The study of esterolytic and oxidative metabolism of the dodecadienoate juvenile hormone analogues by enzymes isolated from the house fly now includes 12 compounds. The results thus far permit certain conclusions to be drawn: all of the compounds are susceptible to metabolism by the microsomal oxidases with no clear evidence of structural preference or of influence on biological activity (except as noted above in connection with resistance); their resistance to hydrolysis by the microsomal esterases varies immensely and this can be correlated with biological activity in the case of the isopropyl esters, i.e., the compounds which resist hydrolysis are probably more potent as a result; this factor is not enough to provide potency with the other compounds studied, however, showing that other conditions must also be met. These conditions are probably related to cuticle penetration, transport within the insect, and conformation with the receptor site.

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Extensive Degradation of Silvex by Synergistic Action of Aquatic Microorganisms

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The herbicide 2-(2,4,5-trichlorophenoxy)propionic acid (silvex) was extensively degraded by a mixed culture of a *Pseudomonas* species and an *Achromobacter* species isolated from farm pond water. Neither of the two organisms separately was able to metabolize the herbicide. In the degradation of silvex by the mixed culture, chlorine was liberated from the herbicide, the aromatic ring was cleaved, and CO_2 was evolved. Except for a minor amount of 2,4,5-trichlorophenol, no silvex metabolites as detected by TLC were found. The mixed culture also readily decomposed 2,4,5-trichlorophenol and 3,5-dichlorocatechol.

The herbicide 2-(2,4,5-trichlorophenoxy)propionic acid (silvex) is used for controlling certain aquatic weeds (Blackburn, 1963; Frank et al., 1963). The metabolic fate of silvex in the aquatic environment is of obvious concern because of the potential toxicity of the herbicide and its metabolites to nontarget organisms and their possible adverse effects on man through his drinking-water supplies. Among the factors which determine the fate of a chemical in natural ecosystems, microbial transformation is one of the most important. Reports on microbial degradation of silvex are few. It has been reported that silvex and a structurally similar herbicide, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are very resistant to microbial degradation (Alexander and Aleem, 1961; Burger et al., 1962). The recalcitrant nature of these chemicals was attributed to the number of chlorines attached to the aromatic nucleus and to the presence of chlorine on the meta position of the ring. A soil fungus, *Streptomyces viridochromogenes*, was capable of cleaving the ether linkage and oxidizing the propionic acid moiety of silvex, but could not degrade the remaining 2,4,5-trichlorophenol (Bounds and Colmer, 1965). A *Brevibacterium* sp. was shown to cometabolize 2,4,5-T to 3,5-dichlorocatechol without any further alteration (Horvath, 1971). In this paper, we report that silvex, a molecule generally considered as recalcitrant, is extensively degraded by the synergistic action of two species of aquatic microorganisms.

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MATERIALS AND METHODS

Isolation of Aquatic Microorganisms. A population of aquatic microorganisms capable of degrading silvex was developed by an enrichment culture technique using farm pond water as an initial source of inoculum. The enrichment medium contained K_2HPO_4, 4.8 g; KH_2PO_4, 1.2 g; NH_4NO_3, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $Ca(NO_3)_2 \cdot 4H_2O$, 0.04 g; $Fe_2(SO_4)_3$, 0.001 g; yeast extract (Difco), 2.0 g; silvex, 0.3 g per liter of distilled water. The pH of the medium was 7.3. Silvex degradation was determined by measuring chloride ion release in the medium (Iwasaki et al., 1952) and by assessing the loss of UV absorbance of the supernatant fluid at 288 nm (λ max for silvex). Once significant loss of silvex became evident, an aliquot from the inoculated flask was transferred to a flask containing the fresh enrichment medium and the procedure was repeated several times. After several transfers, the enriched culture solution was streaked on plates consisting of basal mineral medium and agar supplemented with 300 ppm of silvex and 0.2% yeast extract. The effective organisms were maintained on the basal mineral medium supplemented with 300 ppm of silvex and 0.2% yeast extract.

Degradation of Silvex by Aquatic Microorganisms. To examine the time-course of silvex degradation, the

microorganisms were grown in the mineral medium containing 0.2% yeast extract and 300 ppm of silvex on a rotary shaker at 23 °C. At various intervals after inoculation, aliquots of cell suspension were removed, centrifuged at 12 000*g*, and the supernatant was assayed for chloride and UV absorbance at 288 nm. We also analyzed the supernatant for phenols (Loos et al., 1967) and catechols (Arnow, 1937). Nonbiological degradation of silvex was assessed using the same incubation medium but without the cells.

