Table IX. Release of Diflubenzuron from 1% Attaclay and 1% Sand Granules in Water Treated with 0.1 ppm AI

Formulation	Hour after treatment			
	4	24	48	72
1% Attaclay (lot 1)	0.011[a]	0.015	0.021	0.025
1% Attaclay (lot 2)	0.012	0.0098	0.011	0.016
Av	0.012[b]	0.012	0.016	0.020
1% Sand (lot 1)	0.014	0.026	0.030	0.030
1% Sand (lot 2)	0.016	0.024	0.028	0.047
Av	0.015	0.025	0.029	0.038

[a] Average of two replicates, in ppm. [b] Grand average of both lots.

Application of 1% sand granules at 0.075 lb of AI/acre on Smith's pasture provided 100% control of *A. nigromaculis* larvae, as would be expected at this high rate, but did not generate significant residues in soil or on vegetation (Table VIII). The lack of measurable residues on vegetation was surprising and may be due to use of the upper part of a container of 1% diflubenzuron on sand, with the probable settling of dust into the lower section and therefore not having been placed into the aircraft hopper.

These experiments demonstrate that diflubenzuron can be formulated to provide mosquito control without the production of undesirable residues on vegetation.

Release of Diflubenzuron from Granules. There was greater variation in the release of diflubenzuron from lots of the 1% Attaclay than from lots of the sand granules. It is apparent that release from the sand is faster and also that a greater amount is released during the same period (Table IX). These release properties of the sand granules are considered to be favorable for mosquito control.

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Uptake and Metabolism of Dimethylamine Salt of 2,4-Dichlorophenoxyacetic Acid by Fish

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Bluegills and channel catfish removed less than 0.5% of dimethylamine salt of ^{14}C-ring-labeled 2,4-dichlorophenoxyacetic acid (DMA-[^{14}C]-2,4-D) when exposed in aquaria to water containing 2 ppm of the herbicide. The maximum concentration of 2,4-D in the fish was reached within 24 h of treatment; thereafter it did not change significantly up to 7 days. Catfish removed a smaller amount of the herbicide from the water than bluegills. No evidence for bioaccumulation of 2,4-D in the fish was noted during the duration of the experiment. A major portion of the radioactivity absorbed by the fish was associated with the head and viscera portions with relatively low concentrations in the edible flesh. The fish did not metabolize 2,4-D during the 7 days following treatment. Bluegills administered DMA-[^{14}C]-2,4-D by intraperitoneal injection excreted 90% of the herbicide within 6 h of treatment.

The dimethylamine (DMA) salt of 2,4-dichlorophenoxyacetic acid (2,4-D) is used extensively for controlling aquatic plants such as water hyacinth and Eurasian watermilfoil. This form of the herbicide is relatively nontoxic to fish; the 96-h TL_{50} values for bluegills (*Lepomis macrochirus*) and channel catfish (*Ictalurus punctatus*) are 160 and 125 ppm, respectively (Schultz, 1973). A knowledge of the degree of accumulation of this herbicide by fish is important if they are to be used for human consumption. The herbicide, if accumulated by fish, may undergo metabolic transformation. The nature of these metabolites must be known in order to assess their possible toxicity to fish and man.

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Presently, very little information is available on the uptake and metabolism of 2,4-D by fish. Rodgers and Stalling (1972) studied the uptake and elimination of the ^{14}C-labeled butoxyethanol ester (BEE) of the herbicide in three species of fish. They reported that the maximum residue concentrations were in the fish within 1 to 2 h of exposure. Schultz (1973) examined the uptake and distribution of DMA-[^{14}C]-2,4-D by three species of fish. In these studies, the fish were exposed to the herbicide in plastic pools containing water and a layer of soil at the bottom. The concentration of ^{14}C residues in the edible portion of the fish continued to increase up to 84 days after treatment, but the actual 2,4-D content was negligible, indicating that most of the ^{14}C residue was a metabolite(s) of 2,4-D. When the fish were exposed to DMA-2,4-D in water containing the herbicide but not soil, low levels of non-2,4-D residues were found in them. Since 2,4-D is readily degraded by microorganisms (Loos, 1969) and by photochemical processes (Aly and Faust, 1964; Crosby and

Tutlass, 1966), it is not clear whether the fish actually metabolized the herbicide or if 2,4-D metabolites were first produced in the water and sediment in the pools and subsequently taken up by fish. In order to assess the ability of fish to metabolize 2,4-D, we studied uptake and metabolism of DMA–2,4-D by bluegills and channel catfish under conditions in which degradation of the herbicide due to microbial and photochemical processes was minimal.

