THE BIODEGRADATION OF FENITROTHION AND FENITROOXON BY THE SOIL FUNGUS *Trichoderma viride*

BY

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A THESIS SUBMITTED TO THE DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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ABSTRACT

The biodegradation of fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)-phosphorothioate and fenitrooxon (O,O-dimethyl-O-(3-methyl-4-nitrophenyl)phosphate by the soil fungus *Trichoderma viride* Pers. has been investigated.

Test compounds were incubated with pure cultures of the fungus. The liquid cultures were extracted with ethyl acetate and the residues analysed by gas chromatography. Fenitrothion, fenitrooxon and their hydrolysis product, 3-methyl-4-nitrophenol, were identified in the fenitrothion degradation experiments, while only the hydrolysis product and unreacted starting material was recovered from fenitrooxon test cultures. A clean-up procedure involving ion-exchange chromatography using Amberlyst A-21 resin was developed to facilitate residue analysis.

Evidence indicated that the fungal degradation of fenitrothion involves both oxidative and hydrolytic pathways. Furthermore, the evidence suggested that the 3-methyl-4-nitrophenol hydrolysis product is cometabolised by the fungus.

ACKNOWLEDGEMENTS

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Finally, I thank my wife, Marlene, for her encouragement and understanding during the course of this study.

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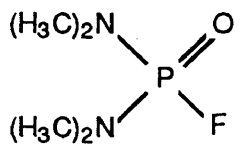
CHAPTER 1 - INTRODUCTION

1.1 HISTORY

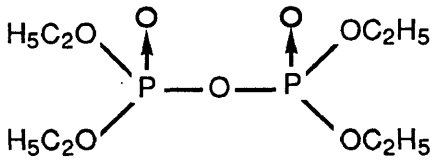
Of present day pesticides, organophosphorus compounds are one of the most important classes, approaching the scale of use of organochlorine compounds. Organophosphorus pesticides not only include insecticides, but have also found use as acaricides, nematicides, herbicides, fungicides and defoliants [1] (references, page 74).

In 1932, Lange and Krueger [2] first observed the abnormal physiological effects of organophosphorus compounds in dialkyl phosphorofluoridates while attempting to find new types of organic pesticides. Rapid progress was made when serious investigations were undertaken into the synthesis of toxic organophosphorus compounds for use as potential nerve gases during the second world war. As a result, Saunders and his colleagues in England developed tetramethylphosphorodiamidic fluoride or Dimefox (I)(structures, **Figure 1-1**, page 2) [3] while Schraders' work in Germany with organophosphorus esters led to the marketing of the first practical insecticide, Bladan, which contained tetraethylpyrophosphate (TEPP) (II), in 1944 [4].

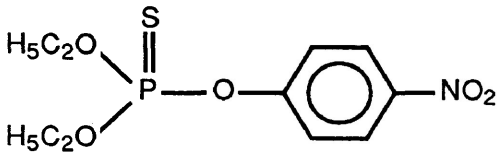
Schraders' subsequent discovery of Parathion (III), O,O-diethyl,O-*p*-nitrophenyl-phosphorothioate, also in 1944, resulted in "great advances in the agricultural practice and scientific knowledge on structure-activity relationships of organophosphorus insecticides" [4]. Parathion is a powerful insecticide with moderate persistency, some systemic activity, and very high mammalian toxicity: LD_{50} (orally in rats) = 6 mg/kg [5]. Less toxic insecticides have been discovered as a result of relatively minor structural modifications of parathion; for example, Chlorthion (1952) (IV), Fenthion (1958) (V), and Sumithion (fenitrothion) (1959) (VI).



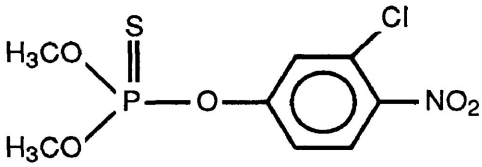
I Dimefox



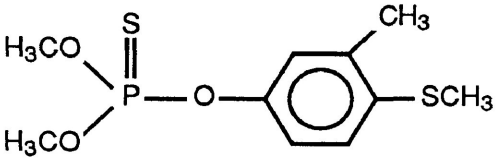
II TEPP (Bladan)



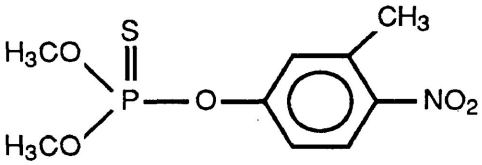
III Parathion



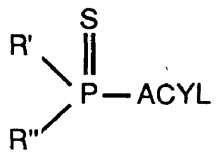
IV Chlorthion



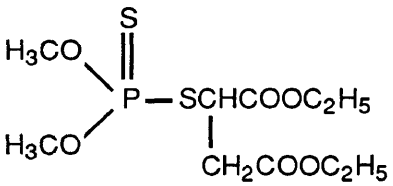
V Fenthion



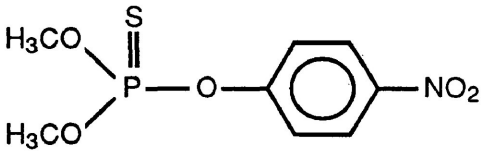
VI Sumithion (Fenitrothion)