Additional information on silvex degradation was obtained using the herbicide labeled uniformly with ^{14}C in the ring. The microorganisms were grown in the yeast extract–mineral medium containing 300 ppm silvex and 2 μCi of [^{14}C]silvex in a biometer flask (Bartha and Pramer, 1965). $^{14}CO_2$ evolved from the incubation mixture was trapped in 0.1 N KOH contained in the sidearm; the CO_2-trapping solution was removed and replaced at appropriate time intervals. One-milliliter aliquots of the CO_2-trapping solution were added to 15 mL of scintillation solution (PPO, 15 g; POPOP, 0.75 g; naphthalene, 240 g; toluene, 1200 mL, ethanol, 720 mL; and scintillation grade 1,4-dioxane, 1200 mL) and counted for radioactivity in a Nuclear Chicago liquid scintillation counter. The radioactivity collected in the KOH trap was verified as $^{14}CO_2$ by acidifying with HCl. Aliquots of cell suspension were also removed periodically and centrifuged, and the supernatant was counted for radioactivity. To determine the total ^{14}C in the cells, the pellet was washed with phosphate buffer and then transferred to scintillation vial for counting ^{14}C.

For chromatographic analysis, the cells and the medium were separated by centrifugation. The supernatant was acidified to pH 2, and then extracted twice with ethyl ether. The amount of radioactivity in the ether extract and aqueous phase was determined by liquid scintillation counting. The ether extract was concentrated under a stream of nitrogen and an aliquot was spotted on thin-layer silica gel plates. The plates were developed in the following two solvent systems: (1) chloroform, and (2) butanol–benzene–water (1:9:10). The radioactive compounds were detected by scanning the chromatograms on a Nuclear Chicago actigraph and by autoradiography; chromatograms were also examined under ultraviolet light to detect possible metabolites. The cell pellet remaining after centrifugation of the cell suspension was extracted with 80% methanol, and the extract was concentrated under vacuum to remove the methanol. The aqueous solution was acidified, extracted with ether, and the ^{14}C in the two phases was determined.

To study the degradation of silvex by resting cells, 5-day old cells grown in a mineral medium containing silvex and yeast extract were harvested by centrifugation, washed twice with 0.1 M phosphate buffer (pH 7.2), and resuspended in the buffer to give 2.5 mg dry weight of cells/mL. [^{14}C]Silvex was then added to the cell suspension, and after the desired periods of incubation, the culture medium was analyzed for [^{14}C]silvex and its possible metabolites using the techniques described above. The activity of each resolved ^{14}C compound on the thin-layer plate was determined by scraping the radioactive zone off the plate into the liquid scintillation fluid containing an added Cab-O-Sil (thixotropic gel suspension powder) and counting it for ^{14}C.

Chemicals. Uniformly ring-labeled [^{14}C]silvex with a sp act. of 5.37 mCi/mL, was purchased from the California Bionuclear Corp., Sun Valley, Calif. The purity of the ^{14}C-labeled compound was greater than 99%, as determined by thin-layer chromatography. Unlabeled silvex was provided by the Dow Chemical Co., Midland, Mich. 2,4,5-Trichlorophenol and 3,5-dichlorocatechol were obtained from the Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

Isolation of Microorganisms Capable of Degrading Silvex. A mixed culture of microorganisms capable of degrading silvex was isolated from the pond water by an enrichment culture technique. The pure-culture isolates included three species of the genus *Pseudomonas*, an *Achromobacter* species, and two unidentified gram negative rods. These isolates were ineffective in degrading silvex when incubated with the herbicide individually or in combination. However, a colony consisting of a *Pseudomonas* species and *Achromobacter* species was able to metabolize the herbicide as indicated by chloride release in the medium and loss of UV absorbance of the supernatant fluid at 288 nm. The culture of *Pseudomonas* species plus *Achromobacter* species (hereafter referred to as mixed culture) could not utilize silvex as the sole source of carbon, but did metabolize the herbicide in the presence of an external carbon source such as yeast extract, which indicates the degradation of the herbicide occurred by a cometabolic process. When pure cultures of the two bacteria were incubated separately with silvex, no degradation of the herbicide was observed, suggesting a synergistic relationship between the two organisms in attacking the herbicide.

