Uniformly ^{14}C-Ring-Labeled 2,4-Dichlorophenoxyacetic Acid: A Metabolism Study in Bluegill Sunfish, *Lepomis macrochirus*†

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The fate of 2,4-dichlorophenoxyacetic acid (2,4-D), a mixture of [*phenyl*(U)-^{14}C]-2,4-D and unlabeled 2,4-D, in bluegill sunfish was investigated after exposure to ∼11 ppm under static conditions for 4 days. Total radioactive residues (TRR) in whole fish increased from 0.41 ppm on day 1 to 0.60 ppm on day 3. TRR levels in fillet (edible) and viscera (nonedible) of treated fish on day 4 were 0.41 and 1.9 ppm, respectively. Most residues in both matrices were acetonitrile soluble; small amounts were hexane soluble or unextractable with solvents. Acid and base hydrolyses with ethyl acetate partitioning were used to release the fillet unextractable residues. The identification of 2,4-D and 2,4-dichlorophenol (2,4-DCP) in the fillet was conclusively confirmed by GC-MS analysis. On the basis of the experimental data from this study, a metabolic pathway for 2,4-D in bluegill sunfish in which the 2,4-D is metabolized to 2,4-DCP and conjugates of 2,4-D and 2,4-DCP is proposed.

Keywords: *2,4-Dichlorophenoxyacetic acid; 2,4-D; bluegill sunfish; herbicide; metabolism*

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is the active ingredient in a variety of herbicidal formulations that are primarily used in pre- and postemergence applications for broadleaf weed control in turf and field crops. The use patterns provide a possible route for entry of 2,4-D residues into the aquatic environment from runoff or other similar mechanisms.

A review of the published literature (*1*−*6*) and the aerobic soil metabolism study (*7*, *8*) indicated that 2,4-D is subject to rapid degradation in soils or soil−water systems due to microbial action. The microbial degradation product is primarily carbon dioxide (*6*−*8*). A review (*5*) of the published studies on the uptake and metabolism of the dimethylamine salt of [^{14}C]-2,4-D by fish suggested that when microbial degradation of 2,4-D occurred in the water to which the fish were exposed, extensive uptake of radioactivity occurred, but the level of 2,4-D in the fish was either not detected or very low (<0.05 ppm) for water concentrations of 2,4-D at 2 ppm (*3*, *6*). When degradation of the 2,4-D was minimized in the water, all radioactivity in the fish was present either as the unchanged compound (*5*) or as the unchanged compound with its conjugates and 2,4-dichlorophenol (2,4-DCP) (*3*).

This paper presents the results of a study designed (1) to minimize the microbial degradation of 2,4-D in order to reduce the amount of ^{14}C-labeled carbon dioxide available for incorporation into the basic biochemicals components of fish tissue, (2) to provide sufficient exposure to 2,4-D to maximize the amount of 2,4-D accumulated in the edible portion of the fish, and (3) to characterize the nature of the ^{14}C residue in the edible tissue.

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MATERIALS AND METHODS

Test System and Aquaculture. The 350 bluegill sunfish (*Lepomis macrochirus*) used in the study were held in culture tanks on a 16-h daylight/8-h darkness photoperiod with a 30-min transition. Fish culture techniques were basically those described by Brauhn and Schoettger (*9*). Observations of the fish were recorded daily during the holding period. During the test periods, the fish received Rangens Salmon Starter ad libitum (∼4.0 g per aquarium per day). Based On the basis of measurements of a representative group, the fish used for this study had an initial mean body weight of 5.5 ± 1.6 g and an initial mean standard length of 56 ± 4.2 mm.

The test was conducted in five glass aquaria: four treated and one control. A small-volume aquarium treated with test material, but not holding any fish, was used as a control-blank to monitor the effect of the test environment on the test material. All culture and dilution water used in the study was from an uncontaminated, deep well source. The total volume of water in the test aquaria was 70 L. Each aquarium was aerated throughout the test. Glass diluter components were used, and the aquaria were covered with glass. Before the test fish were added, the treated, control, and control-blank aquaria were immersed in a water bath that was brought up to 22 ± 2 °C. Measurements of the water quality in subsamples from all aquaria were made daily during the test period.

Preparation of Test Substance and Dosing Solution. The stock dosing solution was prepared by using ∼0.28 g of purified [^{14}C]-2,4-D test substance (26.0 mCi/mmol, radiochemical purity = 99.5% from New England Nuclear) and 2.80 g of nonlabeled 2,4-D test substance in 50.0 mL of acetone [combined concentration = 66.2 mg/mL; specific activity = 2.38 × 10^4 dpm/μg (2.37 mCi/mmol)]. A 10.6-mL aliquot (∼700 mg of 2,4-D) was diluted with ∼4 L of water and added to ∼66 L of water in each treated aquarium. The control aquarium contained only water. A 1-L glass amber bottle with 0.95 mL (∼63 mg) of stock dosing solution was filled with water for the control-blank aquarium.

