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Fungal Degradation of Organophosphorous Insecticides

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DISCLAIMER

Summary

Organophosphorous insecticides are used extensively to treat a variety of pests and insects. Although as a group they are easily degraded by bacteria in the environment, a number of them have half-lives of several months. Little is known about their biodegradation by fungi. We have shown that *Phanerochaete chrysosporium* can substantially degrade chlorpyrifos, fonofos, and terbufos (27.5%, 12.2%, and 26.6%, respectively) during 18-day incubation in nitrogen-limited stationary cultures. The results demonstrate that the chlorinated pyridinyl ring of chlorpyrifos and the phenyl ring of fonofos undergo ring cleavage during biodegradation by the fungus. The usefulness of the fungus system for bioremediation is discussed.

Introduction

Organophosphorus insecticides are used extensively in agriculture to treat a variety of soil pests including corn rootworms, cut worms, and wire worms and to control orchard pests such as the apple flea weevil, the plum curculio, and the coddling moth (*1*, *2*). Many of these compounds are effective as topical sprays and are used to control household pests and aerial insects on field crops and ornamental plants (*1*, *2*). Some organophosphorus insecticides are used in veterinary medicine to control ticks and fleas (*1*, *2*).

Many organophosphorous insecticides appear to function as cholinesterase inhibitors (*1*, *2*). They are typically quite lethal to insects but are often relatively innocuous to fish, mammals, and birds. Compared to certain effective insecticides (DDT, chlordane, and dieldrin, for example), they are not very persistent in the environment. Some organophosphorous insecticides are, however, moderately persistent and have half-lives of several months in soil (*2-4*). Furthermore, substantial amounts of relatively stable and biologically active metabolites may

persist in soils either as free metabolite or tightly bound to soil (*3*, *4*). Mineralization by soil microorganisms appears to be a significant mode of organophosphorous insecticide removal (*3*, *4*).

The wood-rotting fungus *Phanerochaete chrysosporium* can degrade a wide variety of structurally diverse organopollutants to carbon dioxide (*5-10*). Because of its unique biodegradative abilities, considerable interest exists in its role and that of other lignin-degrading fungi in the degradation of agricultural chemicals and their metabolites. In the present investigation, we have shown that *P. chrysosporium* can mediate extensive degradation of three representative organophosphorous insecticides.

Methods

Fungus. *Phanerochaete chrysosporium* BKM-F-1767 was obtained from the U.S. Department of Agriculture Forest Products Laboratory, Madison, Wisconsin. The fungus was stored at room temperature on malt agar slants before use.

Radiochemicals. The following radiolabeled insecticides were a gift from J.R. Coats and L. Somasundaram, Iowa State University to S.N. Kakar: [2,6-phenyl-^{14}C]chlorpyrifos, [U-ring-^{14}C]fonofos, and [methylene-^{14}C]terbufos. The trivial names of the three organophosphorous insecticides are used as a matter of convenience. The following names of these insecticides are recognized by the Chemical Abstracts Service (CAS): chlorpyrifos, phosphorothioic acid O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) ester; fonofos, ethylphosphorodithioic acid O-ethyl-S-phenyl ester; terbufos, phosphorodithioic acid S-[[(1,1-dimethylethyl)thio]methyl]-O-O-diethyl ester. The radiochemical purity of ^{14}C-chlorpyrifos and ^{14}C-fonofos was greater than 98%, while the radiochemical purity of ^{14}C-terbufos was 96%. Structures of these compounds are presented in Figure 1.

Experimental Conditions. Biodegradation studies were performed by using stationary cultures of *P. chrysosporium* grown in the nitrogen-limited medium described by Fenn and Kirk (*11*). Briefly, this medium consisted of 56 mM glucose, 1.2 mM ammonium tartrate, trace elements (*12*) and thiamine (1 mg/L) in 20 mM dimethylsuccinate (sodium) buffer, pH 4.2 (*11*). Cultures received 1 mL of formaldehyde [36% (w/w)] to prevent microbial growth. All cultures were incubated at 37°C.

Cultures (10 mL) were grown in sealed 250-mL Wheaton bottles. The Teflon-lined cap of the bottles was modified by drilling two holes, into which a short and a long piece of glass tubing were inserted. The longer piece of glass tubing extended into the bottle to approximately 3 cm above the fungal mat. Epoxy glue was used to hold the tubing in place. These modified caps served as gas exchange manifolds. Wheaton bottles so modified have been used to demonstrate ^{14}C-carbon dioxide evolution from ^{14}C-labeled organopollutants in several studies (*5-9*). However, we have recently found that 500 mL Erlenmeyer flasks with ground-glass joints fitted with impingers (Ace Glass), originally designed for air sampling, can also be used for gas exchange. In experiments in which 500 mL Erlenmeyer flasks were used it was necessary to increase the volume of the cultures to 20 mL so that the fungal mat completely covered the bottom of the flask.

Radiolabeled compounds were added to cultures immediately after inoculation with fungal spores. Cultures were grown under ambient atmosphere during the first 3 days of incubation. On day 3 and at 3-day intervals thereafter, cultures were flushed with O_2 for 20 min (Wheaton bottles) or 30 min (Erlenmeyer flasks), and the CO_2 evolved was trapped in 10 mL of a solution containing ethanolamine-methanol-Safety Solve scintillation cocktail (Research Products International Corp., Mt. Prospect, Illinois) (1:4:5). The amount of $^{14}CO_2$ trapped was determined by liquid scintillation spectrometry. Mass balance analyses were performed on cultures of *P. chrysosporium* that had been incubated with ^{14}C-chlorpyrifos, ^{14}C-fonofos, or ^{14}C-terbufos for 18 or 21 days.

