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Metabolism of Phosphorothioic Acid, *O,O*-Dimethyl-*O*-(6-ethoxy-2-ethyl-4-pyrimidinyl) Ester (Etrimfos), in Bean and Corn Plants

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The organothionophosphate etrimfos [phosphorothioic acid, *O,O*-dimethyl-*O*-(6-ethoxy-2-ethyl-4-pyrimidinyl) ester] volatilized rapidly from treated primary leaves of bean and corn seedlings. Twenty-one days after treatment with [^{14}C]etrimfos, 36.4 and 29.5% of the total applied radioactivity was recovered from bean and corn seedlings, respectively. Intact etrimfos was by far the principal compound recovered, followed by small amounts of EEHP (6-ethoxy-2-ethyl-4-pyrimidinol) and EDHP (2-ethyl-4,6-pyrimidinediol). Small amounts of the P:O analogue [phosphoric acid, dimethyl *O*-(6-ethoxy-2-ethyl-4-pyrimidinyl) ester] were also present in corn but not in bean leaves. Four additional products were also detected in small quantities in both plants but were not identified.

Etrimfos [phosphorothioic acid, *O,O*-dimethyl *O*-(6-ethoxy-2-ethyl-4-pyrimidinyl) ester] is a new nonsystemic contact and stomach insecticide, effective against pest species of lepidoptera, coleoptera, diptera, and to a variable extent hemiptera (Knutti and Reisser, 1975). Residue field trials on a variety of crops have shown that residues generally ranged from nondetectable amounts to 0.4 ppm at time of harvest (Karapally, 1977). Etrimfos, therefore, is a promising material for the management of pest problems of crops such as vegetables, fruits, and corn.

Knowledge concerning the absorption and the extent and types of biotransformation of etrimfos in plants is lacking; therefore, the metabolic fate and associated residues of etrimfos must be evaluated before the compound can be used for extensive field trials against insect pests. This paper describes the results of our investigations of the fate of [^{14}C]etrimfos in bean and corn seedlings.

MATERIALS AND METHODS

Compounds. Etrimfos labeled with ^{14}C at carbons 4 and 6 of the pyrimidinyl ring was provided by Dr. James Karapally of Sandoz Inc., East Hanover, N.J. The specific activity of the compound was 11 μCi/mg and radiochemical purity as determined by TLC was greater than 99%. The specific activity was diluted to 1.6 μCi/mg with purified nonradioactive etrimfos. The following nonlabeled compounds were also provided by Sandoz Inc.: etrimfos; P:O analogue of etrimfos [phosphoric acid, dimethyl *O*-(6-ethoxy-2-ethyl-4-pyrimidinyl) ester]; EEHP (6-ethoxy-2-ethyl-4-pyrimidinol); and EDHP (2-ethyl-4,6-pyrimidinediol).

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Treatment of Seedlings. *Sieva* variety lima bean seeds (Asgro Seed Company, Kalamazoo, Mich.) were sown in a mixture of vermiculite and sterilized soil (1:1) in plastic trays. Two weeks after germination, the seedlings were transplanted individually in plastic-coated paper cups (8.9 cm diameter × 5.1 cm high). Merit variety corn (Ferris Seed Co., East Brunswick, N.J.) was planted directly in plastic cups (8.9 cm diameter × 10.2 cm high), in a mixture of vermiculite and soil (1:1). Germination took place in 6 days. Plants were subirrigated and held in a greenhouse equipped with plastic lexan windows which did not exclude UV light.

The primary leaves of bean (21 days after germination) and corn plants (11 days after germination) were treated with 10 μL of [^{14}C]etrimfos solution in acetone. The amount of radioactivity applied per leaf was ca. 40000 dpm (11 μg of etrimfos) and was distributed on the leaf surface as evenly as possible.

Extraction and Analysis. At posttreatment intervals of 0, 3, 7, 14, and 21 days, the treated leaves from three plants of corn or bean were cut at the petiole, and the combined leaves were analyzed as described below. The experiment was repeated so that the data are averages of duplicate analyses.

For the leaf rinse, the leaves were placed in a beaker containing 10 mL of 1% v/v Tween 80 in distilled water, and the beaker was shaken for 3 min. This procedure was repeated three times, and the combined rinses were shaken vigorously with 80 mL of chloroform in a separatory funnel. The chloroform was removed and the extraction was repeated two more times. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and then concentrated to 2 mL at 40 °C under reduced pressure. One-half milliliter of the chloroform concentrate was added to scintillation vials, the solvent was removed with the aid of a stream of dry air at room temperature,

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Table I. Solvent Systems Employed for the Development of Thin-Layer Chromatograms and the Basis for Cochromatography