To demonstrate that the fish were exposed to a minimum of 10 ppm of 2,4-D and not to radiolabeled 2,4-D degradates, daily water samples were subjected to HPLC analysis. Three 0.2-mL aliquots from separate regions of each aquarium were analyzed individually by liquid scintillation counting (LSC) on day 0. On subsequent days, the aliquots from the four treated aquaria were pooled and quantitated by LSC in triplicate.

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Water from the control and control-blank aquaria was put in 20-mL borosilicate scintillation vials. At the end of the exposure phase, ~1000 mL of water was collected from each aquarium for the characterization of radiolabeled residues. All samples were stored at ~−20 °C.

Fish Handling and Dosing. The uptake phase was initiated by impartially transferring 70 fish each to the control and four treatment aquaria. The aquaria were kept under constant aeration and in the water bath to maintain the temperature at the target level. These fish were observed at 0 time and twice daily thereafter during the exposure period for any mortality or adverse behavior. Fish were not added to the control-blank aquarium.

Sample Collection, Preparation, and Analysis. On days 1−3, one fish from each treated aquarium and the control aquarium was collected and quickly frozen with dry ice pending homogenization. The four treated fish from each day were pooled for whole-fish ^{14}C residue determination. On day 4, the remaining fish from the control (one fish escaped) and treated (one fish died) aquaria were removed and dissected into fillet and viscera portions. The pooled fillet (edible parts—muscle, skin, and skeleton) and viscera (nonedible parts—fins, head, and internal organs) portions were sectioned. Samples were frozen immediately and stored at ~−20 °C. The whole fish were processed by homogenization with dry ice in a blender. The fillet and viscera frozen individual samples were processed for radioanalysis by homogenization with dry ice in a grinder, the dry ice was allowed to sublime, and the sample was weighed.

Quantification of Radioactivity. Oxidations of the processed tissues were conducted with a Harvey OX-500 biological material oxidizer. Recovery of $^{14}CO_2$ was confirmed by fortifying cellulose powder with a ^{14}C standard ([^{14}C]benzoic acid). The results for samples were corrected for instrument and matrix bias.

Radioactivity was measured by using a TM Analytic Delta 300-6891 liquid scintillation counting system with a Toshiba T100 portable PC. Using the external standard ratio technique counting efficiencies were determined. The radioactive residue levels determined by the combustion radioanalysis method were further corrected for the matrix and machine recoveries.

High-Performance Liquid Chromatography (HPLC). HPLC was performed using a Shimadzu HPLC system equipped with two LC-6A pumps and an SCL-6A system controller, a Shimadzu SPD-6A UV detector at 280 nm, a Raytest Ramona 90 radiodetector with 400 μL of CaF flow cell, and a Gilson model 202 fraction collector with a Nova-Pak C-18 column (8 × 100 mm) (Waters, St. Louis, MO) and acetonitrile/water mobile phases acidified with 0.5−1% (v/v) acetic acid. Chromatographic conditions consisted of two, sequential linear gradients: the first was 30 min from 10% acetonitrile to 50% acetonitrile, and the second was 25 min to 100% acetonitrile, then isocratic for 5 min at 100% acidified acetonitrile at a flow rate of 1.5 mL/min. Retention times for 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-DCAA) (Dow AgroSciences, 95.5%), 4-chlorophenoxyacetic acid (4-CPAA) (Dow AgroSciences, 99.8%), 4-chlorophenol (4-CP) (Dow AgroSciences, 99%), 2,4-D (Dow AgroSciences, 99.5%), 2,4-dichlorophenol (2,4-DCP) (Dow AgroSciences, 99.6%), 2,4-D methyl ester (Dow AgroSciences, 99.7%) and 2,4-dichloroanisole (2,4-DCA) (Dow AgroSciences, 99%) are presented in Table 1. For the preparative scale HPLC purification of the radiolabeled test substance, a Waters Nova-Pak C-18 radial compression column (10 mm i.d. × 25 cm) was used (three sequential linear gradients, the first 25 min from 10% acetonitrile to 35% acetonitrile, then 5 min from 35% acetonitrile to 50% acetonitrile and, finally, 5 min to 100% acetonitrile at a flow rate of 7.0 mL/min).

The chemical nature of recovered radioactive residues was qualitatively determined by HPLC and LSC by comparison with the retention times of analytic standards. The solutions of reference standards were prepared in acetonitrile and analyzed by HPLC to determine their retention times, as recorded by the UV detector. The LSC analysis of the collected fractions showed that [^{14}C]-2,4-D had a retention time of ~27.0 min. A standard reference mixture with 2,4-D, [^{14}C]-2,4-D, 2,4-DCP, and 2,4-DCA was prepared for injection during the HPLC analyses of water samples and tissue extracts.