Cultures (three or five replicates for each compound) were pooled and the individual culture flasks were rinsed with water (40 mL total), which was added to the pooled cultures. The pooled cultures were then extracted three times with methylene chloride (100 mL total). Particulate material (mycelium) was separated from the aqueous fraction by filtration through glass wool. Samples of the particulate material and 1-mL aliquots of the methylene chloride extracts and the aqueous fractions were placed in scintillation vials containing 10 mL of Safety Solve, and radioactivity was determined by liquid scintillation spectrometry.

For thin-layer chromatography (TLC), the solvent was removed from the methylene chloride extracts by evaporation under nitrogen. The residue was redissolved in a minimal amount of methylene chloride. TLC of ^{14}C chlorpyrifos (in hexane:acetone:glacial acetic acid [20:40:1]), ^{14}C fonofos (in chloroform:ethylacetate:hexane [2:2:1]), and ^{14}C terbufos (in nitromethane:acetonitrile:toluene [3:8:9]) was performed as recommended (J.R Coats and L. Somasundaram, personal communication) by using precoated silica gel 60 G F-254 plates [5 x 20 cm, (aluminum backed); thickness, 250 μm; E. Merck AG, Darmstadt, Germany]. After TLC, the elution solvents were evaporated under air. TLC plates were cut into 1-cm strips, which were then moistened with water and placed in scintillation vials containing 20 mL of Safety Solve. With this procedure, the silica gel was desorbed from the aluminum backing and settled on the bottom of the scintillation vial. Radioactivity was then determined by liquid scintillation spectrometry.

Results

The ability of *P. chrysosporium* to degrade ^{14}C chlorpyrifos, ^{14}C fonofos, and ^{14}C terbufos is illustrated in Table 1 and Figures 2-7. In all cases, substantial amounts (27.5%, 12.2%, and 26.6%, respectively) of chlorpyrifos, fonofos, and terbufos were degraded to $^{14}CO_2$ during the 18-day incubation in nitrogen-limited stationary cultures of this fungus. Only the

aromatic ring carbons of chlorpyrifos and fonofos were labeled. Thus, these results demonstrate that the chlorinated pyridinyl ring of chlorpyrifos and the phenyl ring of fonofos undergo ring cleavage during biodegradation by *P. chrysosporium*.

Degradation was also assessed by mass balance analysis. After incubation, cultures were extracted with methylene chloride. Results (Table 1) show that considerable amounts of all of these compounds were converted to metabolites that partitioned into the aqueous phase. Similarly, TLC revealed the presence of several metabolites of these compounds in the methylene chloride fractions (Figures 5-7). Considerable nonbiological degradation occurred in controls as evidenced by metabolite formation in sterile culture medium (data not shown). Controls also suggested that chlorpyrifos, fonofos, and terbufos might be susceptible to air stripping and that volatilization might account for the low mass recovery observed in several experiments and controls.

Because of the low mass recoveries, additional experiments (Table 2) were performed in which cultures (20 mL) were incubated in 500-mL Erlenmeyer flasks with ground-glass joints fitted with a glass impinger for gas exchange. A trap for volatile organics was placed immediately before the carbon dioxide trap. The latex tubing used in this apparatus was assayed for absorbed radioactivity. Results were similar to those reported in Table 1 and are consistent with the conclusion that substantial amounts of all of these pesticides are degraded by *P. chrysosporium*. In general, mass recoveries were improved by using these procedures. However, although some radiolabeled material was retained by the volatile traps and some was absorbed by the latex tubing, we were still unable to account for substantial amounts of mass in some experiments. We suggest that most of this mass may have been lost during manipulations involved with the methylene chloride extraction. In control experiments, the amount of ^{14}C-labeled material found in $^{14}CO_2$ traps was never greater than 1.0% of the radioactivity initially present, demonstrating that $^{14}CO_2$ formation was not a major factor in the nonbiological disappearance observed.

Discussion

Organophosphorus insecticides are useful because they are relatively inexpensive, fairly simple to manufacture, and effective against a wide variety of insect pests (*1, 2*). As a group, they are relatively quickly degraded in the environment (*1, 2*). A number of organophosphorous insecticides do, however, have half-lives on the order of several months and may be thought of as moderately persistent. For example, in a nonsterile Sultan silt loam soil incubated with 50 nM of ^{14}C-chlorpyrifos for 8 months, 26.1% of the radioactivity was recovered as undegraded ^{14}C-chlorpyrifos, 33% was recovered as ^{14}C-3,5,6-trichloro-2-pyridinol, 33% was not extractable from the soil; 1.1% was converted to unidentified water-soluble metabolites, and 6.6% was degraded to $^{14}CO_2$ (*3*). In other words, more than 92% of the ^{14}C-chlorpyrifos originally present remained undegraded, as a biologically active metabolite, or in a form that was tightly bound to soil after 8 months of incubation.

The biodegradation of organophosphorous insecticides has been studied in soils and in bacterial cultures (*1–4*). Less attention has been given to the biodegradation of these compounds by fungi. Fenitrothion [phosphorothioic acid O,O-dimethyl-O-(3-methyl-4-nitrophenyl) ester] is, however, known to be degraded by *Trichoderma viride*, *Mortierella isabellina*, and *Saprolegnia parasitica* (*13*). The present study showed that *P. chrysosporium* degraded chlorpyrifos, fonofos, and terbufos to carbon dioxide. *Trichodrma viride, M. isabellina*, and *P. chrysosporium* are all members of the Deuteromycotina, the Zygomycotina, and the Basidiomycotina, respectively, in the division Amastigomycota, whereas *S. parasitica* is a member of the division Mastigomycota. These limited results suggest that the ability to degrade organophosphorous insecticides may be widespread or at least relatively common among the fungi.

Compared to truly persistent environmental pollutants such as DDT, dieldrin, and chlordane, organophosphorous insecticides in most soils do not present bioremediation problems.