Solvents[a]	Volume, mL	R_f value of the compounds[b]			
		Etrimfos	P:O analogue	EEHP	EDHP[d]
A. Acetone-*n*-hexane	60:40	0.98	0.97[c]	0.47	
B. Benzene-acetone	70:30	0.98[c]	0.66[c]	0.12[c]	
C. Benzene-chloroform-acetic acid	70:25:5	0.58[c]	0.20[c]	0.19	0.05[c]
D. Benzene-chloroform-ethyl acetate-2-propanol	40:20:40:20	0.75[c]	0.61[c]	0.37[c]	0.05[c]
E. Benzene-2-propanol	80:20	0.97[c]	0.76[c]	0.47[c]	0.05[c]
F. Cyclohexane-acetone	50:50	0.95[c]	0.71	0.29[c]	
G. Ethyl acetate-acetone-ammonium hydroxide	60:30:10	1.00	0.35[c]	0.33	0.04

[a] Solvent system E (benzene-2-propanol 4:1) was used for routine separation and quantitation of metabolites. [b] TLC chambers were unsaturated. Solvent front was 15 cm. [c] The basis of cochromatography of various metabolites using different solvent systems. [d] No mobility in systems A, B, and F.

and the residue was radioassayed. The aqueous phase of the rinse was concentrated under reduced pressure at 60 °C to 5 mL. One-half milliliter of the concentrate was added to a scintillation vial, 15 mL of scintillation cocktail containing 10% v/v Biosolve BBS-3 was added, and the radioactivity was determined.

Rinsed leaves were chopped and homogenized for 5 min with 20 mL of chloroform in a VIRTIS 23 homogenizer (The Virtis Co., Inc., Gardiner, N.Y.). The supernatant was allowed to stand for 5 min and was removed with the aid of a pipet. The homogenization was repeated twice in the manner described above. The combined chloroform extracts were filtered and reduced on a rotary evaporator to ca. 5 mL. The leaf residue was resuspended in 15 mL of acetone and homogenized as described above. This step was repeated. The two acetone extracts were pooled and concentrated to ca. 5 mL. This was followed by homogenization of leaf residue in 10 mL of distilled water. This step was also repeated, and the residue was separated by filtration. The aqueous phase was reduced to ca. 10 mL on a rotary evaporator. The leaf residue was dried at room temperature and stored at 5 °C until assayed for radioactive materials.

Plant pigments from chloroform extracts were separated by column chromatography using a Hyflo Super-Cel (Johns-Manville Co., Manville, N.J.) and Attaclay X-250 (Minerals and Chemicals Corp. of America, Metuchen, N.J.) (1:2) column. After a thorough mechanical mixing of these two column materials, a chloroform slurry was prepared. The base of the glass column was first packed with ca. 2 g of anhydrous sodium sulfate, followed by the Hyflo Super-Cel–Attaclay slurry and an additional 2 g of sodium sulfate on the top of the column. The column was packed to a total height of 20.3 cm. The chloroform extract was reduced from 4.5 to 0.5 mL under a gentle stream of dry air. The concentrated extract was applied to the column and was eluted with chloroform under reduced pressure. Five-milliliter fractions were collected. In all extracts, the radioactivity was eluted between 40–70 mL, and these fractions were combined and concentrated on a rotary evaporator. The acetone extracts of the leaves contained very little plant pigments and, therefore, were used directly for TLC. The total radioactivity in chloroform extracts (after removal of plant pigments) and in the acetone and water extracts was determined as described above for leaf rinse fractions.

Chromatography and Radioanalysis. TLC was accomplished on 20 × 20 cm glass plates coated with a 0.5-mm layer of silica gel GF_{254} (Brinkman Instruments Inc., Westbury, N.Y.). For routine analyses the chromatograms were developed in solvent system E, benzene–2-propanol (4:1), since in this system all of the metabolites were clearly separated. Cochromatography was achieved by adding 4 μg of each authentic compound to the extracts prior to the development of the chromatogram in at least six solvent systems of different polarity (Table I). In a separate experiment, using less [^{14}C]etrimfos, pigment-free chloroform and acetone extracts of treated bean and corn leaves (100 μg of etrimfos and ^{14}C activity equivalent to 16000 dpm/leaf) were repeatedly subjected to preparative TLC on 1-mm thick silica gel plates 14 days after treatment. The chromatograms were developed in solvent systems E, D, and C (Table I). The final extracts were subjected to GC-MS analyses at the laboratories of Sandoz Inc., East Hanover, N.J., for confirmation of the identity of the recovered etrimfos and the major metabolite, EEHP.

After TLC of the extracts, radioautography with Kodak RP Royal X-Omat medical X-ray film (Eastman Kodak Co., Rochester, N.Y.) was used to locate the radioactive zones on the developed chromatograms. The exposure time varied between 10 to 20 days. After radioautography the silica gel zones corresponding to the images on the film were scraped and subjected to scintillation counting.