Table 1. Individual HPLC Retention Times for 2,4-D and Standard Reference Compounds

reference standard	UV retention time (min)
2,5-dichloro-4-hydroxyphenoxyacetic acid (4-hydroxy-DCAA)	15.03
4-chlorophenoxyacetic acid (4-CPAA)	21.08
4-chlorophenol (4-CP)	21.63
2,4-dichlorophenoxyacetic acid (2,4-D)	26.97
2,4-dichlorophenol (2,4-DCP)	28.82
2,4-dichlorophenoxyacetic acid methyl ester (2,4-D methyl ester)	35.17
2,4-dichloroanisole (2,4-DCA)	38.55

Mass Spectrometry Analysis. Gas chromatography−mass spectrometry (GC-MS) was performed using a Hewlett-Packard 5970B quadruple mass spectrometer interfaced with a Hewlett-Packard 5890 series II GC. The mass spectrometer was operated in the EI mode with ionizing voltage set at 70 eV. The separations were performed on a J&W DB-1701, 30-m fused silica capillary column. Chromatographic conditions consisted of an injector temperature of 150 °C, a mass spectrometer interface temperature at 265 °C, splitless injection mode, a 2-μL injection volume, a 5 psi column head pressure, and helium carrier gas, with a linear temperature gradient stating at 60 °C (held for 1 min) and rising to 295 °C over 18 min. Retention times for the standard 2,4-D methyl ester and 2,4-dichloroanisole (methylated 2,4-DCP) were 14.3 and 11.2 min, respectively.

Extractions of ^{14}C Residue. *Fillet (edible).* A treated fish fillet subsample was used for extraction and characterization of ^{14}C residues (Figure 1). The subsample was homogenized three times with 75 mL of acetonitrile/0.1 M HCl (4:1) in a Virtishear blender and centrifuged. The acidic acetonitrile volume was measured, and 1.0-mL aliquots (in triplicate) were taken for LSC analyses. The pellet was dried and weighed, and subsamples (0.1 g in triplicate) were oxidized. The acidic acetonitrile was partitioned three times with 30 mL of acetonitrile-saturated hexane. Triplicate aliquots (1.0 mL) of the acetonitrile partition were analyzed directly by LSC. The hexane partition was concentrated under a gentle stream of nitrogen (N_2), and triplicate aliquots (0.2 mL) were analyzed by LSC. The acetonitrile partition was similarly concentrated and analyzed by LSC. The concentrated acetonitrile partition (0.4 mL) was analyzed by HPLC followed by LSC of the collected fractions. A [^{14}C]-2,4-D-fortified (with approximately the same level of radioactivity as found in the treated sample) control fillet was also extracted using the above procedure to validate the method of extraction. The concentrated acetonitrile partition (0.4 mL) was analyzed by HPLC followed by LSC of the collected fractions.

To determine if polar radioactive peaks in the radiochromatogram of the acidic acetonitrile partition were conjugates, an acid hydrolysis of this partition was performed. Aliquots (4 mL) of the fortified control and treated acetonitrile partitions were placed into a solution of 2 N HCl (46 mL) and refluxed for 8 h. The hydrolysates were allowed to cool overnight and then were partitioned with diethyl ether (3 × 20 mL). The aqueous and diethyl ether partitions were analyzed by LSC. The diethyl ether partitions were then concentrated under a stream N_2 and transferred to acetonitrile for HPLC analysis followed by LSC of the collected fractions. The diethyl ether partition of [^{14}C]-2,4-D-fortified control fillet extract subjected to acid hydrolysis was similarly prepared and analyzed.

Diethyl ether partitioning served as a cleanup step in the preparation of the acidic acetonitrile extract for GC-MS analysis. A stream of N_2 was used to remove the acetonitrile from a 20-mL aliquot (5-μg equivalents of [^{14}C]-2,4-D) of the fillet extract. The pH of the extract was reduced to ~1 with 2 N HCl, and the extract was partitioned using diethyl ether (1 × 20 and 3 × 5 mL); the two phases were separated by centrifugation. Both the aqueous and diethyl ether phases

