of *trans*-permethrin by the liver microsomes of both species of fish. Carp microsomes appear to be more active than trout microsomes in hydrolyzing the trans ester. Neither carp nor rainbow trout liver microsomes significantly cleave the ester link

Table II. Metabolism of ^{14}C-Acid Preparations of *cis*- and *trans*-Permethrin by Carp and Rainbow Trout Liver Microsome NADPH Systems

	% of extracted radiocarbon			
	cis-permethrin		*trans*-permethrin	
compd	carp	trout	carp	trout
permethrin	76.1 (95.9)[a]	69.6 (96.6)	20.9 (31.5)	69.8 (82.0)
4'-HO-per	15.8	27.4	7.7	8.6
t-HO-per	2.5	0.4	0.4	0.3
c-HO-per	0	0	1.8	0.1
4'-HO, *t*-HO-per	1.8	0	0	0
Cl_2CA	1.9 (3.6)	0.8 (2.7)	63.5 (68.5)	19.7 (16.8)
t-HO-Cl_2CA	0	0	0.5	0.2
c-HO-Cl_2CA	0	0	1.0	0
c-HO-Cl_2CA-lactone	0.3	0	0.4	0
unknown	1.6 (0.5)	1.8 (0.7)	3.8	1.3 (1.2)

[a] Values in parentheses are the results in the absence of NADPH. Where not stated, the values were 0.

of *cis*-permethrin, i.e., about 95% is unmetabolized in all of the microsomal incubation systems tested in the absence of NADPH.

Permethrin undergoes oxidative metabolism when the microsomal preparations of both carp and rainbow trout are fortified with NADPH. More hydroxyester metabolites are recovered with *cis*- than with *trans*-permethrin. The preferred site of oxidation of both isomers by both carp and rainbow trout microsomes is the 4' position of the phenoxybenzyl moiety. The geminal dimethyl group is hydroxylated to a lesser extent and there is a general preference for the methyl group trans to the carboxy group. Carp microsomes seem more capable of hydroxylating at this position than rainbow trout liver microsomes.

Following ester cleavage, PBalc undergoes a minor degree (<0.5% of the total extracted radiocarbon) of oxidation to PBald (rainbow trout) and PBacid (carp). The oxidation to PBacid is not dependent on fortifying with NADPH.

The small amount of *c*-HO-Cl_2CA-lactone found in extracts of carp liver microsomes may be a degradation product of the unstable metabolite, *c*-HO-Cl_2CA (Unai and Casida, 1977). Most of the unidentified ^{14}C material was retained at the origin after development in the BFE twice × BEM system which suggests that these polar products may be conjugates or decomposed metabolites.

In Vivo Metabolism in Rainbow Trout. Bile from rainbow trout exposed to *trans*-[^{14}C-alcohol]permethrin contained two labeled products, both more polar than the parent compound (Figure 2). One in vivo product did not move from the origin in the BFE solvent but did migrate with Bu/AcA/H_2O as expected for a conjugate(s). Treatment of the bile extract with β-glucuronidase caused most of the radiolabeled polar material to be displaced from the origin and migrate to a less polar region of the TLC plate (Figure 3). Aryl sulfatase had no effect in displacing ^{14}C from the origin.

DISCUSSION

The comparative aspects of in vitro metabolism of the permethrin isomers by fish liver microsomes are summarized in Figure 1. Microsomes of carp and rainbow trout most extensively metabolize *trans*-permethrin by hydrolysis and *cis*-permethrin by oxidation. This pattern, resulting largely from the low level of microsomal esterase activity on *cis*-permethrin, is similar to that found with mammalian systems (Miyamoto, 1976; Soderlund and Casida, 1977a). Oxidative attack strongly favors the 4' position, followed, in general, by the *trans*-methyl and then the *cis*-methyl groups. This study with 1*RS* isomers may obscure stereospecific differences in the preferred methyl

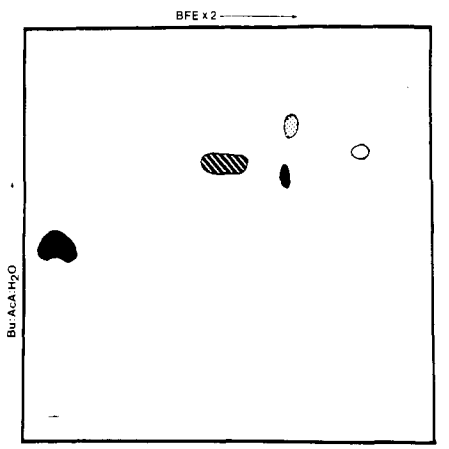


Figure 2. Diagrammatic representation of a two-dimensional thin-layer chromatogram of an ether extract of bile from rainbow trout exposed to *trans*-[^{14}C-alcohol]permethrin. Black spots, ^{14}C metabolites; open spot, authentic *trans*-permethrin; hatched spot, authentic PBalc; dotted spot, authentic PBacid. Note that the origin is positioned in the lower left hand corner.