Radioactive measurements were accomplished with a refrigerated liquid-scintillation spectrometer (Model 6853 Unilux II-A, Nuclear, Chicago). A channel-ratio method was employed for quench corrections. The composition of the scintillation cocktail was according to Knowles and Sen Gupta (1969).

Determination of Unextractable Radioactivity. The total radioactivity in the leaf residue was determined following combustion of a 50-mg sample in an oxygen atmosphere, using an Intertechnique, L.S. Sample Oxidizer (Model IN 4101, Teledyne Isotopes, Westwood, N.J.). The resulting $^{14}CO_2$ was trapped in 20 mL of Oxymix (Teledyne Isotopes, Westwood, N.J.) and radioassayed.

Analysis of Aqueous Extracts. Because of limited solubility in nonpolar organic solvents and high solubility in water, EEHP and EDHP are difficult to extract completely from the water contents of the leaf. Therefore, to identify these products in aqueous extracts, a TLC procedure using the streaking method was employed for the resolution and quantitation of the metabolites (Ahmad and Forgash, 1975).

The nature of conjugated metabolites in the aqueous fractions were studied by incubating these fractions with hydrochloric acid or β-glucosidase (Sigma Chemical Co., St. Louis, Mo.). Aqueous extracts of leaves obtained at 3, 7, 14, and 21 days posttreatment intervals were used for enzyme cleavage studies. The incubation mixture consisted of the following: 2 mL of the aqueous extract, 4 mL

of citrate–phosphate buffer (pH 4.6), and 3 mg of glucosidase (14.1 units). The controls consisted of aqueous extract and buffer. The incubation mixture for acid hydrolysis contained 2 mL of the aqueous extract from 21-day samples and hydrochloric acid was added to yield a final pH of 1.0 (or distilled water for control) in a total incubation volume of 5 mL. Following incubation with shaking for 4 h at 38 °C, the hydrochloric acid was neutralized with sodium carbonate, and the flask contents were then extracted three times with chloroform; the ratio of the volume of chloroform to incubation mixture during each extraction was 2:1. The chloroform extracts were combined and dried over sodium sulfate and the radioactivity in the chloroform and water fractions was determined. The quantitation and identification of metabolites was obtained by pooling all of the samples of each metabolite detected in the chloroform and water fractions of the hydrolysate. The data were averaged from duplicate analyses.

Analysis of Untreated Portions of Seedlings. After the processing of treated primary leaves of bean or corn, the seedlings were divided into two portions: an upper portion consisting of the leaves, petioles, and the supporting stem, and a lower portion comprised of the roots (free of soil) and the non-leaf bearing segment of the stem. Each portion of the seedling was first chopped and then transferred into a Lourdes Multimix. Approximately 100 g of dry ice was added. The low temperature thus obtained in the homogenizer cup greatly facilitated the disintegration of leaves. Within 10 min of blending, a very fine mixture of plant tissues was obtained. The blended tissues were transferred into a glass dish and stored open at −20 °C until all dry ice evaporated. The dish was covered and placed in a desiccator to bring the blended tissues to room temperature. The total activity in the blended tissues was determined following combustion of a 200-mg sample in an oxygen atmosphere and the entrapment of the resulting $^{14}CO_2$ in 20 mL of Oxymix for radioassay as described above.

Evaporation Studies. [^{14}C]Etrimfos was applied in 10 μL of acetone to clean glass petri dishes at a radioactivity level of 40 000 dpm/dish. At selected intervals, the material remaining in the dishes was rinsed with 10 mL of acetone. This procedure was repeated two more times. The combined rinses were concentrated to ca. 2 mL on a rotary evaporator and transferred to a scintillation vial. The remaining acetone was carefully evaporated and the radioactivity counted. Volatilization of [^{14}C]etrimfos from bean and corn leaves was studied in the following manner. [^{14}C]Etrimfos was applied in 10 μL of acetone to primary leaves at a radioactivity level of 40 000 dpm/leaf. At selected intervals, the treated leaves were cut at the petiole and folded and placed in the plastic cup of the Sample Oxidizer. The ^{14}C activity was determined following combustion of the leaves in an oxygen atmosphere and the entrapment of the resulting $^{14}CO_2$ in 20 mL of Oxymix for radioassay as described above.

RESULTS

Volatilization Studies. Volatilization studies of etrimfos were first conducted to determine the role that volatilization might play in the fate of the compound following application to bean and corn leaves. Figure 1 shows the extent of loss of etrimfos from a glass plate surface. The higher initial rate of disappearance occurred during the evaporation of the solvent from the glass surface. The amount volatilized was not only time-dependent, as would be expected, but also depended on the area over which the dose was distributed. The results

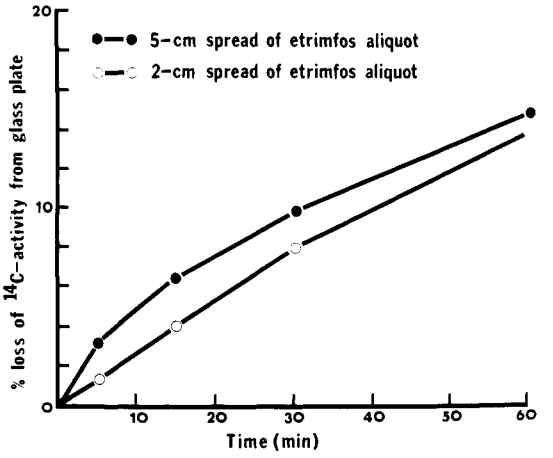

Figure 1. Loss of etrimfos (11 μg) by volatilization of a 10-μL aliquot from glass surface.