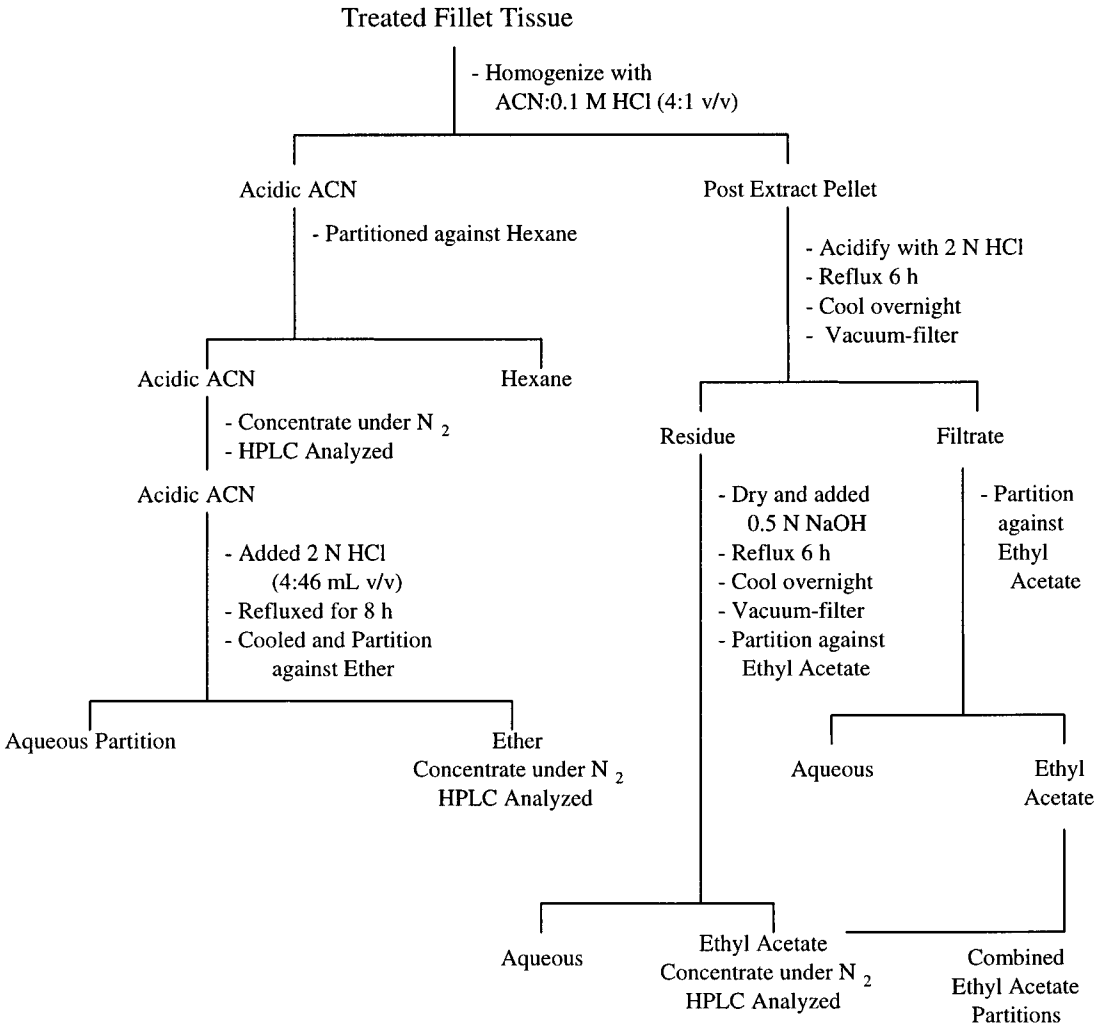

Figure 1. Extraction scheme for the isolation of 2,4-D metabolites in fillet.

were quantitated for total radioactivity by LSC analysis of aliquots (0.5 and 0.2 mL, respectively) in triplicate. The diethyl ether phase was concentrated to near dryness under N_2, transferred to acetonitrile, and concentrated again, and triplicate 0.025-mL aliquots were taken for LSC analysis. The concentrated diethyl ether partition was prepared for HPLC analysis followed by LSC of the collected fractions.

Derivatization of the diethyl ether phase was required to make the radioactive metabolites suitable for GC-MS analysis. To convert 2,4-D to its methyl ester, diazomethane was reacted with the diethyl ether phase for 30 min at room temperature with gentle mixing (*3*). The diazomethane was then removed using N_2. To ensure that the methylation of 2,4-D occurred, the reaction in the same vial was repeated with fresh diazomethane for a total of three times. The methylated product was analyzed by HPLC and GC-MS.

Both mild and harsh hydrolyses were used to release unextractable ^{14}C residue from the postextracted treated fillet pellet. In the mild hydrolysis, ~2.1 g of treated fillet pellet was placed in a solution of 2 N HCl (30 mL) and acetonitrile (5 mL). The hydrolysates were refluxed for 6 h, allowed to cool overnight, and then vacuum filtered. The residues on filter paper were dried in a desiccator, and the filtrates were partitioned with water-saturated ethyl acetate (4 × 20 mL). The aqueous and ethyl acetate partitions were analyzed for radioactivity by LSC (3 × 2 mL). Following mild acid hydrolysis the residue remaining in the pellet was further hydrolyzed in a solution of 0.5 N NaOH (30 mL). The hydrolysates were refluxed for 6 h, allowed to cool overnight, and then vacuum filtered. The filtrates were partitioned with water-saturated ethyl acetate (4 × 20 mL). The aqueous and ethyl acetate partitions were analyzed for radioactivity by LSC (3 × 2 mL). The ethyl acetate partitions after the acid and base hydrolyses were combined and concentrated under a gentle stream of N_2. This concentrated fraction was prepared for HPLC analysis followed by LSC of the collected fractions.

