Identification of [^{14}C]Fonofos Metabolites Isolated from Insecticide-Treated Culture Media of the Soil Fungus *Rhizopus japonicus*

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The degradation of the insecticide [*ring*-^{14}C]fonofos (Dyfonate) and [*ethoxy*-^{14}C]fonofos into water-soluble substances was studied with the soil fungus *Rhizopus japonicus*. After 4 days of incubation of fungal cultures treated with the insecticide, 83% of the applied radioactivity partitioned into the water extraction phase of the culture media, while no radiocarbon could be detected in their hexane extraction phases. In noninoculated controls, however, only 6% of the applied radiocarbon was water-soluble, while 75% still appeared in the hexane extraction phase. After analysis by chromatographic and GC–mass spectrometric methods of water extraction phases of media from [^{14}C]fonofos-treated fungal cultures, the presence of eight compounds was determined of which four were actual metabolites. Their structure, molecular weight, R_f values, and retention times relative to fonofos are shown.

The degradation and metabolism of the organophosphorus soil insecticide fonofos (Dyfonate) was investigated with selected soil fungi by Flashinski and Lichtenstein (1974). Among the nine fungal species tested, *Mucor plumbeus* and *Rhizopus arrhizus* were most active in degrading the insecticide. This activity was evidenced by the production of increasing amounts of water-soluble ^{14}C compounds in the culture media and the disappearance (degradation) of the originally applied insecticide from the fungal cultures. The water-soluble metabolites were nontoxic to mosquito larvae. Hexane-extractable compounds from cultures of the active species were nontoxic to fruit flies, while those from cultures of the less active fungal species were insecticidal. Among the metabolites produced and detected by thin-layer chromatography were primarily dyfoxon, ethylethoxyphosphonothioic acid, ethylethoxyphosphonic acid, methyl phenyl sulfoxide, and methyl phenyl sulfone. Production of the metabolites by the fungi was a function of the live mycelia and was followed by excretion of water-soluble ^{14}C compounds into the culture media. Since the genus *Rhizopus* appeared to be most active in metabolizing fonofos into water-soluble breakdown products, this study was conducted with the species *Rhizopus japonicus* to isolate and identify by instrumental analyses [^{14}C]fonofos metabolites in the fungal culture media.

MATERIALS AND METHODS

Insecticides and Solvents. Analytical grade fonofos (Dyfonate) (*O*-ethyl *S*-phenyl ethylphosphonodithioate), [*ethoxy*-^{14}C]fonofos (specific activity, 2.23 mCi/mmol), [*ring*-^{14}C]fonofos (specific activity, 4.70 mCi/mmol), dyfoxon (*O*-ethyl *S*-phenyl ethylphosphonothiolate), ethylethoxyphosphonothioic acid (ETP), ethylethoxyphosphonic acid (EOP), thiophenol, diphenyl disulfide, and methylphenyl sulfoxide (MPSO) were obtained through the courtesy of the Stauffer Chemical Company. Analyses of an ethanol solution of [*ethoxy*-^{14}C]fonofos by GC–MS indicated, in addition to fonofos, the presence of 3% of other substances of which one had a molecular weight of 280 and a retention time of 220 relative to fonofos = 100. An ethanol solution of [*ring*-^{14}C]fonofos contained, in addition to fonofos, 0.5% diphenyl disulfide, 1.2% of *O*-ethyl *S*-phenyl methylphosphonodithioate and the above mentioned unknown compound with a molecular weight of 280. Solvents used were 95% ethanol, acetone, benzene, anhydrous methanol, and redistilled hexane. For treatment purposes, solutions of radiolabeled fonofos were diluted with nonradioactive insecticide.