VII Schrader proposed structure

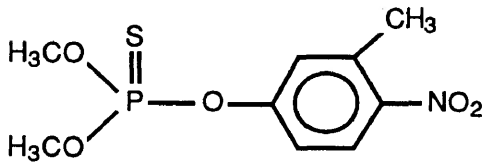


VIII Malathion

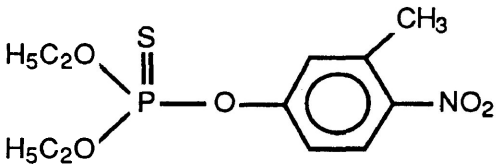


IX Methylparathion

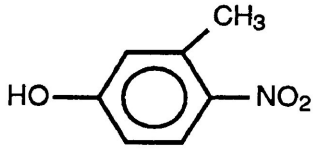
FIGURE 1-1



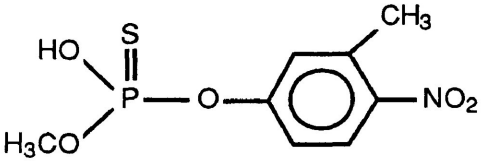
VI fenitrothion



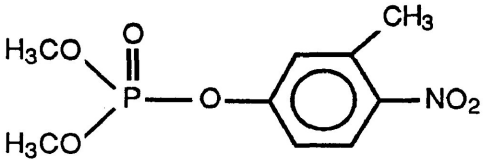
X diethoxyfenitrothion



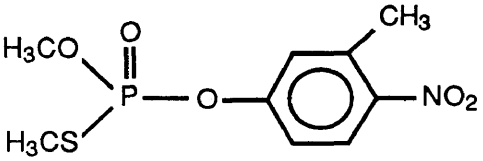
XI 3-methyl-4-nitrophenol



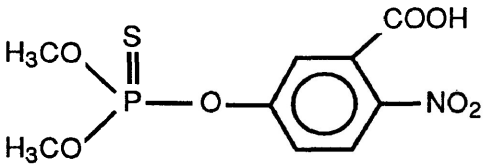
XII demethylfenitrothion



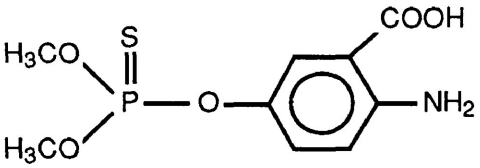
XIII fenitrooxon



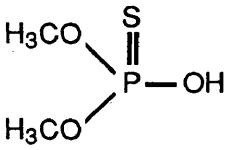
XIV S-methylfenitrothion



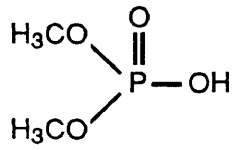
XV carboxyfenitrothion



XVI carboxyamino-fenitrothion

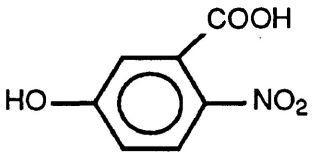


XVII dimethyl-phosphorothioic acid

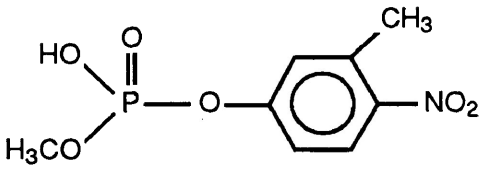


XVIII dimethyl-phosphoric acid

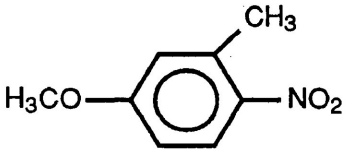
FIGURE 1-1 (continued)



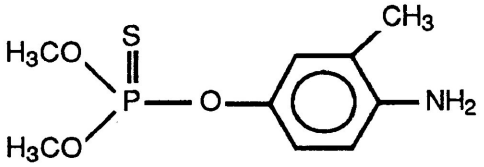
XIX 3-carboxy-4-nitro-phenol



XX demethylfenitrooxon



XXI 3-methyl-4-nitro-anisole



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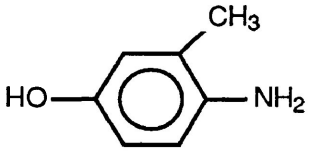
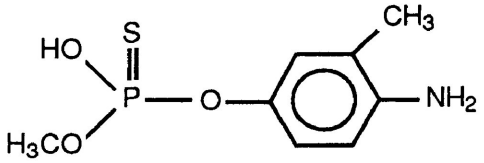