A harsher hydrolysis scheme was used in an attempt to release more radioactivity from the treated fillet pellet. In this scheme, the treated (~10.1 g) fillet pellet was placed in a solution of 2 N HCl (80 mL) and acetonitrile (15 mL). The hydrolysate was refluxed for 16 h, allowed to cool, and vacuum filtered. The residues on the filter paper were dried in a desiccator, and the filtrate was partitioned with water-saturated ethyl acetate (4 × 30 mL). The aqueous (3 × 3 mL) and ethyl acetate (3 × 2 mL) partitions were analyzed for radioactivity by LSC. The ethyl acetate partition was concentrated under N_2 in preparation for HPLC analysis. After the strong acid hydrolysis, the resultant tissue pellet was transferred to a solution of 2 N NaOH (80 mL) and acetonitrile (15 mL). The hydrolysate was refluxed for 16 h, allowed to cool, and vacuum filtered. The residue was dried in a desiccator, and aliquots were combusted to determine the remaining unextractable radioactivity. The filtrate was partitioned with water-saturated ethyl acetate as described above; the aqueous and ethyl acetate partitions were subjected to LSC analysis (3 × 2 mL).

Viscera (Nonedible). Because the viscera were considered to be nonedible, only the organosoluble ^{14}C residues were extracted and characterized. A viscera subsample was homogenized three times with 75 mL pf acetonitrile/0.1 M HCl (4:1) and centrifuged. The acidic acetonitrile extract was separated from the pellet and the extractable residue quantitated by LSC analyses. The pellet was separated, dried, and weighed, and subsamples (~0.1 g in triplicate) were oxidized

Table 2. Distribution of ^{14}C Residues in Extracts of Fillet from Bluegill Sunfish Exposed to 2,4-D for 4 Days

description	HPLC %	% of TRR	ppm
treated fillet		100	0.41
hexane partition		**1.2**	**0.005**
acetonitrile partition (0.328 ppm after concentration)		**84**	**0.34**
polar unknown (4.0 min)	1.8	1.5	0.006
possible conjugate (17.0−18.0 min)	5.4	4.4	0.02
2,4-D (27.5−29.0 min)	87	70	0.29
2,4-DCP (29.0−29.5 min)	5.7	4.7	0.02
acid hydrolysis of acetonitrile partition			
aliquot hydrolyzed		81	0.33
aqueous partition		<MQL[a]	<MQL
ether partition		82	0.33
polar unknown (3.0 min)	1.8	1.5	0.006
2,4-D (26.5−28.5 min)	87	74	0.30
2,4-DCP (28.5−29.5 min)	9.6	7.9	0.03
pellet		**10**	**0.04**
mild acid hydrolysis of pellet			
aqueous partition		<MQL	<MQL
ethyl acetate partition		5.2	0.02
mild base hydrolysis of pellet			
aqueous partition		<MQL	<MQL
ethyl acetate partition		2.5	0.01
combined ethyl acetate partitions (acid and base)		7.1	0.03
polar unknown (3.0 min)	10	0.7	0.003
2,4-D (27.0−29.0 min)	90	6.4	0.03
harsh acid hydrolysis of pellet			
aqueous partition		0.5	0.002
ethyl acetate partition		5.2	0.02
2,4-D (26.0−28.5 min)	74	3.9	0.02
2,4-DCP (28.5−29.5 min)	7.6	0.5	0.002
unaccounted for	18	1.0	0.004
harsh base hydrolysis of pellet			
aqueous partition		2.5	0.01
ethyl acetate partition		0.7	0.003
residue		<MQL	<MQL
total extractable and unextractable		**95**	**0.39**

[a] Value is less than the minimum quantifiable limit.

to determine total ^{14}C residues. The acidic acetonitrile extract was partitioned with 30 mL of acetonitrile-saturated hexane three times. Triplicate aliquots (1.0 mL) of the acetonitrile partition were analyzed directly by LSC. The hexane partition was concentrated under a gentle stream of N_2, and triplicate aliquots (0.2 mL) were analyzed by LSC and HPLC. The acetonitrile partition was concentrated under a gentle stream of N_2 and aliquoted for LSC and HPLC. A [^{14}C]-2,4-D-fortified (with approximately the same level of radioactivity) control viscera subsample was also extracted using the above procedure to validate the method of extraction.

RESULTS AND DISCUSSION

Water Analysis. Water quality conditions maintained during the exposure period were excellent for supporting the bluegill sunfish. The temperature, dissolved O_2, and pH in the control, control-blank, and treated tanks were comparable with values of 21 °C, 7.8 ± 0.5 mg/mL (75−100% saturation at 21 °C), and 8.4 ± 0.2, respectively. The results of HPLC analysis of the daily water samples showed that the bluegill sunfish were exposed to ∼11 ppm of unchanged 2,4-D throughout the 4-day exposure phase of the study.

