Biodegradation of Fenitrothion and Fenitrooxon by the Fungus *Trichoderma viride*

William H. Baarschers* and Holger S. Heitland

The fungus *Trichoderma viride* Persoon was found to effect the hydrolysis of fenitrothion (Sumithion) and fenitrooxon in pure culture. After incubation with the test compounds, liquid cultures were extracted with ethyl acetate and the extracts were analyzed by gas chromatography for residual test compounds and their hydrolysis product, 3-methyl-4-nitrophenol. A cleanup procedure, using Amberlyst A21 resin, was developed to facilitate the analysis. Evidence was found to suggest that this nitrophenol is cometabolized by the fungus.

INTRODUCTION

The environmental chemistry of fenitrothion (Sumithion, see Chart I) has been reviewed extensively (NRCC, 1975). In this review, and in a subsequent status report (NRCC, 1977) it is stated that 3-methyl-4-nitrophenol (nitrocresol) is a major breakdown product of this pesticide. However, chemical hydrolysis does not readily occur between pH 6.3 and 9.0 (Zitko and Cunningham, 1974), and hydrolysis in acidic medium gives primarily demethylfenitrothion (Aly and Badawy, 1982) or a mixture of demethylfenithrothion and the nitrocresol (Greenhalgh et al., 1980). Photochemical hydrolysis of fenitrothion was observed by Miyamoto (1977), and Weinberger et al. (1982) suggest that fenitrothion degradation in aquatic media appears to be predominantly photocatalyzed.

Enzymatic hydrolysis of other thiophosphate pesticides (e.g., parathion) has been reported (Munnecke and Hsieh, 1974; Baumgarten et al., 1982), but these studies involved mainly bacterial cultures. In fact, Hirakoso (1969) states that fungi or yeasts do not deactivate fenitrothion, and Miyamoto (1977) observed that bacteria and some fungi almost exclusively convert this pesticide to aminofenitrothion.

The present study provides direct evidence for the enzymatic hydrolysis of fenitrothion and fenitrooxon by *Trichoderma viride* Persoon. Indirect evidence was found to suggest that this fungus also cometabolizes the resulting 3-methyl-4-nitrophenol in the presence of other nutrients.

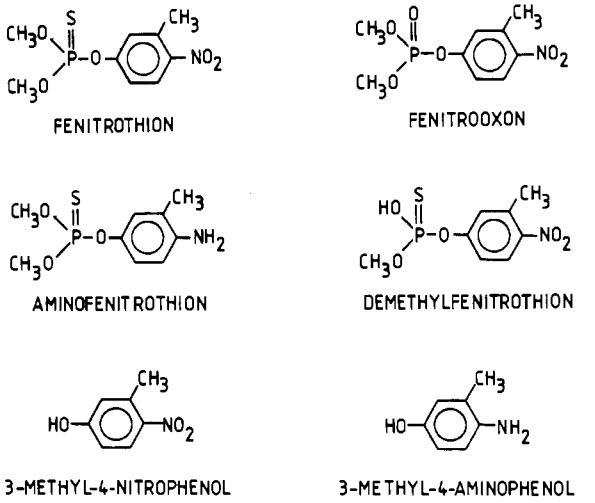
EXPERIMENTAL SECTION

Materials. Technical grade fenitrothion provided by Sumitomo Shoji, Canada, Ltd., was purified by column chromatography (silica gel, benzene). Fenitrooxon was prepared according to Greenhalgh and Marshall (1976). Purity of both compounds was confirmed by mass spectroscopy and proton magnetic resonance. Aminofenitrothion was prepared by zinc/hydrochloric acid reduction of fenitrothion (Sumitomo, 1977). 3-Methyl-4-aminophenol and 3-methyl-4-nitrophenol were obtained from Aldrich Chemical Co.; the former compound was used as supplied, and the latter was recrystallized (water) before use.

Culturing Methods. *T. viride* Persoon, from Dr. J. P. Kutney, Department of Chemistry, University of British Columbia, Vancouver, BC, was maintained on nutrient agar (dextrose 7 g, casaminoacids Difco 1 g, yeast extract 0.5 g, and agar 15 g/L of distilled water). Liquid cultures were grown in 500-mL Erlenmeyer flasks containing 200 mL of nutrient medium (dextrose 5 g, casaminoacids 1 g,

Department of Chemistry, Lakehead University, Thunder Bay, Ontario, Canada P7B 5E1.