Exceptions might exist in cases of inadvertent overapplication or repeated application and in accumulation in soils containing inordinately low levels of microbial activity. In these cases it would be logical to amend the soils with microorganisms having the documented ability to degrade the organophosphorous compound in question. If *P. chrysosporium* were used, a suitable, inexpensive growth substrate would also likely be required, because this fungus typically does not use xenobiotics as growth substrates. Growth of *P. chrysosporium* and degradation of environmentally persistent organopollutants in soils has been demonstrated by using several inexpensive plant residues including ground corn cobs, wood chips, peat, and wheat straw (*14*, *15*).

The ability of lignin-degrading fungi such as *P. chrysosporium* to degrade organophosphorous insecticides may be important from another perspective. Pesticide residues or their metabolites are often incorporated into the insoluble fraction of plants and soils. Some of this insoluble material is undoubtedly lignin or lignin-derived material. Such immobilization represents a form of pesticide inactivation and is sometimes a goal of bioremediation efforts. The immobilized material must eventually undergo turnover and further degradation. Indeed, proponents of immobilization techniques emphasize that release of bound pollutants is expected to occur slowly and at levels that are environmentally insignificant (*16*). *Phanerochaete chrysosporium* can degrade chlorinated anilines covalently incorporated into lignin (*10*). Thus, in soils, lignin-degrading fungi might accelerate degradation of organopollutants that are covalently bound to lignin or lignin-derived material. This technology is promising, but the microbial ecology and biodegradative ability of lignin-degrading fungi in soil require further scrutiny.

Acknowledgments

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Table 1. Mass balance of ^{14}C-radioactivity from *P. chrysosporium* incubated with ^{14}C-chlorpyrifos, ^{14}C-fonofos, and ^{14}C-terbufos for 18 days in Wheaton bottles.

		Recovery in Fraction (%)			
Compound*	Recovery (%)	Methylene Chloride Fraction	Aqueous Fraction	Particulate Material	$^{14}CO_2$
Chlorpyrifos	45.1	9.0	4.4	4.2	27.5
Fonofos	78.2	13.1	50.8	2.1	12.2
Terbufos	60.9	6.9	22.9	4.5	26.6

* The initial concentrations of chlorpyrifos, fonofos, and terbufos were 3.2 µM, 5.6 µM, and 1.4 µM, respectively.

Table 2. Mass balance of ^{14}C-radioactivity from *P. chrysosporium* incubated with ^{14}C-chlorpyrifos, ^{14}C-fonofos, and ^{14}C-terbufos for 21 days in Erlenmeyer flasks

		Recovery in Fraction (%)					
Compound*	Recovery (%)	Methylene Chloride Fraction	Aqueous Fraction	Particulate Material	Organic Trap	Rubber Tubing	$^{14}CO_2$
Chlorpyrifos	61.1	15.1	5.5	0.4	4.0	1.7	34.4
Fonofos	78.3	10.9	50.0	1.0	0.4	0.3	15.7
Terbufos	74.3	6.6	26.2	2.5	2.9	1.3	34.8

* The initial concentrations of chlorpyrifos, fonofos, and terbufos were 1 µM, 7.1 µM, and 0.14 µM, respectively.

Figure legends

Figure 1. Structures of organophosphorous insecticides used in this investigation.

Figure 2. Mineralization of ^{14}C-chlorpyrifos by *P. chrysosporium*. The initial concentration of ^{14}C chlorpyrifos was 3.2 μM. Each point (open squares) represents the mean of five determinations ± the standard deviation. An ininoculated culture served as the control (closed triangles).

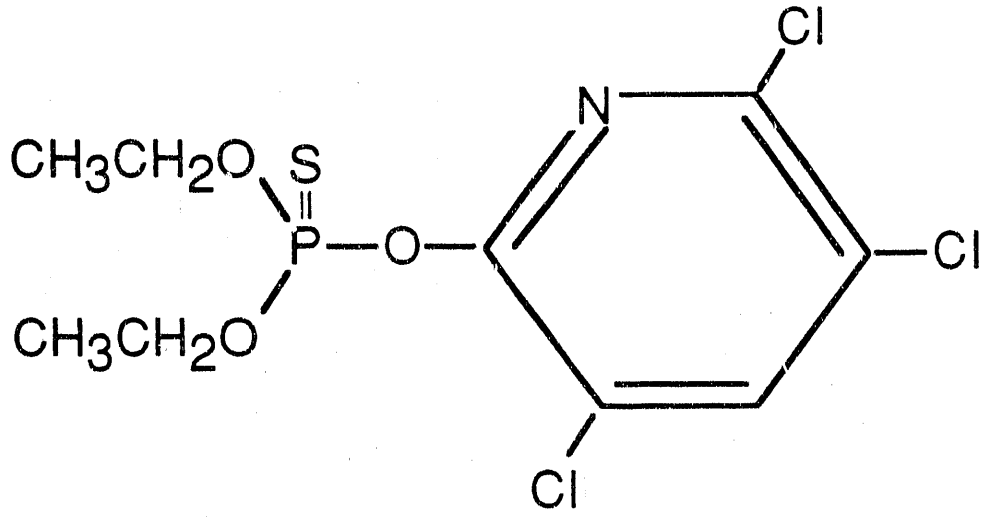
Figure 3. Mineralization of ^{14}C-fonofos by *P. chrysosporium*. The initial concentration of ^{14}C fonofos was 5.6 μM. Each point (open squares) represents the mean of five determinations ± the standard deviation. An ininoculated culture served as the control (closed triangles).

Figure 4. Mineralization of ^{14}C-terbufos by *P. chrysosporium*. The initial concentration of ^{14}C terbufos was 1.4 μM. Each point (open squares) represents the mean of five determinations ± the standard deviation. An ininoculated culture served as the control (closed triangles).

Figure 5. TLC of ^{14}C-chlorpyrifos before incubation (top) and ^{14}C in methylene chloride extract of cultures of *P. chrysosporium* incubated with ^{14}C-chlorpyrifos for 18 days (bottom).