Distribution of Total Radioactivity. Combustions of aliquots of whole fish demonstrated that, within 24 h of exposure to 11 ppm of [^{14}C]-2,4-D, the concentration of the test substance in the whole fish was sufficient to characterize and identify the chemical components (0.41 ppm) and that the levels steadily increased to 0.60 ppm after 72 h of exposure.

The total radioactive residue (TRR) levels of [^{14}C]-2,4-D equivalents present in the treated fillet and viscera were determined to be 0.41 and 1.9 ppm, respectively. The distribution of ^{14}C residues in extracts of fillet and viscera from treated fish are presented in Tables 2 and 3, respectively.

Isolation, Characterization, and Identification of ^{14}C Residues. *Fillet.* Extraction of the fillet from treated fish with acidic acetonitrile released ∼90% of the TRR. The resulting pellet contained ∼10% of the TRR. When the extractable ^{14}C residues were partitioned with hexane, 1.2% of the TRR was present in the hexane partition, and 84% of the TRR remained in the acidic acetonitrile partition. Concentration of the acidic acetonitrile partition from the treated fillet resulted in a loss of ∼3% and an overall recovery of ^{14}C residues in the concentrated acidic acetonitrile of 81% of the TRR. The major component present, by retention time comparison, was 2,4-D (70% of the TRR). In addition, small quantities of 2,4-DCP (4.7% of the TRR) and two relatively polar unknown metabolites (4.4 and 1.5% of the TRR) were present as identified by chromatographic mobility (Figure 2).

The acetonitrile extract was subjected to acid hydrolysis to evaluate the presence of any conjugates. Virtually all of the radioactivity partitioned into the diethyl ether phase. HPLC analysis of the concentrated diethyl ether partition indicated the possible presence of conjugates. The radioactive peak eluting between 17 and 18 min as seen in radiochromatogram of the acidic acetonitrile partition was absent in the HPLC analysis of the concentrated ether partition. In the radiochromatogram of the ether partition, 74% of the recovered radioactivity eluted at a retention time range similar to that of 2,4-D, 7.6% eluted at a range similar to that

Table 3. Distribution of ^{14}C Residues in Extracts of Viscera from Bluegill Sunfish Exposed to 2,4-D for 4 Days

description	HPLC %	% of TRR	ppm
treated viscera		100.	1.9
hexane partition		**4.5**	**0.09**
possible conjugate (16.0 min)	1.5	0.1	0.001
2,4-D (25.5−26.5 min)	31	1.4	0.023
2,4-DCP (26.5−28.0 min)	47	2.1	0.04
2,4-DCA (37.0−38.0 min)	5.4	0.3	0.005
nonpolar unknown (42.0−43.0 min)	5.2	0.2	0.004
unaccounted for	10	0.5	0.009
acetonitrile partition		**106**	**2.0**
polar unknown (3.0−4.0 min)	5.8	5.8	0.11
possible conjugate (17.0 min)	0.9	0.9	0.02
4-CPAA and 4-CP (22.5−26.0 min)[a]	40	40	0.76
2,4-D (26.0−27.5 min)	28	28	0.54
2,4-DCP (28.5−29.0 min)	26	26	0.49
pellet		**8.6**	**0.16**
total extractable and unextractable		**119**	**2.3**

[a] Two peaks quantitated together as they were not resolved at the baseline.

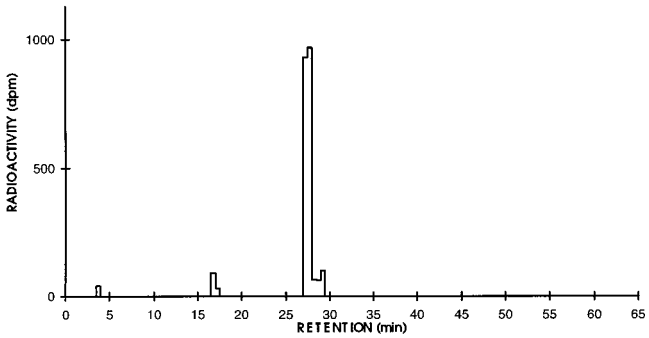

Figure 2. HPLC radiochromatogram of acidic acetonitrile soluble fillet residues.