XXIII 4-amino-3-methyl-phenol



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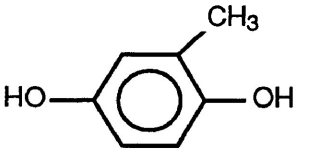
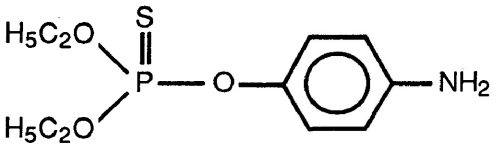
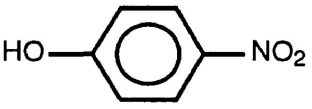


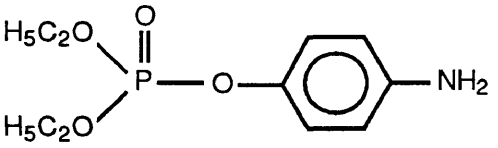
XXV 2-methylhydro-quinone



XXVI aminoparathion

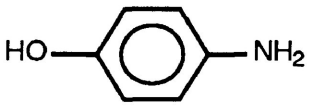


XXVII p-nitrophenol

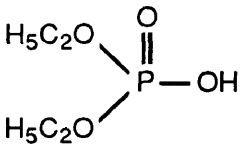


XXVIII paraoxon

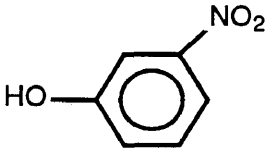
FIGURE 1-1 (continued)



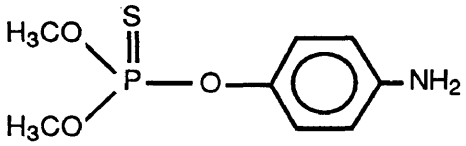
XXIX p-aminophenol



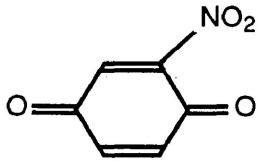
XXX O,O-diethyl-phosphorothioic acid



XXXII m-nitrophenol

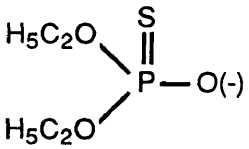


XXXI aminomethylparathion

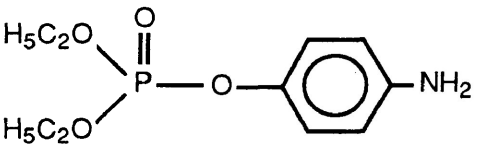


XXXIII nitroquinone

XXXIV hydroquinone

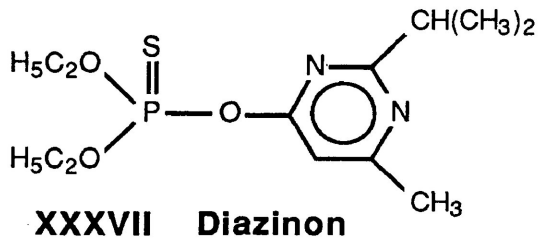


XXXV diethyl-thiophosphate

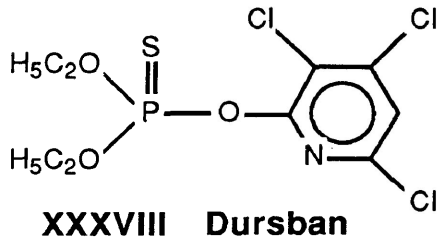


XXXVI aminoparaoxon

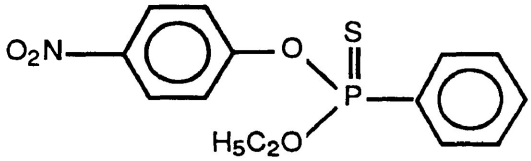
FIGURE 1-1 (continued)



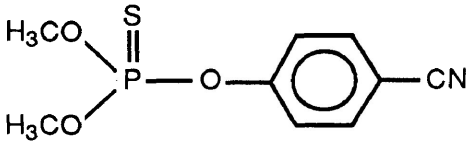
XXXVII Diazinon



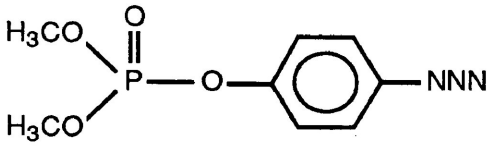
XXXVIII Dursban



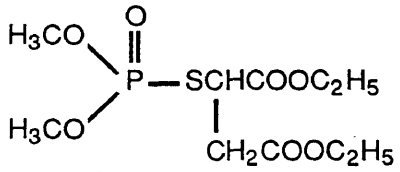
XXXIX EPN



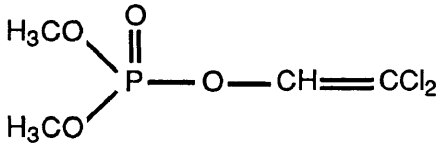
XL Cyanophos



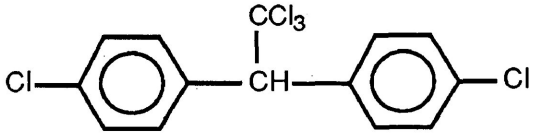
XLI Triazophos



XLII malaoxon



XLIV Dichlorvos



XLIII DDT

FIGURE 1-1 (continued)