Figure 6. TLC of ^{14}C-fonofos before incubation (top) and ^{14}C in methylene chloride extract of cultures of *P. chrysosporium* incubated with ^{14}C-fonofos for 18 days (bottom).

Figure 7. TLC of ^{14}C-terbufos before incubation (top) and ^{14}C in methylene chloride extract of cultures of *P. chrysosporium* incubated with ^{14}C terbufos for 18 days (bottom).



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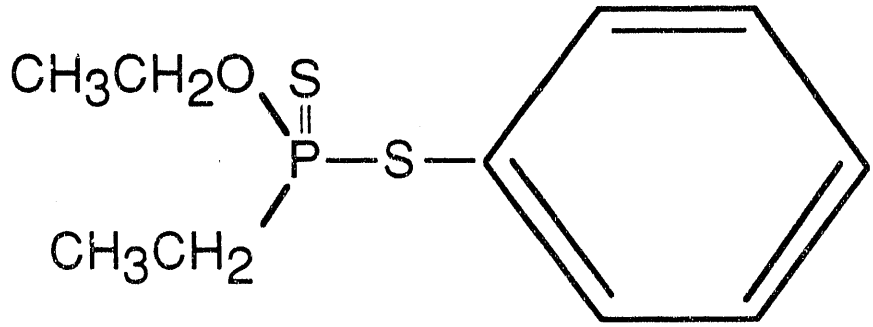

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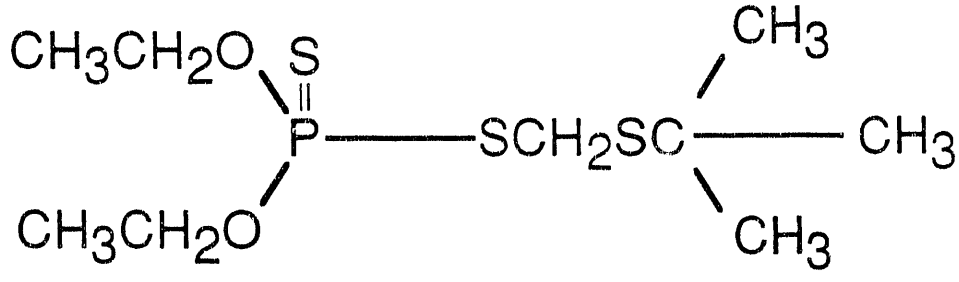