Degradation of Silvex in Growing Culture. In the mixed culture grown in a mineral medium containing 300 ppm of silvex and 0.2% yeast extract, an extensive degradation of silvex was observed (Figure 1a). Degradation of the herbicide started 18–24 h after inoculation, and after 80 h, essentially all of the chlorine in the herbicide was liberated as free chloride and no silvex could be detected in the medium as measured spectrophotometrically. The loss of UV absorbance characteristic of silvex followed essentially the same pattern as the chloride release. We did not detect any phenols or catechols in the medium after incubation of silvex with the culture. A complete loss of the UV absorbance spectrum (240–320 nm) was noticed after 80 h, indicating that the microorganisms had completely destroyed the aromatic ring of silvex.

Evolution of $^{14}CO_2$ from the culture incubated with uniformly ring-labeled [^{14}C]silvex provided additional evidence of ring cleavage (Figure 1b). The level of ^{14}C in the medium started to decrease about 30 h after incubation, and after 80 h, approximately 80% of the ^{14}C from the culture solution had disappeared. The decrease in the ^{14}C in the medium was accompanied by appearance of ^{14}C in the cells and evolution of $^{14}CO_2$. At the conclusion of the experiment, more than 60% of the original activity in silvex had been evolved as $^{14}CO_2$ with about 16% associated with the cells and 20% remaining in the external culture solution.

After 80 h of incubation, the cells and the culture medium were analyzed for silvex and its possible metabolites. The cell suspension was centrifuged, and the supernatant was acidified to pH 2 and then extracted with ethyl ether. More than 99% of the ^{14}C was recovered in the ether extract. Thin-layer chromatographic analysis of the ether extract in (1) chloroform and (2) butanol–benzene–water (1:9:10) did not reveal the presence of any ^{14}C compound other than silvex. The cell pellet remaining after centrifugation of the cell suspension was extracted with 80% methanol, and the extract was concentrated under vacuum to remove the methanol. The aqueous solution was acidified, extracted with ether, and the ^{14}C in the two phases was determined. Essentially all of the

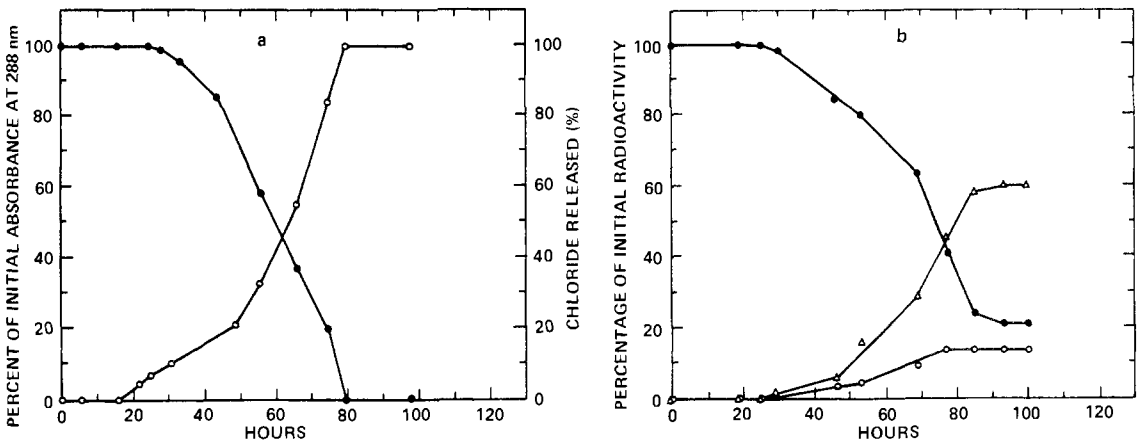

Figure 1. Degradation of silvex by the mixed culture. (a) The cells were incubated with silvex, and the culture solution was assayed for UV absorbance and chloride and at various times after inoculation: (●——●) UV absorbance; (○——○) Cl^-. (b) The cells were incubated with [^{14}C]silvex and the ^{14}C activity in evolved CO_2, culture solution, and cells was measured periodically: (Δ——Δ) $^{14}CO_2$; (●——●) culture solution; (○——○) cells.