MATERIALS AND METHODS

Chemicals. Dimethylamine salt of uniformly ring-labeled [^{14}C]-2,4-D (DMA–[^{14}C]-2,4-D) with a sp act. of 5.38 mCi/mmol was purchased from California Bionuclear Corp., Sun Valley, Calif. This material was judged radiochemically pure by thin-layer chromatography in solvent systems consisting of chloroform and chloroform–methanol (1:1 v/v). Nonradioactive DMA–2,4-D as an aqueous solution containing 524.8 g/L of DMA–2,4-D was provided by Amchem Products, Inc., Ambler, Pa.

Uptake of 2,4-D. The fish (3–4 in. long) were obtained from the National Fish Hatchery, Orangeburg, S.C. They were acclimated to laboratory conditions for 2 weeks before being exposed to 2,4-D. The fish were introduced into fresh springwater containing 2 ppm of DMA–[^{14}C]-2,4-D (pH, 7.6; water temperature, 21 °C). Each liter of water contained one fish (loading ratio 4–6 g/L) and was continuously bubbled with air during the exposure of the fish to the herbicide. All tests were conducted under laboratory light conditions (50 ft-c, provided by fluorescent lamps for 12 h/day). Appropriate controls without fish were also included in the study. Two fish were removed from the treated water at appropriate intervals, rinsed with clean water three times and weighed. To determine the amount of radioactivity in the whole body, the fish were cut into small pieces and homogenized with acetone in a Virtis homogenizer. The slurry was shaken for 30 min, centrifuged, and the supernatant was decanted. The residue was reextracted with 90% methanol. After centrifugation, the two extracts were combined and the amount of ^{14}C in the pooled extract was determined by liquid scintillation counting. The amount of ^{14}C in the tissue residue was determined by solubilizing it in NCS tissue solubilizer (Amersham Searle Corp.) for 48 h at 50 °C, as described by Sikka et al. (1975). Glacial acetic acid (0.003 mL/mL of solubilizer) was added to the solubilized tissue and the solution was counted for ^{14}C using scintillation fluid containing Triton X-100. The samples were stored overnight at 4 °C in the dark before counting. The radioactivity in the methanol extract and in the tissue residue was combined to calculate the ^{14}C concentration in the fish.

To determine the distribution of radioactivity in the fish tissues, the fish were removed from the treated water, rinsed with clean water, and separated into two portions, one containing edible flesh (including attached skin and bones), the other head and viscera. The amount of radioactivity in the edible flesh was determined using the same procedure described for the whole body, while that in the head and viscera portion was measured following solubilization in NCS tissue solubilizer.

Metabolism of 2,4-D. To study the metabolism of 2,4-D by the fish, 30 fish were exposed to 2 ppm of DMA–[^{14}C]-2,4-D. After 7 days, the fish were removed, rinsed with fresh water, and homogenized with acetone. The homogenate was filtered, and the residue was then successively extracted with 90% methanol. The extracts were combined, and the methanol and acetone in the extract was removed under vacuum. The remaining aqueous solution was extracted with chloroform (fraction 1) at pH 8 to remove lipids and other interfering materials. The aqueous layer was then adjusted to pH of approximately 2 and extracted with chloroform twice (fraction 2). The amount of radioactivity in the chloroform extracts (fraction 1 and 2) and the remaining aqueous phase (fraction 3) was determined by liquid scintillation counting. The chloroform extract (fraction 2) was concentrated and chromatographed on thin-layer silica gel plates in the following solvent systems (i) chloroform and (ii) chloroform–methanol (1:1). After drying, the chromatograms were scanned for detection of radioactivity in a Nuclear-Chicago Actigraph. Authentic 2,4-D was cochromatographed for comparison with unknown metabolites in the extract.