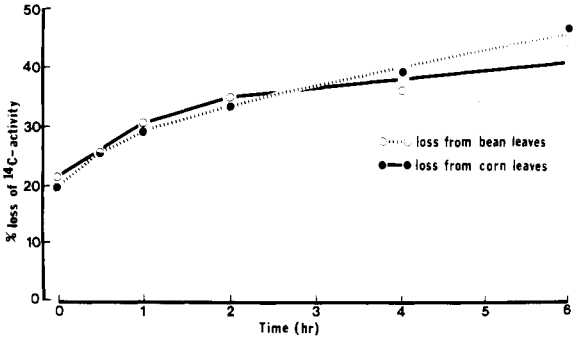

Figure 2. Evaporation of etrimfos (11 μg) from bean and corn leaves.

plotted in Figure 1 pertain to equal amounts of radioactivity spread over circular areas of 2 and 5 cm diameters. The loss was slightly greater from the larger area (Figure 1).

Studies on volatilization of etrimfos from leaf surfaces were conducted by applying [^{14}C]etrimfos to primary leaves of bean and corn plant; the conditions, i.e., age of plants, dosage, manner of application, etc., were the same as in the metabolism studies. It is apparent from Figure 2 that etrimfos volatilized rapidly from both types of leaves. The greatest loss occurred at zero time (ca. 10 min elapsed between the application and radioassay) and amounted to 21% for bean and 19.4% for corn leaves. The reason for the rapid loss of etrimfos from leaf surfaces is not clearly understood at the present. However, similar recoveries of total radiocarbon were obtained at zero time in the metabolic studies in bean and corn.

Metabolism in Bean. The fate of etrimfos applied to bean leaves is given in Tables II and III. The disappearance of etrimfos with time was very rapid. At zero time the total recovery of the radiocarbon was 86.8% and by the third, seventh, fourteenth, and twenty-first day only 43.1, 33.6, 34.7, and 36.4%, respectively, were recovered. Since the radioassay of untreated portions of bean seedlings revealed a maximum of 0.5% of the applied radioactivity by 21 days, it was concluded that translocation did not play a significant role in the disappearance of etrimfos from treated leaves. Evidently, the loss occurred through volatilization of etrimfos from the treated leaves. As discussed above, these results are in agreement with evaporation studies (Figure 2).

Evidence of the occurrence of metabolism–degradation of etrimfos with time was provided by the increasing amounts of ^{14}C activity in acetone and aqueous extracts.

Table II. Distribution of ^{14}C Materials in Bean Seedlings following Treatment of Bean Leaves with [^{14}C]Etrimfos

	Percent of applied radioactivity				
	Number of days after treatment				
Nature of extract	0	3	7	14	21
	Leaf Rinses				
Chloroform	43.0	2.0	0.6	0.3	1.3
Water	2.0	0.3	0.2	0.4	0.5
Total radiocarbon	45.0	2.3	0.8	0.7	1.8
	Leaf Extracts				
Chloroform	39.8	35.1	21.4	15.8	11.6
Acetone	1.0	3.1	6.0	6.0	7.1
Water	0.4	2.2	5.2	11.5	14.8
Total radiocarbon	41.2	40.4	32.6	33.3	33.5
	Leaf Unextractables				
Total radiocarbon	0.6	0.4	0.2	0.4	0.6
	Untreated Portions of Seedlings[a]				
Total radiocarbon	0	<0.1	<0.1	0.3	0.5
Total recovery of radiocarbon	86.8	43.1	33.6	34.7	36.4

[a] Stem and untreated leaves. No ^{14}C activity was detected in root system.

The water-extractable activity in leaves increased from 0.4 (zero time) to 14.8% in 21 days; similarly, the acetone-extractable activity increased from 1.0 (zero time) to 7.1% in 21 days. This was indicative of the formation of polar metabolites which were not extracted by chloroform. Also small amounts of radioactivity were associated with leaf residues and amounted to 0.6% by 21 days.

The etrimfos level in the leaf rinse decreased drastically during the first 7 days, the greatest decrease occurring during the initial 3 days (Table II). The decrement of total radiocarbon in the leaf extracts during the same time period was small in comparison. It is possible that the 8% loss of etrimfos in bean leaves between 3 and 7 days occurred by evaporation of volatile components, presumably the parent compound.