These compounds are all derivative esters of thiophosphoric acid and have the general structural formula for physiologically-active organophosphorus compounds, as was first proposed by Schrader **(VII)** [6], where R' and R" are alkoxy, alkyl, or amino groups, and acyl is any acid residue. Malathion **(VIII)**, synthesized in 1950, is another important organophosphorus compound having low mammalian toxicity and contains a carboxylester grouping.

1.2 MODE OF TOXIC ACTION

The effectiveness of organophosphorus compounds as insecticides, as well as their vertebrate toxicity, results from inhibition of several enzymes, especially the hydrolytic enzyme of acetylcholine, acetylcholinesterase [7]. Lack of effective acetylcholinesterase activity results in the accumulation of acetylcholine, generated at nerve junctions, in the synaptic gap. This interferes with the transmission of nerve impulses and causes loss of muscular coordination, convulsions and ultimately, death. Since acetylcholinesterase is an essential enzyme in both insects and mammals, the basic mechanism of toxic action of the insecticide is thought to be similar in these organisms.

Acetylcholinesterase becomes deactivated because the organophosphorus compound is structurally similar to the natural substrate acetylcholine and binds to the ester-active site of the enzyme. The initially formed enzyme-phosphate complex results in the phosphorylation of the enzyme, in contrast to the acetylated enzyme produced from complexation of the enzyme and acetylcholine. Hydrolysis of the phosphorylated enzyme is much slower than that of the acetylated enzyme, a consequence of the relative strengths of the (substrate)-P--O-(enzyme) and (substrate)-C--O-(enzyme) bonds. In effect, the organophosphate poisons the enzyme by phosphorylation, thereby preventing

efficient hydrolysis of acetylcholine into choline.

As mentioned above, mammalian toxicities have been lowered through slight structural modifications of the more toxic parent homologue. For example, fenitrothion is about 50 times less toxic orally in mice than methyl parathion (**IX**), but has similar insecticidal activity. This has been accounted for by the introducton of the methyl group into the 3-postion of the phenyl ring resulting in lowered enzyme affinity toward phosphate substrate based on steric considerations [8].

The nature of the alkoxy substituents in O,O-dialkyl-O-phenyl phosphorothioates also plays a role in the degree of toxicity. Fenitrothion was found to have 1/40 the toxicity, when orally administered to mice, than the corresponding diethoxy compound (**X**). While enzyme specificity may be partially responsible for this observation, it appears likely that a glutathione S-alkyl transferase reaction is responsible for preferential degradation of the methyl esters before their full toxicity can be exerted in mammals. In comparison with the ethyl and isopropyl esters, mammalian transferase is highly specific toward methyl esters, degrades the methyl esters more rapidly, and results in lowered toxicity of the methyl analogues [9].

1.3 USAGE AND UPTAKE IN PLANTS AND ANIMALS

Both of the above structural modifications have been used to advantage in the development of fenitrothion (**VI**) from parathion (**III**), resulting in an organophosphorus pesticide that has retained the original high insecticidal capability (LD_{50} (house fly): parathion; 1.3 ug/g, fenitrothion; 2.6 ug/g) but has achieved greatly reduced mammalian toxicity (LD_{50} (rat): parathion; 15 mg/kg, fenitrothion; 740 mg/kg) [4]. These qualities, as well as rapidity of action and low residual activity, to be

discussed later (page 11), have led to the extensive use of fenitrothion in order to control grasshoppers, locusts, and caterpillars on pastures and defoliators in forests. It is also effective for the regulation of household and other pests, such as mosquitoes and their larvae, houseflies, poultry mites, cockroaches, lice and bedbugs **[10]**. In Canada, fenitrothion has been used since 1968 when it began to displace DDT **(XLIII)** in the operational control of lepidopterous defoliators in forests.