Water Analysis. The nature of the ^{14}C remaining in the water bathing the fish was also determined. After removing the fish, the water was acidified to pH 2 with 1 N HCl and extracted twice with diethyl ether. The ether extracts were combined, and the amount of radioactivity in the organic and aqueous phases was determined. The ether extract was concentrated and aliquots were chromatographed on thin-layer silica gel plates as described previously.

Table I. Residues of Radioactivity in Bluegills Exposed to Water Containing 2 ppm of DMA-[^{14}C]-2,4-D

Hours after exposure	^{14}C residue[a] (expressed as μg of DMA-2,4-D/g of fresh tissue)		
	Edible flesh	Head and viscera	Total body
6	0.071	1.084	0.528
24	0.078	2.201	0.931
96	0.114	1.712	0.868
168	0.096	1.618	0.819

[a] The values represent mean of two replications.

Table II. Whole Body Residues of Radioactivity in Catfish Exposed to 2 ppm of DMA-[^{14}C]-2,4-D

Hours after exposure	^{14}C residue[a] (expressed as μg of DMA-2,4-D/g of fresh tissue)
8	0.17
24	0.20
48	0.20
72	0.16
96	0.24
120	0.25
144	0.18
168	0.25

[a] The values represent mean of two replications.

RESULTS

Uptake and Distribution of [^{14}C]-2,4-D by Fish. *Bluegills.* Table I shows the concentration of ^{14}C (expressed as 2,4-D equivalent) in edible flesh, head and viscera, and total body at various times after exposure to water containing 2 ppm of DMA–[^{14}C]-2,4-D. The concentrations of ^{14}C-labeled residues reported represent the sum of the radioactivity in the methanol extract and in the extracted residue. The ^{14}C in the methanol extract accounted for 80–90% of the total radioactivity detected in the tissue. The concentration of ^{14}C in the whole fish reached a maximum of about 1 ppm 24 h after treatment. Longer exposure up to 7 days did not result in a significant change in the total ^{14}C concentration. The data showed that the fish removed very small amounts of 2,4-D from the treated water; less than 0.5% of the total amount of the herbicide was absorbed by the fish during a 7-day exposure. At all sampling times, a major portion of the radioactivity absorbed by the fish was associated with the

Table III. Radioactive Residues in Tissues of Catfish Exposed to Water Containing 2 ppm of DMA-[^{14}C]-2,4-D

Hours after treatment	^{14}C residue[a] (expressed as μg of DMA-2,4-D/g of fresh tissue)		
	Flesh	Head and viscera	Whole body
48	0.097	0.49	0.25
168	0.064	0.59	0.32

[a] The values represent mean of two replications.

head and viscera portion; edible flesh accounted for less than 5% of the total ^{14}C residue in the fish.

Channel Catfish. The concentration of ^{14}C (expressed as 2,4-D equivalent) in catfish exposed to 2 ppm of [^{14}C]-2,4-D is shown in Table II. As in the case of bluegills, the concentration of radioactivity in the fish reached an equilibrium within 24 h after treatment. However, catfish removed a smaller amount of the herbicide from the water than bluegills. The maximum concentrations of ^{14}C in catfish and bluegills 24 h after treatment were 0.20 and 0.93 ppm, respectively.

Table III shows the concentration of ^{14}C in edible flesh, head and viscera, and total body 2 and 7 days after exposure to [^{14}C]-2,4-D. As noticed in bluegills, a major portion of the ^{14}C removed by the fish was associated with the head and viscera portion. Edible flesh accounted for about 10% of the total ^{14}C residue in the fish.

Metabolism of 2,4-D by Bluegills and Catfish. In the case of bluegills, extracts of edible flesh and head and viscera were analyzed by thin-layer chromatography to determine the form of the radioactivity, whereas in the case of catfish, the extracts of whole fish were analyzed. It was noticed that essentially all of the radioactivity from the bluegills or catfish exposed to [^{14}C]-2,4-D for 7 days was associated with the chloroform extract at pH 2 (fraction 2). The chloroform extract at pH 8 (fraction 1) and the aqueous phase remaining after chloroform extraction at pH 2 (fraction 3) contained only traces of radioactivity. Thin-layer chromatography analysis of fraction 2 showed that all of the ^{14}C in this fraction was present as a single compound which cochromatographed with authentic ^{14}C-2,4-D in two different solvent systems (R_f of 0.04 in chloroform and 0.71 in chloroform–methanol, 1:1). About 10% of the total ^{14}C with fish was not extractable. This amount may represent 2,4-D and/or its metabolic products.