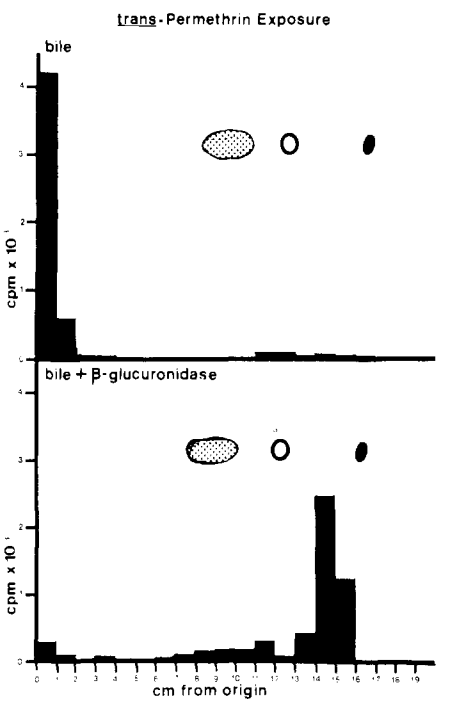


Figure 3. Thin-layer chromatogram of an ether extract of bile from rainbow trout exposed to *trans*-[^{14}C-alcohol]permethrin and treated with β-glucuronidase. The developing solvent was BFE × 2. Black spot, authentic *trans*-permethrin; dotted spot, authentic PBalc; open spot, authentic Pbacid.

group as with mouse microsomes (Soderlund and Casida, 1977b).

There do not appear to be any fundamental qualitative differences in permethrin metabolism between carp liver microsomes and those of rainbow trout although the extent of metabolism was greater with carp. This may mean that carp liver microsomes are more active than rainbow trout liver microsomes; however, no attempt was made in these studies to accurately compare the kinetics of liver microsomal metabolism by these two species of fish.

Fish metabolize a variety of foreign compounds (Dewaide, 1971) and may excrete the metabolites in the bile as conjugates (Lech, 1973). Rainbow trout metabolize permethrin in vivo since the bile of trout exposed to *trans*-[^{14}C-alcohol]permethrin contains polar metabolites which are probably conjugates(s). The majority of the conjugate fraction is cleaved on treatment with β-glucuronidase, suggesting that most of the polar material is a glucuronide conjugate. This study, establishing that permethrin undergoes in vitro and in vivo metabolism in fish, lays the background for more detailed investigations as to the identity of metabolites in the bile and the pharmacokinetics of permethrin uptake, metabolism, and excretion.

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Toxicity and Fate of Nine Toxaphene Fractions in an Aquatic Model Ecosystem

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The acute toxicity (96 h LC_{50}) of nine toxaphene fractions to bluegill fish (*Lepomic macrochirus*) varied from 2.6 to 29.0 μg/L (5.0 to 7.8 μg/L for unfractionated toxaphene). Bluegill fish, snails (*Helisoma* sp.), daphnids (*Daphnia magna*), and algae (*Oedogonium cardiacum*) were exposed to two water concentrations of unfractionated toxaphene and three of its fractions (representative of different toxicities) for 1–32 days in aquatic model ecosystems. Only slight differences were observed between the three fractions and unfractionated toxaphene in total amounts accumulated by each species. Bioaccumulation potential for bluegill fish was much higher than for snails (average of 6000 vs. 480 times water content, respectively). Thin-layer chromatographic analysis of fish and snail extracts taken after 3, 15, and 32 days of exposure indicated considerable metabolism by snails of all compounds but little metabolism by fish.

The widely used insecticide, toxaphene, is a complex mixture of at least 177 ten-carbon polychlorinated compounds (Casida et al., 1974). Attempts to fractionate and identify compounds within this mixture have met with limited success. Two compounds that have been identified, commonly referred to as toxicants A and B, are several times more toxic to mice and houseflies than the toxaphene mixture (Khalifa et al., 1974). The toxaphene mixture is also quite toxic to nontarget organisms, particularly aquatic organisms (Plimmentel, 1971), but little is known about the toxicity of individual compounds in the mixture. Thus, the structure, relative toxicity, and environmental behavior of the compounds in the toxaphene mixture are only beginning to be known. An obvious problem in studying such a complex mixture of compounds is to determine which part of the mixture to investigate first. We therefore initiated this study to determine if different fractions of the toxaphene mixture differed significantly in (1) acute toxicity to fish (test more species only if major differences

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