Chart I



yeast extract 0.5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, vitamin stock 0.2 mL, minerals stock 2.5 mL/L of distilled, deionized water) on a rotary table shaker (12-cm stroke, 90 rpm) from 10 to 20 days and at a temperature from 20 to 23 °C. Liquid cultures were started, in triplicate, with standard size inocula (o.d. 9 mm) cut from previous agar plates. The growth of some blank cultures was monitored by the dry-weight method used previously (Baarschers et al., 1980). Maximum dry weight (500 mg/200 mL) was obtained after 3 days, followed by a gradual decrease in dry weight to approximately 350 mg/200 mL.

Addition and Extraction Methods. Pesticide (4 mg/200 mL, 20 ppm) or nitrocresol (1 mg/200 mL, 5 ppm) was added in acetone (0.5 mL) to liquid medium after autoclaving. Pure acetone (0.5 mL) was added to control cultures. After the desired growth/incubation time all cultures were acidified to pH 3 (0.5 M aqueous HCl) and extracted with ethyl acetate (3 × 50 mL), the combined extracts of each culture were dried (Na_2SO_4), and the solvent was evaporated on a rotary evaporator at room temperature. Resulting residues were transferred, either by direct means or after cleanup (see below), to 10-mL sample vials with a minimum of solvent, blown dry with nitrogen, and made up to volume with a solution of an internal standard in ethyl acetate for analysis.

Controls and Extraction Efficiencies. For hydrolysis studies the pH of liquid cultures of *T. viride* was measured at regular intervals by sterile withdrawal of 5-mL aliquots. The observed pH changes (i.e., decrease from starting pH 5.0 to a minimum pH 4.2 (day 6) followed by gradual increase to maximum pH 7.1) were used to adjust the pH in a set of control flasks (pH controls), containing only nutrient medium and pesticide, by sterile addition of acid

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Table I. Enzymatic Hydrolysis of Fenitrothion

expt no.	% recd (ee)[a]	mg hydrolyzed[b]	% hydrolyzed (ee)[c]	balance[d]
1	60.3 (80.0)[e]	0.83	10.4 (56.2)	29.0
2	54.4 (93.2)	0.54	6.7 (93.6)	38.9
3a	59.1 (85.0)	0.64	8.0 (75.7)	32.9
3b	85.0 (85.0)	0.74	9.3 (75.7)	5.7
4	46.4 (85.5)	0.94	11.6 (81.5)	42.0

[a-e] See notes with Table II.

Table II. Enzymatic Hydrolysis of Fenitrooxon

expt no.	% recd (ee)[a]	mg hydrolyzed[b]	% hydrolyzed (ee)[c]	balance[d]
5a	29.9 (82.6)[e]			
5b	61.0 (82.6)			
6a	43.1 (84.8)			
6b	83.0 (84.8)			
7a	26.3 (86.6)			
7b	57.7 (86.6)			
8	33.1 (96.5)	2.26	28.3 (80.3)	33.7
9	47.9 (84.0)	2.05	25.6 (80.3)	20.2
10	29.0 (89.0)	2.46	30.8 (81)	37.2

[a] Percentage fenitrothion/oxon recovered based on extraction efficiency (ee, as percent recovery from controls). [b] Milligrams of fenitrothion/oxon hydrolzyed per gram of dry weight mycelium. [c] Percent fenitrothion/oxon hydrolyzed (ee extraction efficiency of nitrocresol as percent recovered from controls). Both b and c as calculated from amount of nitrocresol found. [d] Percent fenitrothion/oxon unaccounted for. [e] All values result from triplicate cultures and analyses. [f] Nitrocresol not measured.

or base. Also for each experiment two sets of triplicate cultures were grown without pesticide. To one of these sets (extraction efficiency controls) pesticide was added at the end of the incubation period, and the mixtures were allowed to equilibrate on the shaker for 2 h. Extraction and analysis of this set allowed for assessment of the recovery efficiency of each compound. The final set of cultures (blanks) was extracted and analyzed so as to allow interpretation of all gas chromatograms in terms of pesticide breakdown products and/or natural metabolites of the fungus.