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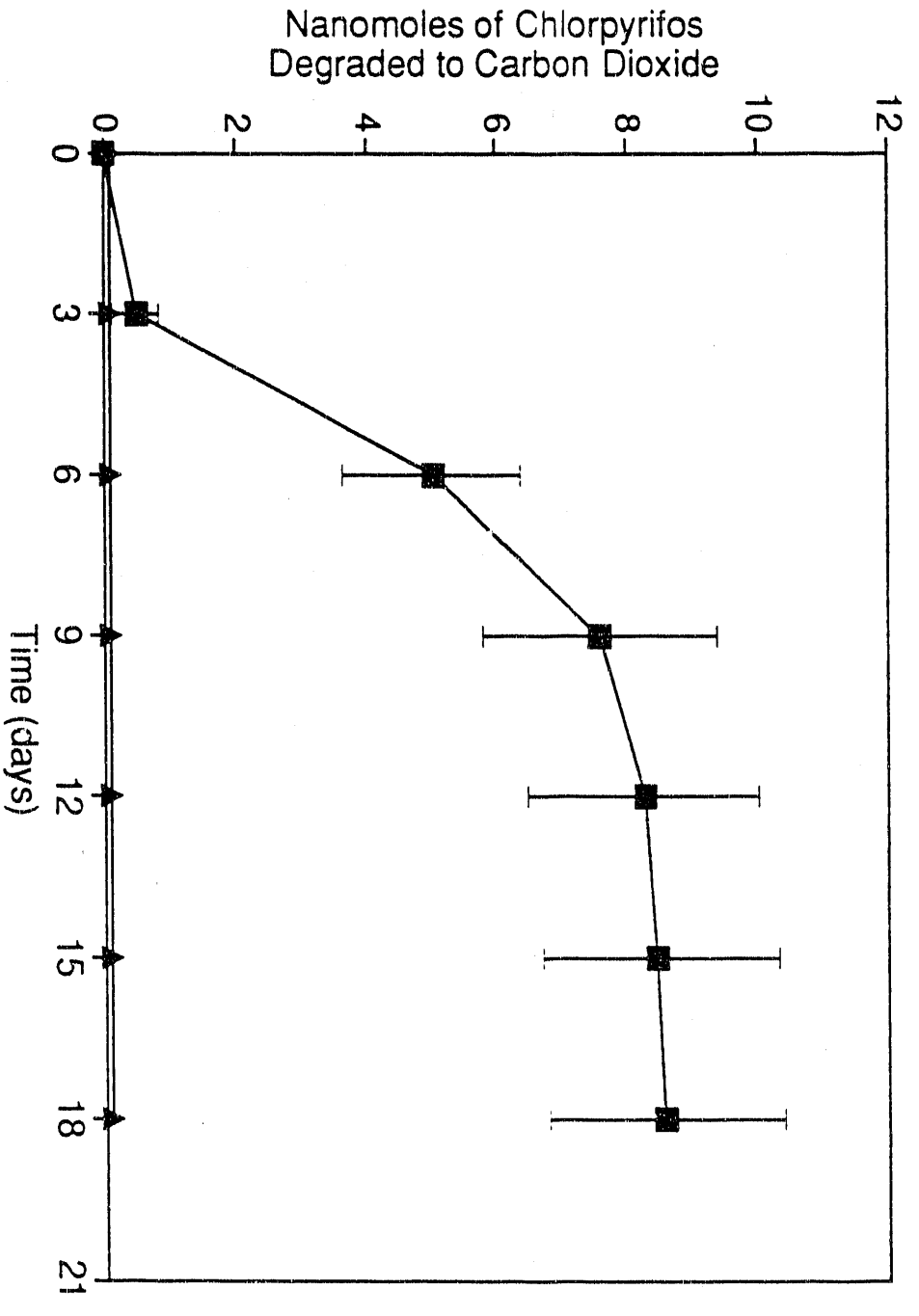
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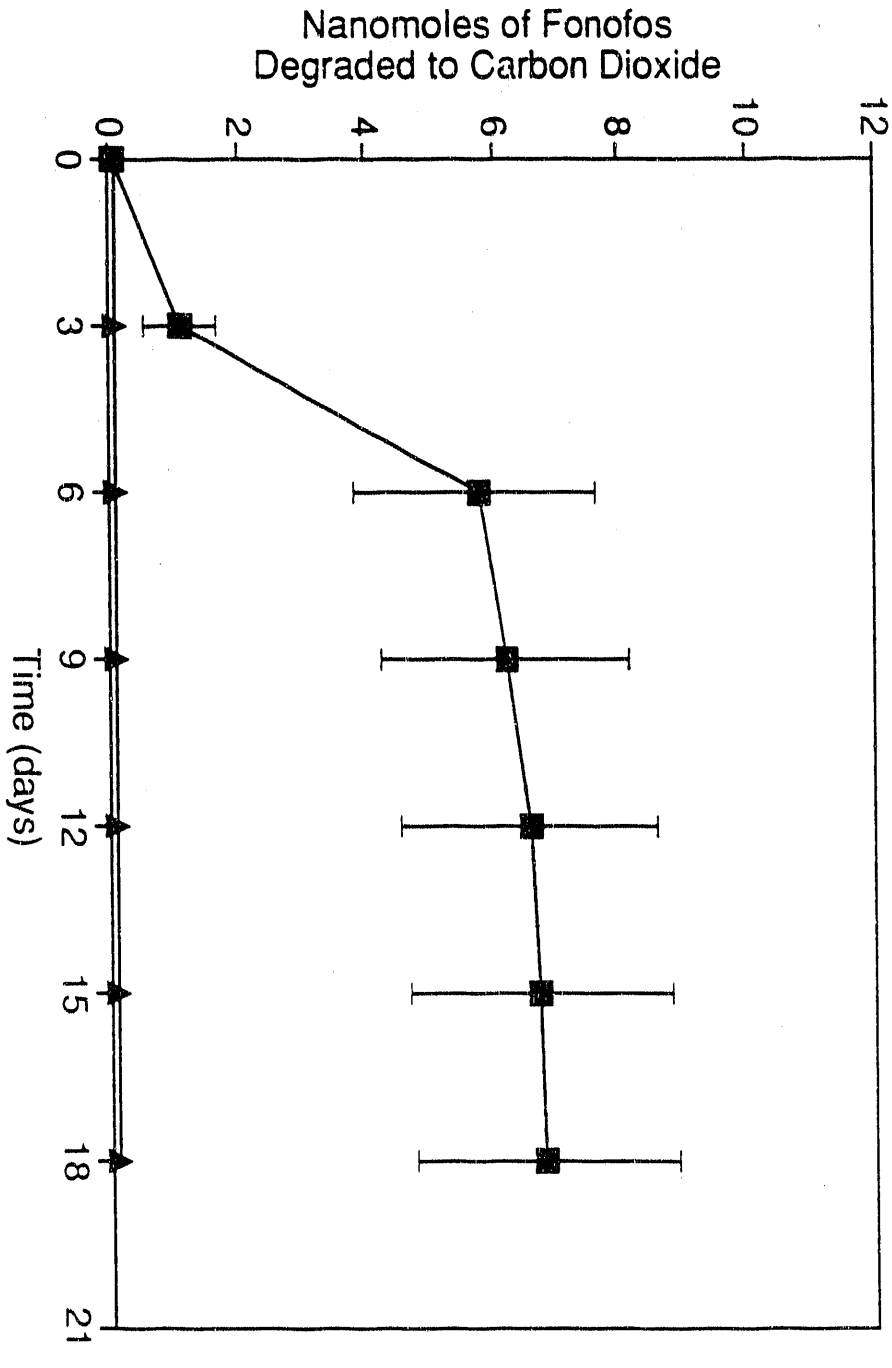
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