Fungal Culture. The soil fungus *Rhizopus japonicus* was obtained through the courtesy of Dr. P. R. Wallnöffer at the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau in Munich, Germany. The nutrient medium (Wegener et al., 1967) contained per liter of distilled water 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $Fe_2(SO_4)_3$, 2 g of $(NH_4)_2SO_4$, 0.005 g of $ZnSO_4 \cdot 7H_2O$, and 50.0 g of glucose. The final pH was adjusted to 6.8. Since, in the course of glucose degradation by the fungus, the pH of the medium drops considerably, 0.375 g of sterile $CaCO_3$ was added to each 25 mL of autoclaved medium to act as a neutralizing agent.

Insecticide Treatment and Culture Inoculation. Rate of Degradation of [*ethoxy*-^{14}C]Fonofos by *Rhizopus japonicus*. To study the rate of degradation of [*ethoxy*-^{14}C]fonofos by the fungus *Rhizopus japonicus*, fungal cultures were incubated with the insecticide over a 1 to 4 day period. For that purpose, [*ethoxy*-^{14}C]fonofos was added in 0.5 mL of ethanol to 25 mL of culture media and $CaCO_3$ in each of six 100-mL Erlenmeyer flasks. The insecticide concentration amounted to 0.4 ppm. Standard spore suspensions were prepared from agar slants and added to each medium. The cultures were then incubated for 1, 2, or 4 days on a reciprocal shaker (120 rpm) in the dark at 27 ± 1 °C. For control purposes 6–25-mL portions of media each were treated with the insecticide as described, but no inoculation was performed. Determination of the radiocarbon content in the water and hexane extraction phases of the culture media and the controls was conducted as described below with two flasks, each, after 1, 2, and 4 days of incubation.

Production of [^{14}C]Fonofos Metabolites by *Rhizopus japonicus* during 4 Days of Incubation. To produce sufficient quantities of [^{14}C]fonofos metabolites, thirty 100-mL Erlenmeyer flasks, each containing sterilized medium and $CaCO_3$, were treated with the insecticide. [*ethoxy*]-^{14}C]Fonofos or [*ring*-^{14}C]fonofos were added in 0.5 mL of ethanol to the media in each of the 30 flasks to yield a concentration of 4 ppm of fonofos. Inoculation of the insecticide-treated media was performed as described

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above, followed by incubation for 4 days at 27 ± 1 °C. At that time, no $CaCO_3$ was visibly present in the fungal cultures. For control purposes 30 flasks, as described above, were prepared and incubated except that no insecticide had been added.

Extraction Procedures. After incubation of the fungal cultures, they were vacuum filtered to separate the mycelia from the media. Since preliminary experiments with *Rhizopus japonicus* indicated that 70 to 80% of the applied radiocarbon was, after 4 days of incubation, associated with the water extraction phase of the media, it was these mycelia-free media which were primarily investigated in this study. These media were twice extracted with hexane at a 1:1 ratio, resulting in a hexane and a water extraction phase.

Analytical Procedures. The amounts of radiocarbon in the two extraction phases were determined by liquid scintillation counting. After that, the pooled water extraction phases were freeze-dried for 56 h. The resulting yellowish powder was refluxed for 2.5 h with acetone, which was then filtered and concentrated at 35 °C to 1–2 mL. Determination of ^{14}C before and after freeze-drying indicated that no appreciable radiocarbon had been lost. The hexane extraction phases were concentrated to a volume of 2 mL on a rotary evaporator at 30 °C. Water and hexane extraction phases of the controls were utilized as background information for analyses by gas-liquid chromatography.

Gas-liquid chromatography (GLC) was performed with a Carlo-Erba (Factovap 2200) gas chromatograph equipped with an FID detector. A 200-cm Pyrex glass column (1.5 mm i.d.) containing 3% OV-1 on Chromosorb W-AW-DMCS 80–100 mesh was used at 130–230 °C for temperature programming. The injector was held at 250 °C and the detector at 300 °C. Flow rate of nitrogen was 40 mL/min. Injection volume was 1–3 μL and the solvents were acetone or hexane.