Cleanup Procedures. Amberlyst A-21 ion-exchange resin (free base form) was treated with methanol (100 mL/25 mL of resin, 2 bed volumes/h) and then with ethyl acetate in the same way. Treated resin, stored in ethyl acetate, was used to clean up some extracts containing the nitrocresol. Dry extracts (see above) in ethyl acetate (0.5 mL) were passed through resin (1.5 mL) in a glass column (12 mm i.d.), followed by a further 3.5 mL of the same solvent. The combined eluate of each extract was evaporated to dryness and analyzed for nonacidic material (e.g., fenitrothion). The resin was then washed with methanolic HCl (1.5 M, 6 mL) to recover the nitrocresol; the acidic eluents were blown dry (nitrogen) and prepared for gas chromatography as above.

Gas Chromatography. A Perkin-Elmer Model 3920 B gas chromatograph was used with a mixture of OV-17 and OV-101 (3% each) on Gas Chrom Q (100–120 mesh) in glass columns (1.80 m × 2 mm i.d.) and flame ionization detectors. Nitrogen was the carrier gas (30 mL min^{-1}) at isothermal column temperatures from 190 to 220 °C (injection and detector temperature; column temperature −15 °C). Dibutyl sebacate was used as internal standard for analysis of fenitrothion and fenitrooxon, and diethyl sebacate was the standard for the nitrocresol. For each experiment, solutions of the internal standard containing known amounts of the test compound were analyzed for the preparation of calibration curves (peak height ratio vs. concentration), which then allowed for the quantitative analysis of all extracts. For some analyses of 3-methyl-4-nitrophenol, standard solutions and extracts were silylated (Tri-Sil Z, Pierce) prior to addition of the internal standard solution.

RESULTS AND DISCUSSION

Preliminary experiments indicated the formation of 3-methyl-4-nitrophenol in extracts of pure cultures of *T. viride*, *Mortierella isabellina*, and *Saprolegnia parasitica*, which had grown in the presence of fenitrothion. *T. viride* was found to be the most effective in the formation of this hydrolysis product and was therefore chosen for the experiments reported here.

The results of monitoring of pH changes in *T. viride* cultures containing either fenitrothion or fenitrooxon were used to prepare a set of pH controls for each experiment. Recovery of test compounds from these pH controls was 90% for fenitrothion (i.e., the same as extraction efficiency controls, Table I) and 80% for fenitrooxon (slightly lower than extraction efficiency, Table II). This confirms that hydrolysis of the test compounds was not pH mediated and did not result from interaction with the nutrient medium.

The results of several hydrolysis experiments with fenitrothion and fenitrooxon are summarized in Tables I and II, respectively. The amounts of test compounds added (20 ppm) have no other significance than to ensure the presence of an excess of these compounds in the cultures at all times, while at the same time keeping toxic effects (Baarschers et al., 1980) to a minimum. Thus, in order to assess the extent of hydrolysis, the amount of test compound hydrolyzed (calculated from the amount of nitrocresol formed) is expressed per unit weight of biomass (columns 3 of Tables I and II).

Expressing the extent of hydrolysis of fenitrothion as a percentage of the test compound added (column 4, Table I) together with recoveries of unchanged starting material revealed a significant discrepancy in the material balance. In experiments 1, 2, 3a, and 4 an average 36% of fenitrothion added is unaccounted for. However, addition of the pesticide to 7-day-old cultures (experiment 3b) gave approximately the same amount of nitrocresol after a further 10-days incubation (i.e., 0.74 mg of fenitrothion hydrolyzed/1 g of mycelium) but led to a much higher recovery of starting material, suggesting that other factors affect the fate of the pesticide during the first few days. The observation of trace amounts of fenitrooxon in the extracts resulting from experiments 1, 2, 3a, and 4 may mean that an oxidative process is operative, in contrast with the usual reduction to aminofenitrothion found with bacterial cultures (Miyamoto, 1977). The oxon is not expected to accumulate in these cultures if its ease of enzymatic hydrolysis parallels its fast chemical hydrolysis as compared to fenitrothion (Zitko and Cunningham, 1974).