^{14}C activity in the cell extract was present in the form of water-soluble products unextractable with ether.

Degradation of Silvex by Resting Cells. Since no metabolites of silvex could be detected in studies with growing cells, attempts were made to accumulate the metabolites produced during silvex degradation by incubating the herbicide with a resting-cell suspension of the silvex-adapted cells. The suspension of the silvex-adapted cells (2.5 mg dry wt/mL) was incubated in 0.02 M phosphate buffer containing 300 ppm of silvex and 2 μCi of [^{14}C]silvex in a biometer flask. After 4 h of incubation, 93% of the bound chlorine was released as chloride in the medium and about 65% of the initial radioactivity was evolved as $^{14}CO_2$. The culture medium was analyzed for [^{14}C]silvex and possible degradation products by thin-layer chromatography after 2 and 4 h of incubation. It was observed that most of the residual radioactivity in the ether extract of the culture medium was present as unchanged silvex. A small amount of ^{14}C (1–5% of the initial ^{14}C) was present in the form of a ^{14}C metabolite which cochromatographed with authentic 2,4,5-trichlorophenol.

Degradation of 2,4,5-Trichlorophenol and 3,5-Dichlorocatechol by the Mixed Culture. Our findings showed that 2,4,5-trichlorophenol may possibly be a metabolite produced during degradation of silvex by the mixed culture. Another intermediate in the pathway of silvex degradation may be 3,5-dichlorocatechol. It was of interest to determine whether the mixed culture could degrade these two compounds which are likely intermediates in the degradation of the herbicide. A washed suspension of silvex-adapted cells was incubated with 2,4,5-trichlorophenol or 3,5-dichlorocatechol and the degradation of the chemicals was determined by measuring chloride ion liberation. It was noticed that within 4 h of incubation of the cell suspension with 10 ppm of 2,4,5-trichlorophenol or 3,5-dichlorocatechol, 87 and 97% of the bound chlorine was released as inorganic chloride, respectively.

Degradation of Silvex by Pure Cultures of *Pseudomonas* Species and *Achromobacter* Species. The *Pseudomonas* species or the *Achromobacter* species isolated from the mixed culture failed to metabolize silvex or 2,4,5-trichlorophenol when the washed cells of each orgainsm were incubated separately with the chemicals; neither did the organisms degrade the chemicals when the cells of the two organisms were mixed in different proportions. Presently, we can not offer an explanation for the inability of individual or intentionally mixed cultures of the two bacteria to metabolize silvex or 2,4,5-trichlorophenol. It is likely that the organisms underwent some changes during their isolation from the mixed culture which may have resulted in a loss of their ability to degrade the chemicals. Additional work is required to establish the nature of the interaction between the two bacteria.

DISCUSSION

The present investigation has shown that silvex is extensively degraded to the extent of ring cleavage by the action of certain aquatic microorganisms. Prior to this work, no microorganisms had been reported to be capable of causing ring cleavage of silvex or of a structurally similar molecule. Our findings are in contrast to those of other workers (Alexander and Aleem, 1961; Burger et al., 1962) who reported that silvex as well as other phenoxy herbicides which also have a chlorine in the meta position on the aromatic nucleus were highly resistant to microbial degradation in soil. Their failure to show microbial degradation of these chemicals may have resulted from their having used soil inocula in which the appropriate microorganisms may have been absent.

Although the results clearly showed biodegradation of silvex, no metabolites, except a trace amount of 2,4,5-trichlorophenol (identified on the basis of TLC), could be found. This is probably due to the fact that the intermediates were degraded as rapidly as they were formed so that subsequent concentrations were too small to be detected by the analytical methods used. This suggestion is supported by the finding that 2,4,5-trichlorophenol and 3,5-dichlorocatechol, the two possible silvex metabolites, were degraded quite readily by the mixed culture without a lag. The appearance of 2,4,5-trichlorophenol in the culture fluid together with the capacity of the cells for dissimilating it suggests that it forms part of the pathway of silvex degradation. The cells also extensively destroyed 3,5-dichlorocatechol, suggesting that it is a likely intermediate in the pathway of silvex degradation. On the basis of these findings, we postulate that silvex metabolism by the mixed culture involves a cleavage of the ether linkage to give rise to 2,4,5-trichlorophenol which is then dehalogenated and hydroxylated resulting in the formation of 3,5-dichlorocatechol. The latter may then be degraded by the pathways described by other workers (Bollag et al., 1968; Evans et al., 1971; Gaunt and Evans, 1971; Tiedje et al., 1969).