The present spruce budworm *(Choristoneura fumiferana)* infestation began in eastern Canada in the 1950s and was first treated with DDT in 1952. Although some protection was achieved against defoliation, the intensity and area of the infestation increased. As a result of increasing concern regarding the longterm bioactivity and persistence of DDT, its use in Canada was restricted in 1969 and instead fenitrothion was used in the 1970 budworm spray program. By 1976, 30 million hectare in New Brunswick, Quebec and Ontario, as well as smaller pockets in eastern Canada and Maine, USA, were severely infested **[11]**. While fenitrothion was effective in controlling defoliation, prolongation and increasingly rapid recurrence of severe budworm infestation almost certainly resulted from preservation of the food supply **[12]**. Although fenitrothion is not registered for agricultural use, the quantity sprayed in 1971 in New Brunswick exceeded 43% of the total major insecticide usage in agriculture in Canada **[11]**. Recently, budworm control programs have employed the biological insecticide Bt *(Bacillus thuringiensis)* partially a result of the concern surrounding the possible relationship between fenitrothion and Reye's syndrome. Fenitrothion, however, still finds extensive usage in Quebec and eastern Canada, and it is from this perspective that dispersal and persistence of fenitrothion in the environment is of interest.

Studies by Hurtig *et al.* **[13]**, of spray operations using application rates of 140 to 280 g/hectare, suggest that only 40% of the pesticide reaches the tree-top level. The other

60% must either remain in the atmosphere or settle in areas outside the target zone. After such a spray, Yule and Duffy **[14]** found the concentration of fenitrothion to be 0.04 ppm in the top 15 cm of forest soil while coniferous foliage absorbed 2-4 ppm. Spray cards placed near trees had coverage of 0.07-14 ug/sq cm **[15]**. Findlay *et al.* **[16]** placed cages of Japanese quail *(Coturnix coturnix japonica)* under trees during spray operations. Studies of the birds' crop concentrations led them to suggest that the major route of uptake into the quail body resulted from direct contact with the insecticide rather than through ingestion of poisoned insects.

Eidt and Sundaram **[17]** estimated a maximum concentration of 25 ppb in a forest stream 15 cm deep after spraying at 210 g/ha. Ingestion of contaminated insects appears to be a relatively important contributor toward the residue concentrations in fish. Rainbow trout *(Salmo gairdneri)*, which were held in cages that excluded food organisms, had average body concentrations of 0.4-0.6 ppm one day after spraying at 210 g/ha. Dace *(Chrosomus eos* and *C. neoganeus)* and brook stickleback *(Culaea inconstans)* however, were captured outside of cages and contained 4.8 ppm fenitrothion six hours after spraying and 13.7 ppm the next day **[18]**.

1.4 DEGRADATION

Rainfall after spraying does not result in the heavier fenitrothion loading of streams. This has been accounted for by the observation that fenitrothion is readily adsorbed by silty loams and in this state resists leaching by water **[19]**. Baarschers *et al.* **[20]** studied the soil adsorption characteristics of fenitrothion **(VI)** and its hydrolysis product, 3-methyl-4-nitrophenol **(XI)**, showing that the amounts of both compounds adsorbed are directly proportional to the organic matter content of the adsorbent. In

addition, these authors argued that these soil adsorbed compounds would not be very susceptible to photodegradation on the forest floor, and therefore it was essential that more attention be directed toward further studies on the microbial degradation of these compounds.

1.4.1 Chemical Degradation

Sundarham **[21]** studied the degradation of fenitrothion in aqueous systems and found the compound to be relatively stable to hydrolysis under acidic conditions, but hydrolysis increased rapidly with alkalinity. Hydroxide ion selectively attacks the phosphorus atom and displaces 3-methyl-4-nitrophenol **(XI)** anion resulting in cleavage of the P--O(aryl) bond and formation of phosphate diester **[4]**. Some discrepancy exists concerning the nature of pH 7 and lower hydrolysis products: Greenhalgh *et al.* [22] identified 3-methyl-4-nitrophenol **(XI)** and demethylfenitrothion **(XII)** mixtures while Aly and Badawy **[23]** found dealkylated parent material and methanol but no phenolic derivatives. The latter authors reported fenitrothion half-lives of 247.5, 86.1, and 4.3 days at pH 5, 7, and 9, respectively.

1.4.2 Biodegration - Plants and Animals

The fate of fenitrothion in aquatic microcosms, with respect to the role of aquatic plants, was investigated by Weinberger *et al.* **[24]**. They suggested that fenitrothion uptake and rapid degradation by aquatic macrophytes and microphytes was photocatalysed. In light, fenitrothion degradation products included fenitrooxon **(XIII)**, demethylfenitrothion **(XII)**, S-methylfenitrothion **(XIV)**, carboxy-

fenitrothion (**XV**) and carboxyaminofenitrothion (**XVI**), while in the dark only hydrolysis products were isolated. Greenhalgh and Marshall [**25**] irradiated oxygenated solutions of fenitrothion with UV light and confirmed that oxidation was the major photolytic degradation process of the parent compound in solution.