Water Analysis. In order to determine if any metabolites resulting from 2,4-D metabolism in fish were excreted into the water, the nature of the ^{14}C remaining in the water bathing the fish was examined. Essentially, all of the radioactivity in the water was extractable with diethyl ether at pH 2. Thin-layer chromatographic analysis of the ether extracted indicated the presence of only one spot with an R_f value corresponding to that of authentic [^{14}C]DMA–2,4-D. The results from the controls (water containing [^{14}C]DMA–2,4-D but no fish) showed no detectable loss of ^{14}C from the water over the 168-h test period.

Metabolism of 2,4-D by Bluegills Following Intraperitoneal Injection. On account of low uptake of 2,4-D by bluegills exposed to the herbicide in water, it was decided to examine the ability of the fish to metabolize 2,4-D administered by intraperitoneal injection. DMA–[^{14}C]-2,4-D was dissolved in distilled water and 50 to 100 μL of the solution was injection into the peritoneal cavity. The fish were transferred to fresh water which was periodically monitored for ^{14}C. At the end of the experiment, the water was acidified to pH 2 and extracted with diethyl ether, and the ether and water phases were counted for radioactivity. The ether extract was concentrated and analyzed by TLC as described earlier.

Table IV. Time Course of ^{14}C Excretion Following Intraperitoneal Injection of DMA-[^{14}C]-2,4-D

Time after injection, h	^{14}C excreted (% of ^{14}C injected)[a] 2,4-D dosage	
	1.0 ppm	2.5 ppm
0–1	62.4	57.7
1–3	22.1	24.0
3–6	4.2	7.2
Total	88.7	88.9

[a] The values represent mean of two replications.

It was observed that 2,4-D was rapidly excreted from the fish following intraperitoneal injection. About 90% of the initial ^{14}C was excreted by the fish within 6 h of treatment (Table IV). When the water was acidified and extracted with ether, essentially all of the ^{14}C was present in the ether extract. Thin-layer chromatography of the ether extract in two different solvent systems revealed only the presence of 2,4-D.

DISCUSSION

The results of this study show that the uptake of DMA–2,4-D by bluegills and channel catfish is very small and the herbicide does not bioaccumulate in the fish. The residues of 2,4-D detected in the fish in the studies are below the established tolerance limit for 2,4-D of 1.0 ppm in fish (U.S. Environmental Protection Agency, 1975). A low uptake of DMA–2,4-D by the fish may be explained by the fact that the herbicide in the water was mostly present in an ionized form which is less likely to partition from water into fish. Similar results have been reported on the uptake of other water-soluble pesticides and their metabolites (Sanborn, 1974; Sikka et al., 1975). The dimethylamine salt and butoxyethanol ester of 2,4-D differ considerably with respect to their uptake by fish. In comparison to our results, Rodgers and Stalling (1972) observed a substantially higher uptake of BEE of 2,4-D by fish. In their studies, the herbicide concentration in the fish was many times higher than in the ambient water. The higher lipid solubility of the ester compared with the DMA salt of 2,4-D may account for the greater uptake of the former by the fish. Neely et al. (1974) reported a straight-line relationship between the partition coefficient (an index of lipid solubility) of a chemical and its ability to bioconcentrate in fish.

Our findings demonstrate that bluegills and catfish do not metabolize 2,4-D. In contrast to our results, Schultz (1973) reported that most of the radioactivity in fish exposed to [^{14}C]DMA–2,4-D in the presence of hydrosol was present as metabolites of the herbicide. When fish were exposed to DMA–2,4-D in water alone, low levels of 2,4-D metabolites were also noted. However, his studies did not indicate whether the ^{14}C metabolites detected in the fish were produced by the fish themselves or if the the 2,4-D was metabolized outside the fish as a result of microbiological or nonbiological reactions and the metabolites were then absorbed by the fish. Since 2,4-D is known to be readily degraded by microorganisms (Loos, 1969) and by photolytic process (Aly and Faust, 1964; Crosby and Tutlass, 1966), we speculate that the ^{14}C metabolites found in the fish in the studies reported by Schultz (1973) originated in the water surrounding the fish as a result of microbial and/or photochemical activity and were subsequently removed by the fish. The results of our

studies support this speculation. Under the conditions of our experiments where no degradation of [^{14}C]2,4-D was observed in the water, all the radioactivity in the fish was present as the unchanged herbicide.