The data presented herein demonstrate the significance of a synergistic relationship between microorganisms in

the decomposition of recalcitrant molecules. The inability of pure cultures of microorganisms to degrade a chemical can not be taken as proof that the substance is resistant to microbial attack; it may, in fact, be readily destroyed by the combined action of two or more organisms. A synergistic relationship between microorganisms has also been noted in the degradation of cycloparaffinic hydrocarbons (Beam and Perry, 1974) and other pesticides (Bordeleau and Bartha, 1971; Gunner and Zuckerman, 1968; Pfaender and Alexander, 1972).

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Fate of 2-Chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene (Oxyfluorofen) in Rats

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Two albino rats were dosed orally with [^{14}C]trifluoromethyl-labeled 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene for 7 consecutive days. Only trace amounts of radioactivity (2–4%) were recovered in the urine and tissues. The major route of dose elimination was through the feces (~95%). About 75% of the fecal radioactivity was the unchanged dosed compound. Other compounds present in feces were: 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrophenol, 4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-ethoxybenzenamine, *N*-[4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-ethoxyphenyl]acetamide, and *N*-[4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-hydroxyphenyl]acetamide.

The use of diphenyl ethers, such as fluorodifen and nitrofen, as herbicides has been reported (Rogers, 1971; Nakagawa and Crosby, 1974; Yih and Swithenbank, 1975). The diphenyl ether, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene, is an experimental herbicide of broad spectrum activity known as RH-2915 (hereafter compound A). The Weed Society of America has adopted the common name of oxyfluorfen and a trademark of GOAL has been established for RH-2915. Compound A has been shown to provide a high degree of weed control and a great versatility of application (Yih and Swithenbank, 1975). In order to determine the fate of ^{14}C-labeled compound A in an animal system, a material balance and metabolism study was conducted with rats using ^{14}C-labeled material.

MATERIALS AND METHODS

Synthesis of Compounds. *Compound A. 2-Chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene.* Compound A was synthesized by a procedure similar to that of Yih and Swithenbank (1975) with a ^{14}C label in the CF_3 group. Radiopurity was established as 99% using thin-layer chromatography and radioautography. The compound was used at a sp act. of 0.99 mCi/g or 2220 dpm/μg.

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Compound B. 5-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-nitrophenol. 1,3-Bis[2-chloro-4-(trifluoromethyl)phenoxy]-4-nitrobenzene (51.2 g, 0.1 M) was treated with potassium hydroxide (26.4 g, 0.4 M) in water (200 mL) at 50–60 °C for 4 h in dimethyl sulfoxide (800 mL). The mixture was diluted with water, acidified with dilute acid, and extracted with ether. The extract was extracted with 10% potassium hydroxide solution and this extract acidified and extracted with ether. The ethereal solution was dried with magnesium sulfate and the solvent removed to give a residue which was distilled at 0.4 mm up to 100 °C to give 2-chloro-4-trifluoromethylphenol. The distillation residue was recrystallized from benzene–hexane to give 3-[2-chloro-4-(trifluoromethyl)phenoxy]-4-nitrophenol (21.5 g). The solvent was thoroughly removed from the above mother liquors and this residue extracted with warm hexane (40 °C, 500 mL) and the extract cooled to 10 °C to give B (9.1 g), mp 68–70 °C. Anal. Calcd for $C_{13}H_7ClF_3NO_4$: C, 46.78; H, 2.10; Cl, 10.63; F, 17.09; N, 4.20. Found: C, 46.28; H, 2.07; Cl, 10.61; F, 17.69; N, 4.05.

Compound C. 4-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-ethoxybenzenamine. Compound A (50.0 g, 0.138 M), platinum(II) oxide (200 mg), and 1000 mL of absolute ethyl alcohol were charged to a 2-L Parr shaking autoclave. Hydrogen was added at room temperature at an initial pressure of 40 psi and was recharged until a constant pressure was maintained. The reaction mix was filtered and the filtrate concentrated at 100 °C and 20 mm to yield