White pine *(Pinus strobus)* seeds, exposed to a solution of fenitrothion, were monitored for residues during early growth. Hallet *et al.* [**26**] found absorption and accumulation of fenitrothion, fenitrooxon (**XIII**) and S-methlyfenitrothion (**XIV**) in the perisperm and embryos. Moreover, the residues did not adversely affect later pine seedling growth. Metabolism of fenitrothion has also been observed in stored wheat. Abdel-Kader and Webster [**27**] identified demethylfenitrothion (**XII**), 3-methyl-4-nitrophenol (**XI**) and dimethyl- phosphorothioic acid (**XVII**) in wheat grain stored for 12 months after treatment with fenitrothion. Using ^{32}P-labelled fenitrothion, Miyamoto and Sato [**28**] showed that 50% of applied material penetrated into rice plant tissues within 24 hours and that it was rapidly decomposed, decreasing to less than 5% after one week. Fenitrooxon (**XIII**) was detected but disappeared more quickly than fenitrothion. Demethylfenitrothion (**XII**) and dimethylphosphorothioic acid (**XVII**) were also identified.

Fenitrothion is rapidly degraded and eliminated from mammalian systems. Hollingworth *et al.* [**29**] investigated the metabolism of ^{32}P-labelled fenitrothion in mice and identified several oxidation and hydrolysis products. More than 75% of material administered at dosages up to 200 mg/Kg, was recovered as metabolites in the urine within 24 hours; after 72 hours, recovery was at least 90%. The authors showed that both the P--O(alkyl) and P--O(aryl) bonds of fenitrothion, as well as those of fenitrooxon (**XIII**), are readily broken. Metabolites resulting from oxidation of the ring methyl group, hydroxylation of the ring, or reduction of the nitro group were not

isolated and it was concluded that these reactions are not important in the detoxification of fenitrothion. Guinea pigs and rats, intravenously injected with ^{32}P-labelled fenitrothion, quickly absorbed the material from the blood. Fenitrothion and fenitrooxon were identified in the tissues and decreased rapidly in concentration while demethylfenitrothion **(XII)**, dimethylphosphorothioic acid **(XVII)** and dimethylphosphoric acid **(XVIII)** were found in the liver and kidneys **[30]**. Metabolites are rapidly excreted as mainly 3-methyl-4-nitrophenol **(XI) [31]**, and also 3-carboxy-4-nitrophenol **(XIX) [32]**, demethylfenitrothion **(XII)** and demethylfenitrooxon **(XX) [29]**.

1.4.3 Biodegradation - Microorganisms

Numerous studies have been undertaken to determine the degradation dynamics of organophosphorus pesticides in soils containing mixed microbial populations. Degradation studies with sterile and non-sterile soil samples have established that microbial metabolism is largely reponsible for parathion inactivation in soil and aquatic environments **[33]**. These studies have involved comparison of degradation efficiency in natural soil and the same soils sterilised by a number of methods including autoclaving, gamma-irradiation, treatments with sterilants such as sodium azide, methyl bromide and detergents, as well as Millipore filtration. The evidence is such that persistence of parathion can be linked to decreased microbial activity in the sterilised soils. As stated earlier, microbial degradations of parathion tend to involve either hydrolysis or reduction. This appears to be the case in both pure and mixed cultures, although numerous parameters, such as soil pH, soil type, soil moisture, nutrient content and organic matter, may determine a particular degradation pathway.