Table III shows that the only compound in the rinse extracted by chloroform was etrimfos, accounting for 95.6% (of the total rinse ^{14}C activity) at zero time and 72.2% at 21 days. The balance of the activity was associated with the aqueous phase which was also analyzed for metabolites (Table III). It is clear that at zero time the major component in the aqueous phase of the rinse was again the parent compound, indicating that the extraction of the parent compound by chloroform was not complete. Small amounts of the EEHP were present in the aqueous phase of the rinses. The formation of EEHP in the leaf rinse could have resulted from the following: (1) spontaneous degradation of the parent, (2) hydrolysis, and (3) photolysis.

Analysis of metabolites by TLC (Table III) showed that, whereas the parent compound was the only material present in the chloroform extract, the acetone extracts contained both etrimfos and EEHP plus five additional minor metabolites. GC–MS analyses confirmed the presence of etrimfos and the EEHP metabolite in the chloroform and acetone extracts of bean plants, respectively. In addition, the radioactivity associated with the TLC origin increased to 2.7% of the applied dose in 21 days, indicating the presence of very polar metabolites.

Metabolism in Corn. The fate of [^{14}C]etrimfos following treatment of corn leaves is presented in Tables IV and V. The total recoveries of the applied radiocarbon and the pattern of distribution of ^{14}C activity in various fractions and extracts of leaf (with time) were remarkably similar to that found for beans. However, unlike the bean leaf extracts that contained about the same quantity of radioactive materials from day 7 to 21 (32.6 and 33.5%, respectively, Table II), in corn leaves the amount of ^{14}C materials dropped from 31.6% at 7 days to 24.3% in 21 days (Table IV). Also, there was a difference in the quantity of radioactive materials in the untreated portions of the two seedlings. In corn, this ranged from 0.5% at 3 days to 0.8% at 21 days (Table IV), whereas in bean the range was somewhat lower, <0.1 to 0.5% (Table II).

In addition to the metabolites detected in bean plants, small quantities of the P:O analogue were detected in rinses of corn leaves; there was 0.7% present initially and this decreased gradually to zero by 21 days. Chloroform extracts of the rinses also contained the EEHP. Again, as in the case of bean, the presence of etrimfos and the EEHP metabolite in corn leaf extracts was confirmed by GC–MS analysis. TLC analysis of the rinses showed that the parent material had disappeared from the surface of the treated corn leaf by 14 days (Table V). TLC analysis

Table III. Composition of ^{14}C Materials in Rinses and Extracts of Bean Leaves Treated with [^{14}C]Etrimfos

		Percent of applied radioactivity				
		Number of days after treatment				
Nature of extracts	Nature of radiocarbon[a]	0	3	7	14	21
		Rinses				
Chloroform	Etrimfos	43.0	2.0	0.6	0.3	1.3
Water	Etrimfos	1.9	0.2	0.1	0.2	0.2
	EEHP	0.1	0.1	0.1	0.2	0.2
	TLC origin	0	0	0	0	0.1
	Total (water)	2.0	0.3	0.2	0.4	0.5
		Extracts				
Chloroform	Etrimfos	39.8	35.1	21.4	15.8	11.6
Acetone	Etrimfos	0.8	0.2	3.7	0.4	0.3
	EEHP	0.1	0.5	0.2	0.3	0.1
	Unknowns					
	TLC zone A	0	0.3	0.3	0.7	0.6
	TLC zone B	0	0.2	0.1	0.1	0.2
	TLC zone C	0	0.4	0.3	0.6	0.6
	TLC zone D	0	0.4	0.4	1.0	0.9
	TLC zone E	0	0	0.3	1.7	1.7
	TLC origin	0.1	1.1	0.7	1.2	2.7
	Total (acetone)	1.0	3.1	6.0	6.0	7.1

[a] TLC solvent system E (Table I). The R_f of the unknown metabolites associated with TLC zones A, B, C, D, and E were 0.32, 0.27, 0.22, 0.08, and 0.05, respectively.

Table IV. Distribution of ^{14}C Materials in Corn Seedlings following Treatment of Corn Leaves with [^{14}C]Etrimfos

Nature of extracts	Percent of applied radioactivity: Number of days after treatment 0	3	7	14	21
Leaf Rinses					
Chloroform	40.9	1.4	0.6	1.0	0.6
Water	3.6	1.7	2.3	3.5	3.7
Total radiocarbon	44.5	3.1	2.9	4.5	4.3
Leaf Extracts					
Chloroform	36.1	34.9	15.9	6.3	3.8
Acetone	2.9	3.8	6.4	6.9	6.7
Water	0.3	3.0	9.3	12.6	13.8
Total radiocarbon	39.3	41.7	31.6	25.8	24.3
Leaf Unextractables					
Total radiocarbon	0.1	0.2	0.1	0.3	0.1
Untreated Portions of Seedlings[a]					
Total radiocarbon	0	0.5	1.5	0.8	0.8
Total recovery of radiocarbon	83.9	45.5	36.1	31.4	29.5

[a] Stem and untreated leaves. No ^{14}C activity was detected in root system.

of the aqueous phase of the rinse also showed the absence of the parent material by the fourteenth day. Also compared to bean leaves, there was a greater amount of total EEHP present in the corn leaf rinse. From this it was concluded that the degradation of etrimfos on corn leaves was greater than on bean.