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Metabolism of Cytrolane Systemic Insecticide (Mephosfolan), Propylene (Diethoxyphosphinyl)dithioimidocarbonate, in Cotton Plants

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Cytrolane Systemic Insecticide (mephosfolan), labeled with carbon-14 at the imino carbon atom, was applied foliarly to cotton plants in the greenhouse. After 42 days, 58.2% of the applied dose was recovered from the cotton plants. Most of the radioactivity, 53% of the applied dose, was recovered from the treated leaves while small amounts of radioactivity were recovered from the untreated leaves and the stems (including the petioles and roots), 1.1 and 4.1%, respectively. Of the applied radioactivity that was recovered from the treated leaves, 41.5% was in the ethanol extract and 11.5% was in the plant marc. The major residue that was found on the leaves, 60.9% of the radioactivity in the extract after 42 days (25.3% of the applied dose), was identified as mephosfolan. Thiocyanate ion and the glucose conjugates of (hydroxymethyl)ethylene (diethoxyphosphinyl)dithioimidocarbonate and (hydroxymethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate were identified as metabolites of mephosfolan. These metabolites accounted for 0.2, 2.1, and 4.6% of the applied dose, respectively. Two additional metabolites were found, accounting for 1.9 and 0.9% of the applied dose, which had two-dimensional TLC properties that were similar to the two metabolites derived from the carbon-14 labeled iminodithiolane intermediate, propylene dithioimidocarbonate hydrochloride. Therefore, these two metabolites may not retain the diethoxyphosphinyl moiety of mephosfolan.

Mephosfolan (I) [propylene (diethoxyphosphinyl)dithioimidocarbonate], the active ingredient in Cytrolane (trademark of American Cyanamid Co.) Systemic Insecticide, is an organophosphate insecticide effective for the control of both sucking and chewing insects that attack crops of economic importance. Field evaluation of mephosfolan on cotton grown in Egypt revealed excellent control of the cotton leafworm *Spodoptera littoralis* (Zeid et al., 1968; Kamel and Mitri, 1970), the pink bollworm *Pectinophora* spp., and the spiny bollworm *Earias insulana*. The compound is also effective against several major pests of beets, carrots, celery, corn, rice, and sugarcane.

A metabolism study of carbon-14 labeled mephosfolan was conducted with cotton plants (Delta pine smooth leaf variety) in a greenhouse in order to determine the nature and toxicity of the plant metabolites. This paper describes the isolation, identification, and synthesis of the cotton plant metabolites of mephosfolan.

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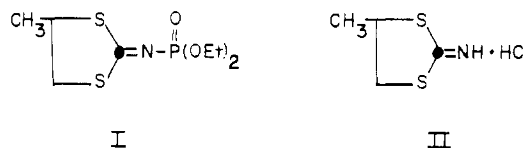
MATERIALS AND METHODS

Radiochemicals. Carbon-14 labeled mephosfolan was synthesized from [^{14}C]cyanogen chloride and 1,2-dimercaptopropane by New England Nuclear Corp. (Boston, Mass.) following a procedure developed by Addor (1964, 1965). The radiopurity of the preparation was 99% as ascertained by two-dimensional TLC in chloroform–ethyl acetate–dioxane (150:30:30) vs. chloroform–acetone–acetic acid (129:15:6). The sp. act. was 5.38 mCi/mmol (20 μCi/mg, 44.4 × 10^3 dpm/μg). The radiolabeled synthesis intermediate, propylene [^{14}C]dithioimidocarbonate hydrochloride (II), was obtained with a sp. act. of 5.38 mCi/mmol (32 μCi/mg).



• ^{14}C CARBON

Synthesis of Metabolites. The 1,2-dimercaptopropane, 2,3-dimercaptopropanol, diethyl phosphoro-