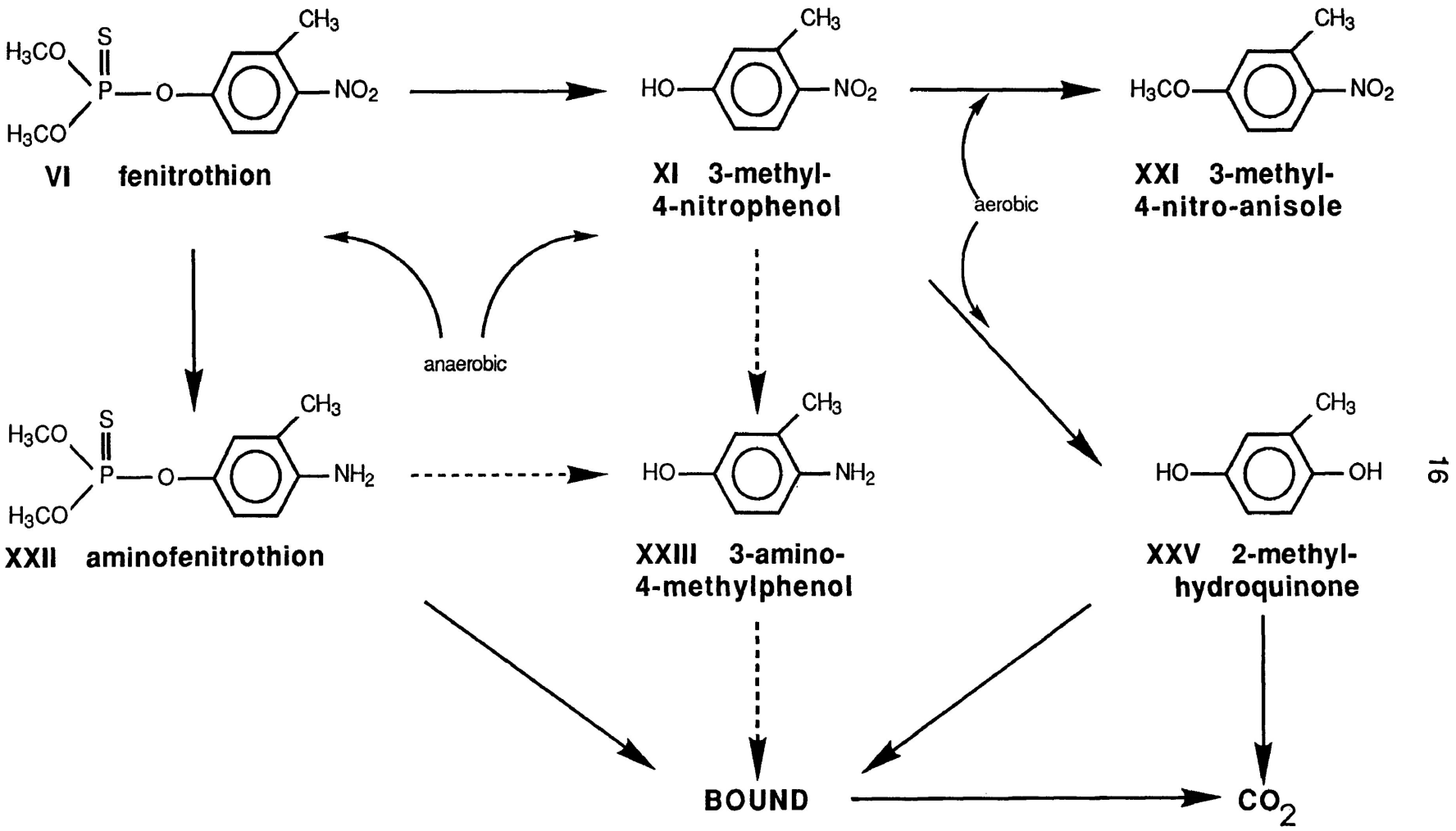
Gorder and Lichtenstein [34] investigated the degradation of ^{14}C-labelled parathion (III) assayed with microorganisms isolated from a cranberry bog and provided evidence that oxygen concentration and various carbon sources changed degradation pathways. After establishing that microbial activity was indeed responsible for parathion inactivation, the microorganisms were grown in a basal salts medium and shown to utilise parathion as the sole carbon source. 56.4% of applied (phenyl ^{14}C) parathion gave rise to ^{14}C carbon dioxide after 192 hours incubation while (ethyl ^{14}C) parathion produced only 0.2% radiocarbon as carbon dioxide. The indication here is that cranberry soil microorganisms metabolise the phenyl carbons of parathion and liberate carbon dioxide. No evidence was presented to show whether the carbon dioxide originates directly from parathion or from a cleavage product such as *p*-nitrophenol (**XXVII**).

A plot of the rate of ^{14}C carbon dioxide evolution against time was similar to that of a microbial growth curve. Addition of glucose to the medium depressed the formation of radioactive carbon dioxide, as did the amendment with yeast extract, although to a lesser extent. Aminoparathion (**XXVI**) and traces of aminoparaoxon (**XXXVI**) were detected in the yeast-fortified medium. Simulations of moist and flooded soil using this medium indicated parathion reduction to aminoparathion (**XXVI**) is greatly enhanced under anaerobic conditions relative to the aerobic situation. Furthermore, inocula isolated from 18-23 cm depth of soil exhibited greater reductive capability than microorganisms isolated from the soil surface (0-5 cm). In a later study, Lichtenstein *et al.* [35] showed that mixing of these soil layers contributed to an increase in parathion persistence, and also that the amino derivatives were strongly adsorbed by the soil. It was suggested that non-flooded sandy cranberry soil was more oxidatively active than loam soil, which was reductively more active, and that degradation pathway differences

were likely due to the physical properties of the soils and the differing populations of soil microorganisms.