Analysis of the leaf extracts showed comparable amounts of parent material in the chloroform and acetone extracts of corn and bean (39.0 and 40.8%, respectively). However, only 3.8% of the parent material was recovered from extracts of corn leaves compared to 11.9% from beans. This difference appears to be related to a lower recovery of total radiocarbons from corn (29.5%) than from bean (36.4%). This is presumably due to the greater volatilization of ^{14}C materials from corn leaves.

Hydrolysis Studies. In the hydrochloric acid hydrolysis experiments, the controls from aqueous bean extracts showed the following composition of ^{14}C activity: TLC origin > unknowns > EEHP (Table VI). Acid hydrolysis showed an increase in the amount of EEHP which correlated with decreased amounts of the unknowns. Presumably, these materials were transformed to EEHP by the acid treatment. In corn, no EEHP was detected either in the control or acid hydrolysate and most of the activity appeared to be due to unknowns followed by materials at the TLC origin (Table VI). Hydrochloric acid caused a slight increase in the amount of total unknowns with a concomitant decrease in the ^{14}C activity at the TLC origin. The small amount of radioactivity present in these extracts, together with the nature of the TLC streaking method, did not permit radioautography and thus the results of the acid hydrolysis are not clear.

The results of β-glucosidase hydrolysis of aqueous extracts are even more complex and difficult to interpret (Table VII). Unlike the controls for acid hydrolysis, the controls of glucosidase hydrolysis were acidic (pH 4.6). Therefore, the metabolite composition was altered. This becomes clear by comparing the composition of 21-day controls of the acid (Table VI) and enzymic hydrolysis (Table VII). In the case of bean, some increase at 21 days is apparent in the amounts of EEHP and unknown materials at zone E, indicating that these materials may be involved in the formation of a glucoside. In corn, no EEHP was detected by the fourteenth day, and unlike the situation in beans, an appreciable decrease in the amounts of materials at zone E was apparent 21 days after treatment (Table VII). A concomitant increase in the amounts of other unknowns and activity at the TLC origin was also apparent. Exactly how glucosidase affected these transformations is unknown at the present.

Further Analyses of ^{14}C Activity at TLC Zone E. Of all the metabolites of unknown structure, the ^{14}C activity associated with TLC zone E (R_f 0.05, development using solvent system E, benzene–2-propanol, 4:1) appeared to be a major component in the aqueous phase of the treated leaves (Tables III, V, and VII). Although this R_f corresponded to the authentic EDHP metabolite, the homogeneity or the identity of this ^{14}C activity could not be

Table V. Composition of ^{14}C Materials in Rinses and Extracts of Corn Leaves Treated with [^{14}C]Etrimfos

Nature of extracts	Nature of radiocarbon[a]	Percent of applied radioactivity: Number of days after treatment 0	3	7	14	21
		Rinses				
Chloroform	Etrimfos	39.6	0.9	0.3	0	0
	P:O analogue	0.7	0.2	<0.1	0.3	0
	EEHP	0.6	0.3	0.3	0.4	0.3
	TLC origin	0	0	0	0.2	0.3
	Total (chloroform)	40.9	1.4	0.6	0.9	0.6
Water	Etrimfos	3.2	1.2	1.3	0	0
	EEHP	0.4	0.5	0.9	2.4	2.8
	TLC origin	0	0	0.1	1.1	0.9
	Total (water)	3.6	1.7	2.3	3.5	3.7
		Extracts				
Chloroform	Etrimfos	36.1	34.9	15.9	6.3	3.8
Acetone	Etrimfos	2.9	0	0	0	0
	EEHP	0	1.7	2.0	1.3	0.4
	Unknowns					
	TLC zone A	0	0.2	0.3	0.6	0.2
	TLC zone B	0	0.1	0.4	0.2	1.0
	TLC zone C	0	0.1	0.2	0.1	0.2
	TLC zone D	0	0.5	0.9	0.6	0.4
	TLC zone E	0	0.6	2.1	3.8	3.8
	TLC origin	0	0.6	0.5	0.3	0.7
	Total (acetone)	2.9	3.8	6.4	6.9	6.7

[a] TLC in solvent system E (Table I). R_f of the unknown metabolites associated with TLC zones A, B, C, D, and E were 0.32, 0.27, 0.22, 0.08, and 0.05, respectively.