Initial attempts to carry out similar delayed addition experiments with fenitrooxon were partly unsuccessful. It appeared that the presence of the oxon, which is substantially less toxic to *T. viride* than fenitrothion at 20 ppm (Baarschers et al., 1980), stimulates the production of a natural metabolite of the fungus, observed in trace amounts in blank cultures, with the same retention time as the nitrocresol. Although nitrocresol recoveries were therefore not determined in experiments 5a, 6a, and 7a (Table II), delayed addition of fenitrooxon did again result in considerably higher recoveries of starting material (experiments 5b, 6b, and 7b). Development of a cleanup method (see the Experimental Section) allowed for nitrocresol analysis (experiments 8–10), which confirmed that enzymatic hydrolysis of fenitrooxon by this fungus is indeed more effective than that of fenitrothion, although the

Table III. Nitrocresol Recoveries from *T. viride* Cultures

	% nitrocresol recd:[a] addn at day				
expt no.	0	3	5	7	14 (ee)
11	55.3[b]	57.0	70.3		102.6[c]
12	63.8	63.3	74.6	77.2	99.0

[a] No cleanup of extracts; nitrocresol silylated for analysis. [b] All values result from triplicate cultures and analyses. [c] After 2 h equilibration, represents extraction efficiencies.

difference between the two compounds was nowhere near the several orders of magnitude difference observed (Zitko and Cunningham, 1974) with alkaline hydrolysis.

The above observations would account for the hypothesis that (i) enzymatic hydrolysis of the test compounds proceeds throughout the incubation period and (ii) during the period of growth (i.e., days 1–4) the test compounds and/or the nitrocresol formed are cometabolized by the fungus.

Rather than carrying out further delayed addition experiments with the oxon, it was decided to incubate *T. viride* cultures directly with 3-methyl-4-nitrophenol. Addition of this compound at day zero (i.e., with inoculation) and at days 3, 5, and 7 gave results as summarized in Table III. It is clear that a substantial portion of the nitrocresol "disappears" during the first few days and that progressively higher nitrocresol recoveries are obtained when this compound is added after day 4. This suggests that the missing material in experiments 1, 2, 3a, and 4 is primarily due to cometabolism of the nitrophenol after hydrolysis rather than to direct consumption of fenitrothion. The complete absence of any other known metabolites or other unidentified compounds in the extracts of experiments 11 and 12 is a strong indication that a substantial portion of the nitrocresol is consumed by cometabolism.

These results complement work done on malathion and parathion as reviewed extensively by Mulla et al. (1981). These authors cite several reports of microorganisms, primarily bacteria, utilizing these thiophosphate pesticides as a sole course of carbon, phosphorus, and nitrogen. The present results obtained with fenitrothion and a fungus, and indicating hydrolysis as well as cometabolism in the presence of abundant alternate nutrients, would appear to be more relevant from an environmental point of view.

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Photooxidation of Thioether Pesticides on Soil Surfaces

Kirk Gohre and Glenn C. Miller*[1]

Four thioether-containing pesticides undergo photosensitized transformation to the corresponding sulfoxides when sorbed on soil surfaces and exposed to sunlight. The pesticides examined were disulfoton, methiocarb, butocarboxim, and fenthion. Disulfoton underwent the most rapid loss on the three soils examined, and half of the originally applied pesticide was degraded in 1–4 days. Methiocarb generally degraded the most slowly. The probable oxidant forming the sulfoxides is singlet oxygen, although other sensitized oxidation pathways are also possible. The production of the sulfoxides was demonstrated not to be due to metabolic processes. The lowest organic content soil (0.79%) gave the most rapid degradation rates. The rates of conversion of these compounds to the sulfoxides were sufficiently rapid that photooxidation is likely an important transformation pathway of these compounds on soil surfaces.

INTRODUCTION

The production of singlet oxygen ($^1O_2$) on irradiated soil surfaces using chemical traps has previously been demonstrated (Gohre and Miller, 1983). The rapid degradation of the parent chemicals and generation of singlet oxygen products suggest that singlet oxygen may be a important oxidant on irradiated soil surfaces and may play a significant role in the transformation of electron-rich xenobiotics exposed to sunlight on the surface of soils.

Many sulfur-containing pesticides in use today have thioether or thioketone groups susceptible to singlet oxygen oxidation (Tamagaki and Hotta, 1980; Arjunan et al., 1984; Liang et al., 1983). Studies have examined the photochemistry of thioether-containing pesticides on silica gel and in aqueous solution, organic solution, plant, and glass

Department of Biochemistry, University of Nevada, Reno, Nevada 89557.
[1] Present address: Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720.

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