Analyses by thin-layer chromatography (TLC) was conducted with precoated silica gel plates (Merck, Darmstadt), thickness 0.23 mm. Plates were developed in benzene (for compounds 1–7, Figure 2) and with benzene–methanol (1:1), or benzene–acetic acid (5:1) (for compounds 8 and 9, Figure 2) until the front migrated 17 cm.

Measurements of radioactivity in solvents or on thin-layer plates were conducted with a liquid scintillation counter (Type Betazint 5000, Berthold Frieseke Co.) or with a thin-layer scanner (Type II, Berthold Frieseke Co.).

Structural analyses were conducted with a GC–MS (LKB 9000 S, 70 eV) instrument. Data obtained by mass spectrometry were interfaced (WDV Company, Munich) with a digital computer (IBM 1130), and the corresponding bar spectra were plotted by means of a Benson III-Plotter.

RESULTS AND DISCUSSION

Rate of Degradation of [*ethoxy*-^{14}C]Fonofos by *Rhizopus japonicus*. After incubation of fungal cultures with [*ethoxy*-^{14}C]fonofos for 1, 2, and 4 days, respectively, analyses of the water and hexane extraction phases of the culture media indicated the rapid degradation of the insecticide by the soil fungus (Figure 1). It was noticed that on the 4th day of incubation, no $CaCO_3$ was visibly present in the fungal cultures. Results showed that after 4 days, 83.4% of the applied radioactivity was water soluble and that no ^{14}C could be determined in the hexane extraction phase. This then indicated that the major portion of the insecticide had been degraded. In non-inoculated controls, however, only 6.4% of the applied radiocarbon was water soluble, while 75.4% still appeared in the hexane extraction phase.

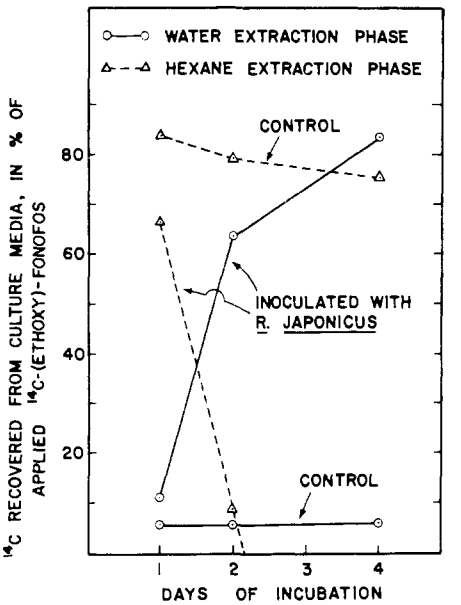


Figure 1. Degradation of [*ethoxy*-^{14}C]fonofos by the soil fungus *Rhizopus japonicus*.

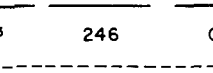
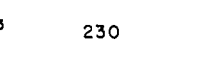
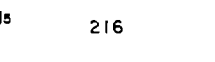
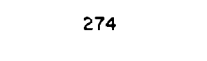
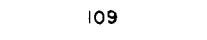
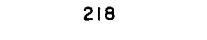
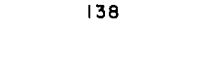
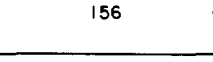
Production of [^{14}C]Fonofos Metabolites by *Rhizopus japonicus* during 4 Days of Incubation. To test the efficiency of the fungus in these experiments, two culture media were analyzed as described after 3 days of incubation. At that time, culture media contained 8.9 ± 2.6% of the applied radioactivity in the hexane extraction phase and 54.5 ± 0.6% in the water extraction phase; in addition, 21.7 ± 2.9% of the applied radioactivity were associated with the mycelium as determined by combustion to $^{14}CO_2$ in an Intertechnique Oxymat, Plasisir, France, utilizing the methods described by Peterson et al. (1969).