Table VI. Composition of ^{14}C Metabolites in the HCl Hydrolysates of the Aqueous Extracts of Bean and Corn Leaves at 21 Days

	Percent ^{14}C material[b]			
	Bean		Corn	
Metabolites[a]	Control	HCl	Control	HCl
EEHP	15.2	25.1	0	0
Unknowns[c]	38.8	26.2	74.0	76.4
TLC origin	46.0	48.3	26.0	23.6

[a] TLC in solvent system E (Table I). No parent or P:O analogue was detected. [b] Total water-extractable activity at 21 days in bean was 14.8% (Table II) and in corn 13.8% (Table IV). [c] Corresponding to TLC zones A, B, C, D, and E (solvent system E, Table I).

resolved by using the other solvent systems listed in Table I, because in all of these systems, the material(s) remained close to the TLC origin. Therefore, we conducted additional experiments, using other highly polar eluting systems in which the EDHP metabolite is resolved at higher R_f's. A procedure involving repeated chromatography and counting of ^{14}C activity associated with these zones was employed, using aqueous phase aliquots derived from the same batch of [^{14}C]etrimfos-treated bean and corn leaves that were used for GC–MS analyses for confirmation of the identity of the major metabolites. As a first step, the aqueous phase was concentrated to ca. 0.5 mL on a rotary evaporator and 1 mg of authentic EDHP metabolite was added to the concentrate. The material was then streaked on 1-mm silica gel plates and a chromatogram was developed using solvent system E. The TLC zone corresponding to the EDHP standard (R_f 0.05) was scraped and extracted three times with 5-mL aliquots of water. Ninety percent of the activity associated with the zone was extracted by this procedure. The extracts were combined, reduced once again on the rotary evaporator, and subjected to a second TLC using a chloroform–methanol–ammonium hydroxide (6:4:1) mixture for the development of the chromatogram. The TLC zone corresponding to the EDHP metabolite (R_f 0.42) was scraped and extracted three times with water. The efficiency of extraction of ^{14}C activity by water was 95%, and the same efficiency was obtained in the third and fourth TLC separations. The solvents for the development of chromatograms employed in the third and fourth TLC runs were butanol–methanol–ammonium hydroxide–water (12:4:1:3), yielding EDHP at R_f 0.60, and chloroform–methanol–ammonium hydroxide (6:4:1) with an EDHP R_f of 0.42. The results of these successive TLC separations of ^{14}C activity are presented in Table VIII. It is clear that a greater portion of ^{14}C activity associated with TLC zone E (solvent system E, EDHP R_f 0.05) is due to material(s) other than EDHP. Furthermore, based on the fourth TLC separation (Table VIII) we conclude that EDHP is formed in both bean and corn leaves, although the amounts are small. Thus, 14 days

Table VII. Results of β-Glucosidase Hydrolyses of Aqueous Extracts of Bean and Corn Leaves Treated with [^{14}C] Etrimfos

	Percent ^{14}C material[b]							
	Enzyme treatment				Control			
	Days after treatment				Days after treatment			
Nature of ^{14}C materials[a]	3	7	14	21	3	7	14	21
				Bean				
EEHP	39.1	13.6	26.9	18.1	38.9	18.6	25.5	8.0
Unknowns TLC zone E[c]	16.7	22.8	21.8	25.8	19.6	22.0	26.0	18.6
Unknowns[d]	36.2	36.3	42.6	41.4	34.7	40.4	41.0	58.0
TLC origin	8.0	27.3	8.7	14.7	6.8	19.0	7.5	15.4
				Corn				
EEHP	22.4	9.1	0	0	26.5	8.6	0	0
Unknowns TLC zone E[c]	22.9	39.8	44.9	47.3	38.7	37.9	43.2	67.7
Unknowns[d]	17.5	21.6	25.4	29.1	17.7	23.9	29.9	17.4
TLC origin	37.2	29.5	29.7	23.6	17.1	29.6	26.9	14.9

[a] TLC in solvent system E (Table I). [b] For total water-extractable ^{14}C activity at indicated posttreatment periods see Tables II and IV. [c] Corresponding to TLC zone E (solvent system E, Table I). [d] Corresponding to TLC zones A, B, C, and D (solvent system E, Table I).