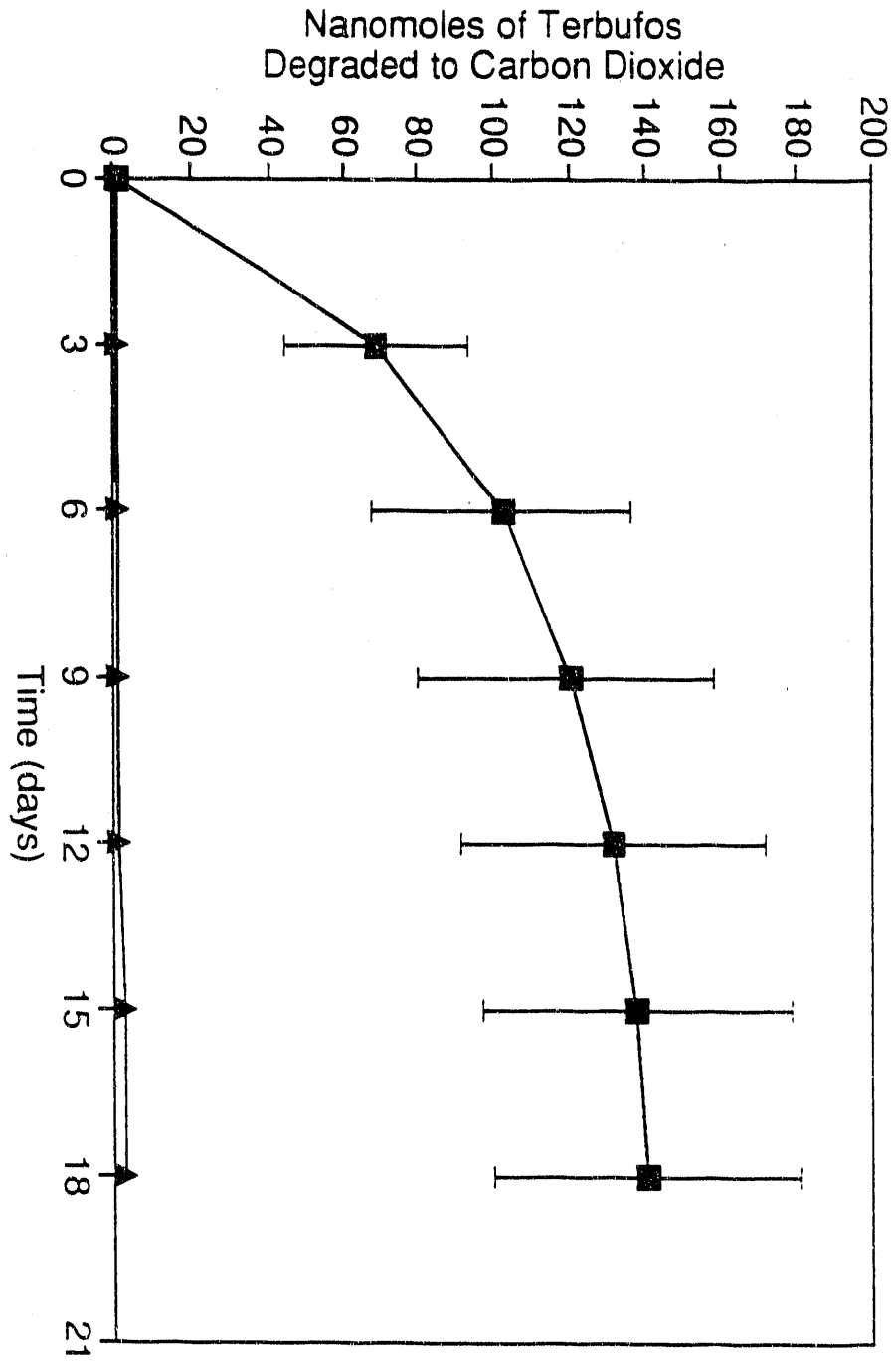
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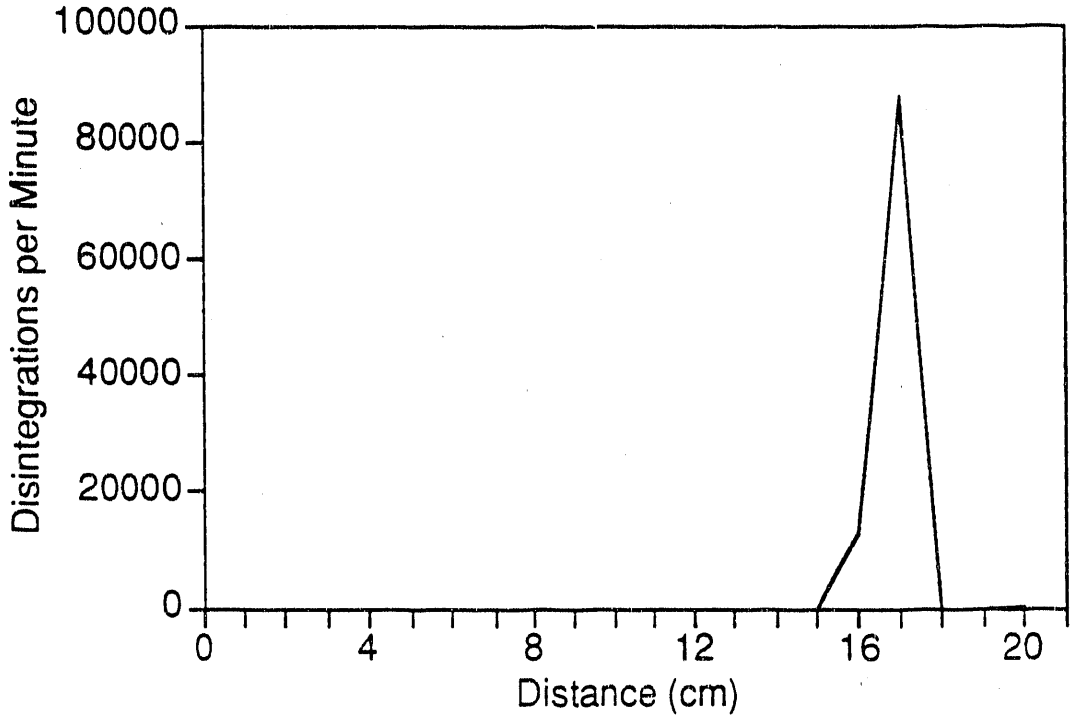
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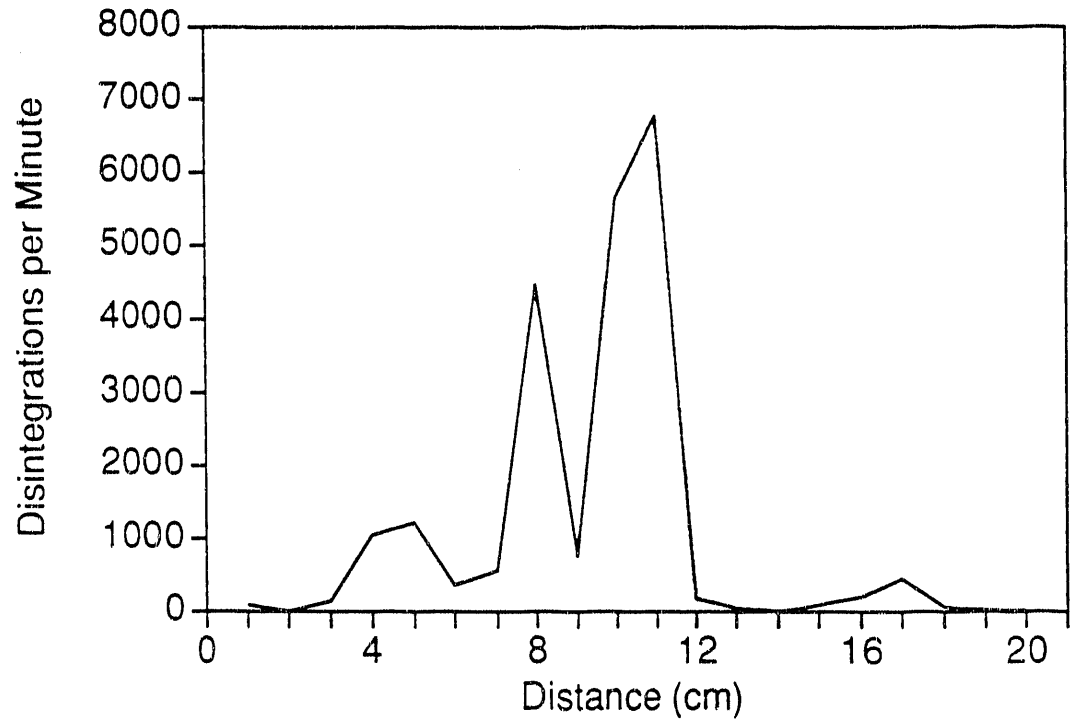