After 4 days of incubation, all culture media were extracted as described. Subsequent analyses indicated that at that time 68.6% of the applied radioactivity was associated with the water extraction phase and only 3% with the hexane extraction phase. The radiocarbon content in the mycelia at that time was not determined. Water extraction phases prepared from 28 replicates of (a) [*ethoxy*-^{14}C]fonofos, or of (b) [*ring*-^{14}C]fonofos treated cultures were then analyzed as described. Analyses of the treatment solutions by GC–MS indicated that solution (a) contained, in addition to fonofos, 3% of other substances, one of which had a molecular weight of 280 and a retention time of 220 relative to fonofos = 100. Solution (b) contained, in addition to fonofos, 0.5% diphenyl disulfide and 1.2% of *O*-ethyl *S*-phenyl methylphosphonodithioate and the above mentioned unknown compound with a molecular weight of 280.

Investigation of the extracts by instrumental analyses indicated the presence of eight compounds (no. 2–9) whose suggested structure, molecular wight, R_f values, and retention times relative to fonofos (no. 1) are shown in Figure 2. The relative retention times were determined by mixed injections.

[*ethoxy*-^{14}C]Fonofos-treated culture media were extracted, and compounds in the water extraction phase were separated by TLC with benzene as the development solvent. The scanning radiogram showed two radioactive areas, of which compound 8 at the origin and compound 2 (dyfoxon) could be extracted with benzene and ethanol.

[*ring*-^{14}C]Fonofos-treated culture media were also analyzed by TLC as described. Nine areas (I to IX) could

No.	COMPOUND	MOLECULAR WEIGHT	Rf (BENZENE)	Rt (FONOFOS=100)
1		246	0.80	100
2*		230	0.94	75
3		232	0.92	85
4		216	0.95	86
5		274	0.75	130
6*		109	0.88	41
7		218	0.92	93
8*		138	0.00	–
9*		156	0.14	–

* = ACTUAL METABOLITES PRODUCED

Figure 2. Compounds isolated (2–9) from culture media of the soil fungus *Rhizopus japonicus* after a 4-day incubation period with [^{14}C]fonofos. R_f was obtained on silica gel, relative retention times by FID gas chromatography.

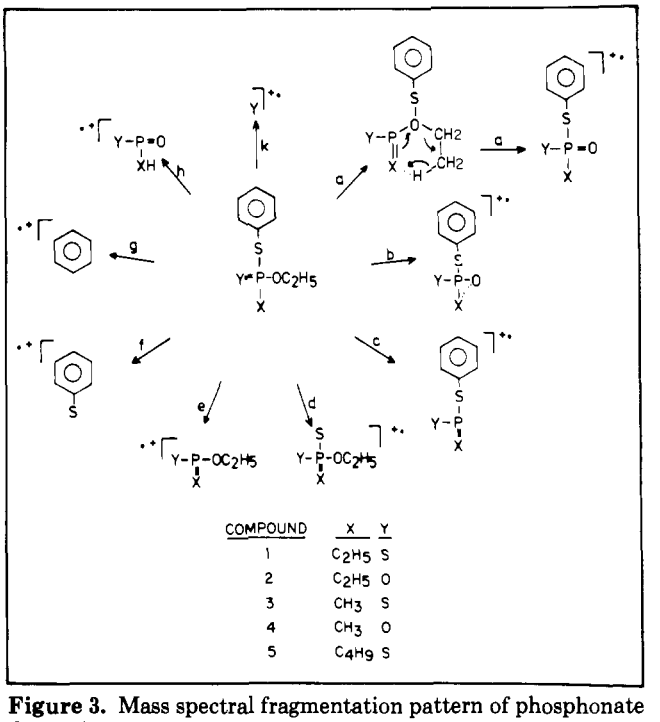


Figure 3. Mass spectral fragmentation pattern of phosphonate derivatives.