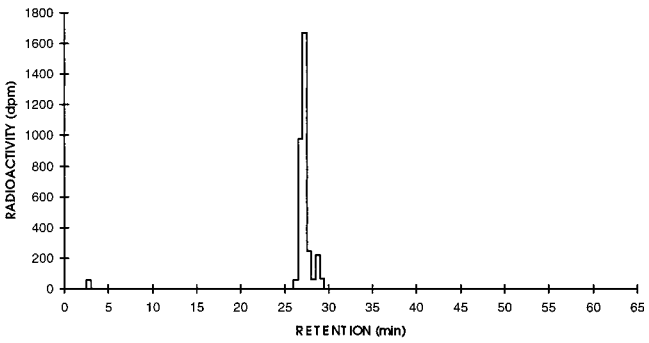

Figure 3. HPLC radiochromatogram of ether partition of acid-hydrolyzed acidic acetonitrile soluble fillet residues.

of 2,4-DCP, and 1.5% eluted in the void volume (Figure 3). The change in the elution profile of the radioactive residue following acid hydrolysis indicated a mixture of 2,4-D and/or 2,4-DCP conjugates.

When the ^{14}C residue present in the ether phase was subjected to esterification, HPLC analysis suggested that methylation had occurred. The components that eluted at the retention time for 2,4-D and 2,4-DCP had shifted to longer retention times. The new retention time was consistent with those for standards of 2,4-D methyl ester and 2,4-DCA. GC-MS data confirmed the presence of methyl esters of 2,4-D and 2,4-DCP. Full scan analysis of the 2,4-D methyl ester reference standard and the tentatively identified component from treated fillet revealed that both showed a characteristic fingerprint (*m*/*z* 234, 199, and 175). The characteristic chlorine contribution was observed for each mass unit at 236/238, 201, and 177. The full scan spectrum of the 2,4-DCA reference standard and the methyl ether of 2,4-DCP showed that both displayed the same characteristic fingerprint and characteristic chlorine atom contribution (*m*/*z* 176/178/180, 161/163/165, and 133/135/137).

Mild acid and base hydrolyses extracted an additional 76% of the total radioactivity remaining in the acidic acetonitrile extracted pellet. When the ^{14}C residues released as a result of the acid and base hydrolyses (7.1% of the TRR) were pooled, concentrated, and subjected to HPLC analysis (Figure 1), the majority (90%) of the radioactivity eluted at the time range characteristic of 2,4-D and represented 6.4% of the TRR. On the basis of LSC analyses, harsher hydrolysis conditions did not appear to liberate any additional radioactive residue from the acidic acetonitrile extracted fillet pellets. After harsh acid hydrolysis, the ethyl acetate partition contained 5.2% of the TRR, and after base hydrolysis, the ethyl acetate partition contained only 0.7% of the TRR. The HPLC analysis of the ethyl acetate partition after harsh acid hydrolysis showed that 74% of the recovered radioactivity had an elution range comparable to 2,4-D and represented 3.9% of the TRR, and 7.6% of the radioactivity had an elution range similar to that of 2,4-DCP and represented 0.5% of the TRR. The total radioactivity recovered from the treated fillet pellet was 89%.

Viscera. When the initial acidic acetonitrile extract was partitioned with hexane, 4.5% of the TRR (0.086 ppm) was partitioned into the hexane, and 106% of the TRR remained in the acidic acetonitrile partition. Approximately 8.6% of the TRR remained unextracted in the pellet.

HPLC analysis of the hexane partition of the viscera (Figure 4) from the treated fish showed the presence of two major components: one component (2.1% of the TRR) eluting at a retention time similar to that of 2,4-DCP and the second (1.4% of the TRR) eluting with a retention time similar to that of 2,4-D. A minor component (0.3% of the TRR) eluted with a retention time similar to that of 2,4-DCA. A very nonpolar unknown metabolite (0.2% of the TRR) eluted at 42.0−43.0 min, and a minute quantity (0.1% of TRR) eluted at 16.0 min. Both DCP and DCA have been observed in soil metabolism studies (*8*). Possibly their presence in the hexane extract of the viscera was a result of the degradation of 2,4-D by microbes located in the viscera.

The HPLC analysis profile of the acidic acetonitrile partition could represent 4-CPAA and 4-CP (40% of the TRR), 2,4-D (28% of the TRR), and 2,4-DCP (26% of the

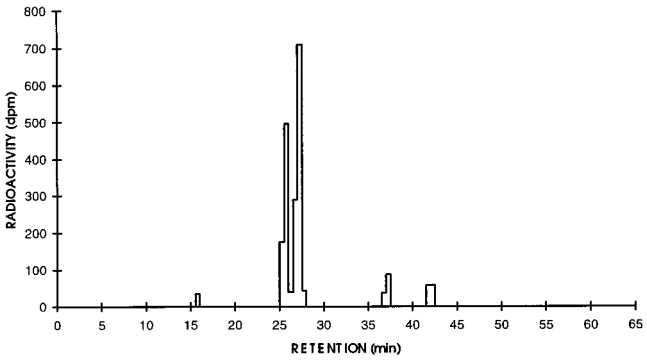

Figure 4. HPLC radiochromatogram of hexane partition of acidic acetonitrile soluble viscera residues.