Table VIII. Results of Successive TLC Separations of ^{14}C Activity Associated with the EDHP (2-Ethyl-4-hydroxypyrimidinediol) Metabolite of Etrimfos in the Aqueous Phases of Bean and Corn Seedlings

			^{14}C activity[a]			
			Bean		Corn	
TLC steps	Solvents for development of chromatograms	R_f of EDHP metabolite	% of aqueous phase[b]	% of total applied[c]	% of aqueous phase[b]	% of total applied[c]
First	Benzene-2-propanol (4:1)	0.05	25.04	3.13	41.92	5.63
Second	Chloroform-methanol-ammonium hydroxide (6:4:1)	0.42	4.14	0.52	21.53	2.89
Third	Butanol-methanol-ammonium hydroxide-water (12:4:1:3)	0.60	2.90	0.36	10.77	1.45
Fourth	Chloroform-methanol-ammonium hydroxide (6:4:1)	0.42	2.80	0.35	8.16	1.10

[a] Recovered fourteenth day after treatment with [^{14}C]etrimfos. The data were corrected for losses during extraction steps by taking into account the extraction efficiencies. [b] ^{14}C activity in the aqueous phase amounted to 12.5 and 13.43% in bean and corn, respectively. [c] One hundred leaves of each plant treated at the rate of 100 μg of etrimfos (16 000 dpm)/leaf.

after treatment of seedlings with [^{14}C]etrimfos, only 0.35 and 1.1% of the total applied activity appeared as EDHP in the aqueous extracts of bean and corn, respectively.

DISCUSSION

Metabolism–degradation of etrimfos in bean and corn leaves was remarkably similar. Evaporation experiments, as well as metabolic studies, indicated that 21 days after treatment, about two-thirds of the etrimfos was lost by volatilization from treated leaves of both bean and corn. There was no evidence for significant translocation of etrimfos in either plant. No radioactivity was associated with the lower untreated part of the seedling, i.e., the roots and the non-leaf bearing segment of the stem. The small amount of ^{14}C activity associated with the top leafy portion of the seedling could have resulted from the condensation of etrimfos volatilized from the treated leaf surfaces.

The P:O analogue of etrimfos is reportedly very unstable and presumably rapidly degrades to EEHP (Karapally, 1975). Small amounts of the P:O analogue were detected in corn leaf rinse; the amount decreased with time with a concomitant increase in the amount of EEHP. In bean leaf rinse, however, the amount of EEHP relative to corn rinse was always lower and no P:O analogue was found, although in some preliminary studies trace amounts (ca. 0.1%) of the P:O material were detected at zero time. The quantities of the P:O material found in corn leaves may have been higher than that detected, considering its instability and apparent degradation during the processing of the extracts. Due to the instability of this material, no attempts were made to confirm its presence in corn extracts by GC–MS analysis.

It is possible to elucidate the initial steps involved in the metabolism–degradation of etrimfos in bean and corn leaves. It apparently follows the established pathways for organothionophosphate insecticides in animals and plants (O'Brien, 1967). Etrimfos is desulfurated oxidatively to the P:O compound, and etrimfos and the P:O compound are converted to the pyrimidinol, EEHP, via the cleavage of the pyrimidinyl bond.

Several additional metabolites that appear to be more polar than EEHP (based on chromatograms developed in solvent system C, D, E, and G) were detected in relatively small quantities. The chromatographic behavior and amount of ^{14}C activity at TLC spots A, B, C, and D (R_f's 0.32, 0.27, 0.22, 0.08 in solvent system E, Table I) indicate that activity at each location was the result of very small quantities of single metabolites.

The ^{14}C activity associated with TLC zone E close to the TLC origin (R_f 0.05 in solvent system E) was of particular interest in that the aqueous phases of the leaf extracts always showed higher quantities at this zone relative to the other unknown metabolites and an authentic standard of EDHP was found to cochromatograph with this zone. Repeated cochromatography, using highly polar TLC solvent systems that mobilized EDHP appreciably (Table VIII), indicates that EDHP may be a minor component of this activity. Because of the low yield, poor solubility (practically insoluble in most organic solvents), and absence of a suitable derivatization procedure, the confirmation of this material by other analytical methods was not attempted.

Results of the acid and glucosidase hydrolyses of aqueous extracts are difficult to interpret. Considering the low amount of aglycons released in the HCl treatment, it appears that the total conjugated materials comprise a very small fraction of the entire ^{14}C activity of the aqueous phase. There was some indication that small amounts of EEHP glycoside may have been present in bean plants. Since EDHP has been tentatively identified as a minor component of the aqueous phase (2.8% in bean and 8.16% in corn at 14 days), it is possible that a small amount of conjugated EDHP was also present. Clearly, more work is needed to determine whether conjugation mechanisms play a significant role in the metabolism of etrimfos and its degradation products.

Because the plants were placed in a greenhouse with plastic windows which allow transmission of UV radiation, degradation of etrimfos, particularly on the bean and corn leaf surfaces, may have resulted from photolysis. In this respect, it is interesting to note that none of the unknown metabolites found in leaf extracts were detected in the leaf rinses; therefore, these materials in the leaf extracts were either the products of plant metabolic processes, or formed by photooxidation and were absorbed by the leaves. If, indeed, these metabolites were the products of photooxidation, then their absence from leaf rinses may be attributed to their loss from leaf surfaces through volatilization.

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