be differentiated by radioscanning of the thin-layer plates. Eight of these areas had distinctive peaks. They were removed from the thin-layer plate and extracted with benzene, followed by a second extraction with benzene–methanol (1:1). The remaining, nonradioactive area was handled the same way. Each of these extracts, including the one from area VII which did not contain measurable amounts of radioactivity, was then concentrated under vacuum to dryness and redissolved in 1.5 mL of acetone and analyzed by GC–MS. The radioactivity in percent of total recovered was 28.6% for area I (origin), 45.4% for area II, 0.5% for area III, 1.8% for area IV, 4.1% for area V, 3.9% for area VI, none in area VII, 6.5% for area VIII, and 9.2% for area IX. Because of the presence of biological material, as shown in the controls, it was impossible to identify compounds in areas IV, V, and VI. However, area I, containing 28.6% of the ^{14}C isolated from the thin-layer plate, contained a compound of unknown structure with a molecular weight of 190. Zone II, containing 45.4% of the total ^{14}C, showed a compound with a molecular weight of 156. Its structure is methyl phenyl sulfone (no. **9**, Figure 2), since its spectrum compared favorably with that of an authentic sample. Area VII contained compounds **1** and **5**, while area IX contained compounds **2**, **3**, **4**, and **6**, as shown by instrumental analyses (Figure 2).

Determination of the Structure of Compounds 2–9. GC retention times, R_f values, and mass spectrometric fragmentation patterns obtained with compounds **2**, **6**, **7**, and **9** (Figure 2) were identical with the available authentic samples. Since it was very difficult to isolate compound **8** by GLC, it was only compared with an authentic sample by TLC. The proposed structures for compounds **3**, **4**, and **5** are based on mass spectrometric investigations, which made it possible to identify the various functional groups. Figure 3 shows the most important fragmentation patterns of this group of substances. A characteristic fragmentation (path a) is the McLafferty rearrangement (McLafferty, 1957), similar to that known for carbonyl, oxime, alkyl, and benzene compounds (Spiteller et al., 1966; Budzikiewicz et al., 1966; Djerassi et al., 1966).

This γ-H migration proceeds via a six-center transition state, including —P=S and —P=O double bonds. This yields ions at m/e 218, 202, 204, 188, and 246 for compounds **1** through **5**, respectively. The cleavage of the ethyl group (path b) yields, with compound **1**, an ion at m/e 217. Compound **3** shows an analogous behavior, since the ion m/e 203 of medium intensity was registered. Spectra of compounds **1** and **2** showed an ethoxy cleavage (path c); this cleavage was also observed with compounds **3**, **4**, and **5**.

The elimination of the aryl radical (path d) was registered in the spectra of compounds **1** and **3** with ions of m/e 169 and 155, respectively. The cleavage of the C_6H_5-S group (path e) yields strong peaks at m/e 137 (compound **1**), 121 (compound **2**), 123 (compound **3**), and 165 (compound **5**).

Based on previous experience, compounds 1 to **5** yield the ions of m/e 109 (Figure 3, path f) and 77 (path g). It also appears that the fragment m/e 93 of compound **2**, 95 of compound **3**, and 137 of compound **5** indicate the path as shown in h. The simple cleavage of the alkyl radical (path k) yields the ion of m/e 29 with compounds **1** and **2**, the ion of m/e 15 with compounds **3** and **4**, and the ion of m/e 57 with compound **5**.

Based on this fragmentation pattern compound **3** differs from fonofos (compound **1**) only in its alkyl side chain, where the ethyl group was replaced by a methyl group. Although biological material interfered with the fragmentation pattern of compound **4**, it was still possible to define its structure since a well-defined molecular ion was registered at m/e 216 and fragments at m/e 188, 109, and 77. The structure of the alkyl side chain C_4H_9 of compound **5** was not definitely determined, since the mass spectrum alone did not define the nature of the isomer.