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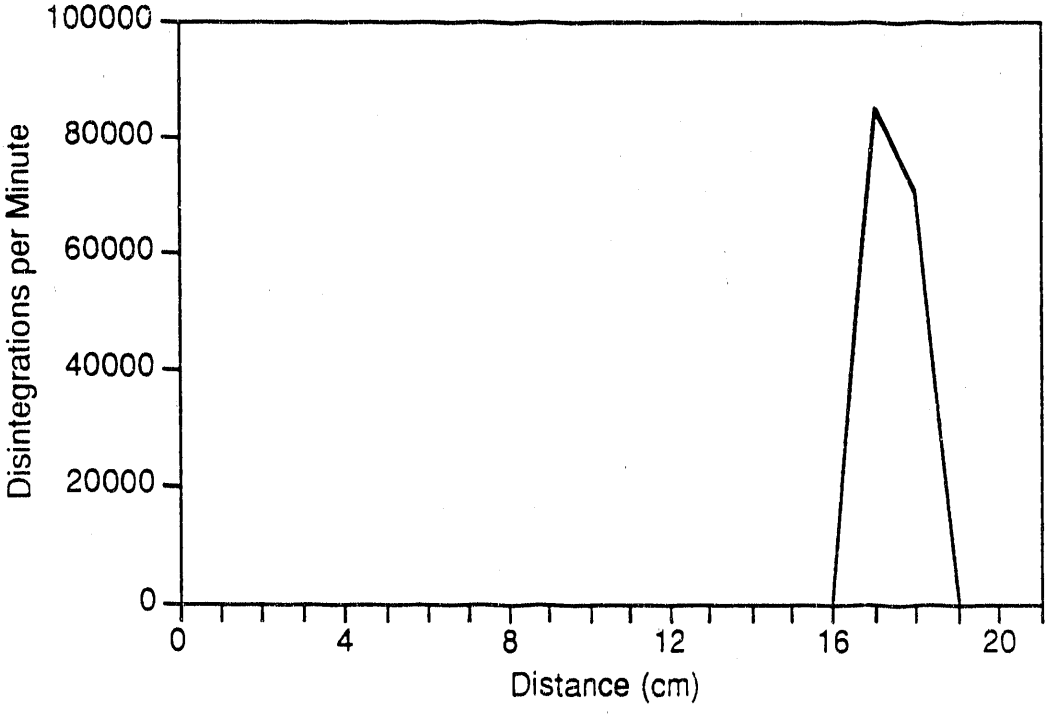
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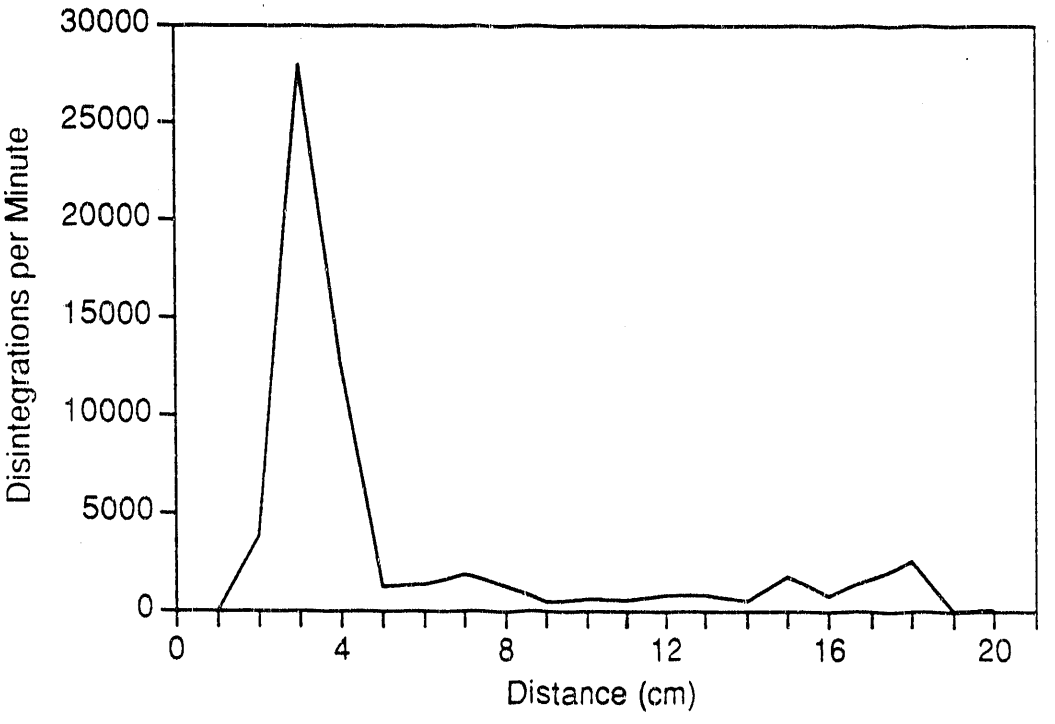