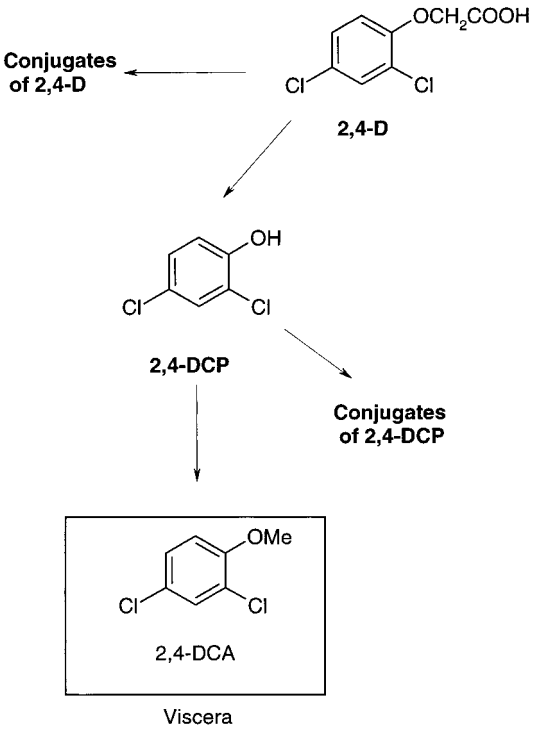

Figure 5. Proposed metabolic pathway for 2,4-D in bluegill sunfish.

TRR). The presence of 2-chlorophenoxyacetic acid and 2-chlorophenol cannot be ruled out because reference standards were not available for chromatographic comparison. Also present in small quantities were a possible conjugate eluting at 17 min (0.9% of the TRR) and a very polar unknown metabolite (5.8% of the TRR) at 3.0−4.0 min. Microbial action in the viscera could also be the source of the monochlorinated degradates of 2,4-D (*10*).

An aliquot of control fish fillet, fortified with [^{14}C]-2,4-D to a TRR level of 0.46 ppm, was subjected to similar extraction procedures. When the acidic acetonitrile was further partitioned with hexane, 0.2% of the TRR was partitioned into hexane, and 97% of the TRR remained in the acidic acetonitrile. Only 2,4-D was identified in the fortified control fillet. A small amount of a polar unknown (1.8% of the TRR) with a retention time of 3.0 min was found in the ether partition of the acid-hydrolyzed acetonitrile partition. Approximately 5.5% of the TRR remained unextracted in the pellet. The overall mass balance was 103% in the fortified control fillet extracts. Stability of 2,4-D to these hydrolytic conditions was demonstrated by subjecting 2,4-D to the acid hydrolysis conditions. The Radiochromatogram of the resulting fortified control partition revealed 98% of the recovered radioactivity eluted at the characteristic time range of 2,4-D. The results of the fortified control viscera extraction were very similar. Of the fortified radioactivity, 0.4% of the TRR (0.008 ppm) was present in the hexane partition, 94% of the TRR (1.7 ppm) remained in the acidic acetonitrile partition, and 8.1% of the TRR (0.145 ppm) remained in the pellet, for a total recovery of 102% (1.84 ppm). These data demonstrated that 2,4-D was stable during the extraction, fractionation, and characterization methods conducted to evaluate the fate of 2,4-D in fish.

On the basis of the experimental data from this study, we propose a metabolic pathway (Figure 5) of 2,4-D in bluegill sunfish in which the 2,4-D is metabolized to 2,4-DCP and conjugates of 2,4-D, and the 2,4-DCP is subsequently metabolized to conjugates of 2,4-DCP. 2,4-D and 2,4-DCP were present in the fillet (edible portion), and the identification was conclusively confirmed by GC-MS analysis comparing known standards with isolated metabolites.

Conclusion. The composition of the residues isolated from bluegills (fillet and viscera) in this study differed from those reported by Stalling and Huckins (*6*). Stalling and Huckins reported that 2,4-D rapidly degraded in their aquatic test system, which led to the metabolic incorporation of the ^{14}C label into specific natural biochemicals. The experimental conditions described in this report did not lead to the degradation of the 2,4-D, thus resulting in the evaluation of the uptake and metabolism of 2,4-D by bluegills. The results of our investigation demonstrate that 2,4-D increases in the tissue of bluegill sunfish with time of exposure. The viscera (1.9 ppm, 2,4-D equivalents) contained significantly more residues than found in the edible fillet (0.41 ppm, 2,4-D equivalents). The major residue was 2,4-D, with the remaining residue components consisting of 2,4-DCP, acid labile conjugates of 2,4-D and 2,4-DCP, and bound residues.

ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; 2,4-DCA, 2,4-dichloroanisole; 4-CPAA, 4-chlorophenoxyacetic acid; 4-CPA, 4-chlorophenol; HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; GC-MS, gas chromatography−mass spectrometry; TRR, total radioactive residue; MQL, minimum quantifiable limit.

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