It is evident that among the compounds listed in Figure 2, only compounds **2**, **6**, **8**, and **9** represent actual metabolites of fonofos. As indicated previously, standard solutions of both [*ring*-^{14}C]- and [*ethoxy*-^{14}C]fonofos contained 2–3% impurities. In addition, three ring-labeled metabolites exist but could not be identified from areas IV, V, and VI because of interference by biological ma-

terial. Since compounds 3 and 7 were also isolated from the [*ring*-^{14}C]fonofos standard solutions, the question of their metabolic formation cannot be answered at this time. Compound 4, which is directly formed from compound 3, is probably not formed from fonofos (compound 1).

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Degradation of Tetrachlorvinphos and Its Major Metabolite 2,4,5-Trichlorophenacyl Chloride in Aqueous Media

M. Humayoun Akhtar

The degradation of the insecticide tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate] and its major metabolite, 2,4,5-trichlorophenacyl chloride, was investigated in buffered aqueous media at various pHs. Rate constants and half-life studies revealed that these compounds were stable in acidic and neutral solution but unstable under alkaline conditions. The major mode of decomposition of tetrachlorvinphos in both acidic and basic media is dephosphorylation which results in the formation of 2,4,5-trichlorophenacyl chloride. In alkaline media the chloride undergoes hydrolysis, followed by aldol condensation resulting in the formation of 1-(2,4,5-trichlorobenzoyl)-2-(2,4,5-trichlorophenyl) glycerol.

Organophosphorus and carbamate insecticides are widely used in animal production. These compounds are known to degrade chemically and/or biochemically in a relatively short period after their application (Sheets, 1967). Tetrachlorvinphos [2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate], an organophosphate insecticide, is an effective foliar insecticide for a wide range of crops and is particularly active against adult and larval forms of lepidopterous pests. In animal production it is used in and around agricultural premises to control external parasites—flies, mites, ticks, etc. (Beynon et al., 1973).

The metabolism of tetrachlorvinphos in dogs and rats (Akintonwa and Hutson, 1967), dairy cows (Gutenmann et al., 1971), and the breakdown in plants and soils (Beynon and Wright, 1969) have been investigated. While hydrolysis is considered to be one of the main detoxification mechanisms for organophosphates (Muhlmann and Schrader, 1957), no information is available on the fate of tetrachlorvinphos in aqueous media. The present work was undertaken to obtain information on degradation of tetrachlorvinphos and its major metabolite 2,4,5-trichlorophenacyl chloride, in aqueous media at various pHs.

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EXPERIMENTAL SECTION

Chemicals. All solvents were of pesticide grade and used as received. Analytically pure tetrachlorvinphos was prepared by the procedure described by Whetstone et al. (1966). 2,4,5-Trichlorophenacyl chloride was prepared from tetrachlorvinphos by acid hydrolysis and was purified by column chromatography on neutral alumina (BDH Chemicals) and finally recrystallized from hexane. 2,4,5-Trichlorophenacyl alcohol was prepared by hydrolysis of 2,4,5-trichlorophenacyl acetate (Cebrian, 1948). 2,4,5-Trichloroacetophenone was obtained by the reduction of 2,4,5-trichlorophenacyl iodide.

Stock solutions of tetrachlorvinphos (1.5 mg/mL) and 2,4,5-trichlorophenacyl chloride (0.5 mg/mL) were prepared in acetone and stored at -20 °C. Buffer solutions ranging from pH 6–9 were prepared by titrating 0.1 M disodium hydrogen phosphate with 0.1 M sodium hydroxide and 0.1 M phosphoric acid to the desired pH. All solutions were brought to room temperature (~25 °C) before mixing.

Rate of Hydrolysis. The hydrolysis reactions were conducted at 27 ± 2 °C in the dark. One milliliter of stock tetrachlorvinphos or metabolite solution was pipetted into a 500-mL volumetric flask covered with aluminum foil.