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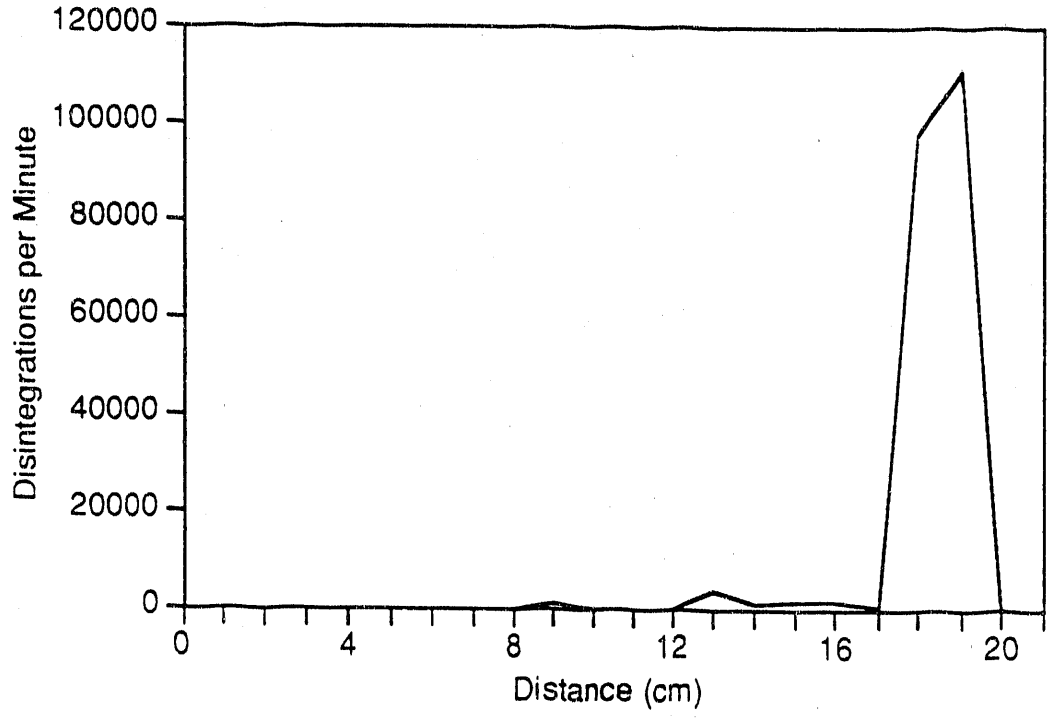
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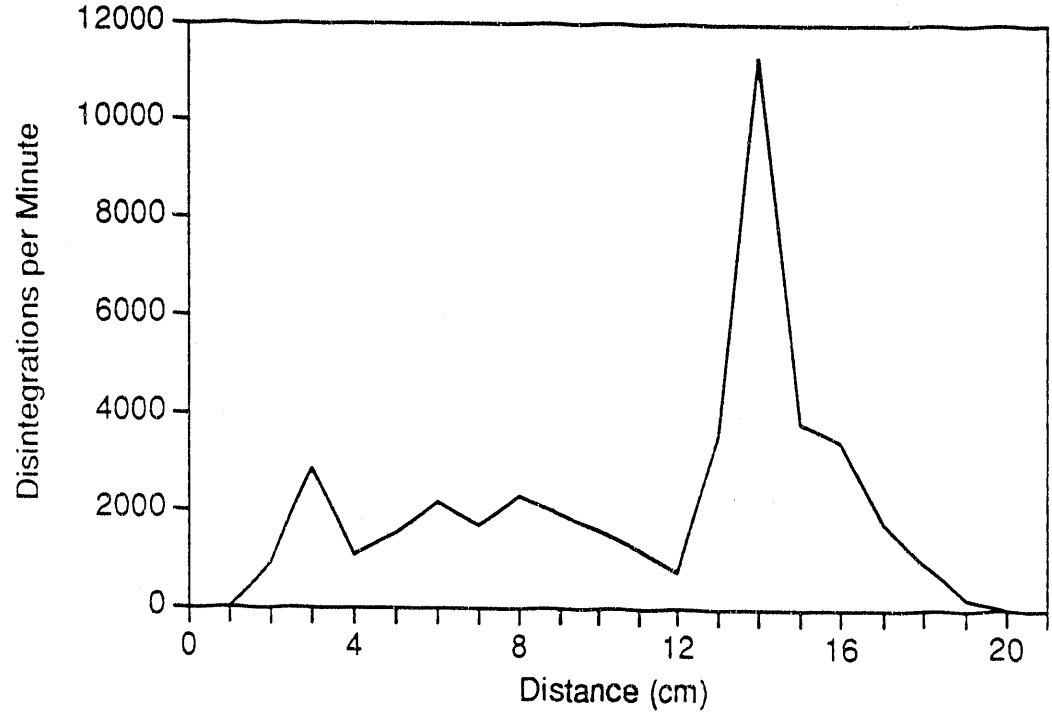
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