Spillner *et al.* [36] considered the degradation of (phenyl ^{14}C) fenitrothion in two forest soils and its impact on the soil microbes. The results were similar for the two soils and indicated that 50% of the fenitrothion was deactivated within the first 3 days of incubation. This was accompanied by formation of 3-methyl-4-nitrophenol (**XI**) and the rapid evolution of ^{14}C carbon dioxide and the accumulation of soil-bound radiocarbon as terminal products. These findings are similar to those of Takimoto *et al.* [37] who followed the degradation of fenitrothion under aerobic conditions. Spillner *et al.* [36] identified 3-methyl-4-nitroanisole (**XXI**) as the only other metabolite and postulated that the primary degradation product 3-methyl-4-nitrophenol (**XI**) gave rise to carbon dioxide and soil-bound radiocarbon, as demonstrated by incubation of the phenol with soil: carbon dioxide arose from complete oxidative degradation of the phenyl ring, while the soil-bound radiocarbon appeared to be associated with the organic material of the soil through covalent bonding. The majority of the bound material was postulated as originating from 2-methylhydroquinone (**XXV**), an oxidative product of 3-methyl-4-nitrophenol (**XI**), which readily formed free radicals, subsequently polymerized, and became incorporated into the soil. Strongly soil-adsorbed radiocarbon was shown to be non-toxic to mosquito larva. **Scheme 1-1** is a proposed metabolism scheme for fenitrothion in forest soil, and describes both aerobic and anaerobic degradation pathways.

Spillner *et al.* [38] studied effects of fenitrothion on microorganisms in forest soils. Fenitrothion at 1 or 5 ppm did not significantly affect microbial respiration or microbial population . Higher doses of fenitrothion (274-640 ppm) also did not alter population numbers or respiration of soil microflora according to Salonius [39].



SCHEME 1-1 Proposed aerobic and anaerobic metabolism routes of fenitrothion in forest soils. Solid arrows denote major routes. After [36]

The interaction of some organophosphorus pesticides with pure cultures of algae, bacteria and fungi has also been studied. Kikuchi *et al.* **[40]** studied ^{14}C fenitrothion interactions with three species of algae. While the uptake of fenitrothion from solution was rapid, the concentration of adsorbed material decreased quickly when the algae were transferred to pesticide-free medium. *Ananbaena flos-aquae* most efficiently degraded fenitrothion to fenitrooxon **(XIII)**, demethylfenitrooxon **(XX)**, demethylfenitrothion **(XII)** and 3-methyl-4-nitrophenol **(XI)**. *Chlorella vulgaris* decomposed fenitrothion to chiefly demethylfenitrothion **(XX)** and 3-methyl-4-nitrophenol **(XI)**. Mackiewicz *et al.* **[41]** studied parathion **(III)** metabolites of *Chlorella pyrenoidosa* and found mainly aminoparathion **(XXVI)** and a trace of *p*-nitrophenol **(XXVII)**, but no paraoxon **(XXVIII)** or *p*-aminophenol **(XXIX)**.

Several species of bacteria have been shown to interact with organophosphorus pesticides. Mick and Dahm **[42]** incubated *Rhizobium japonicum* and *R. meliloti* with parathion **(III)** and found that 85% of the original parathion was reduced to aminoparathion **(XXVI)**. Hydrolysis of parathion to O,O-diethylphosphorothioic acid **(XXX)** accounted for 10% of the parent material, but no paraoxon **(XXVIII)** was identified. Reductive degradation of parathion was also found by Parasnis and Dhala **[43]**. The bacterium *Nocardia alba* degraded the pesticide to aminoparathion **(XXVI)**, but apparently no further degradation took place. Up to 61% of parathion was degraded after 8 days in shake cultures with traces of aminoparathion appearing within 24 hours of innoculation.

Bacillus subtilis effectively degrades a number of related organophosphorus pesticides as shown by Miyamoto *et al.* **[44]** and Yasuno *et al.* **[45]**. Here again, a reductive mechanism yielding aminoparathion **(XXVI)**, aminomethylparathion **(XXXI)** and aminofenitrothion **(XXII)** from parathion **(III)**, methylparathion **(IX)** and fenitrothion

(**VI**), repectively, was found. Miyamoto *et al.* [**44**] reported the isolation of demethylfenitrothion (**XII**), dimethylphosphorothioic acid (**XVII**) and demethylaminofenitrothion (**XXIV**) residues, in addition to the major metabolite aminofenitrothion (**XXII**) (65% of parent compound) when the bacterium was incubated with fenitrothion. No fenitrooxon (**XIII**) was detected. Methylparathion (**IX**) yielded the methylparathion analogues of the above compounds in a similar experiment, but was reduced more rapidly than fenitrothion.

Rapid hydrolysis of organophosphorus pesticides was found with two species of bacteria by Adhya *et al.* [**46**]. A *Flavobacterium* sp. was able to hydrolyse parathion (**III**) and Diazinon (**XXXVII**) and the dimethyl compounds methylparathion (**IX**) and fenitrothion (**VI**) while a *Pseudomonas* sp. only hydrolysed the diethyl compounds. The authors concluded that the phosphorus alkyl substituent was more important than the aromatic moiety in determining bacterial hydrolytic activity. These researchers also considered the fate of hydrolysis products. The *Pseudomonas* sp. efficiently degraded the *p*-nitrophenol (**XXVII**), but the nature of the end product depended on culture medium. In glucose amended medium, a reductive mechanism yielded *p*-aminophenol (**XXIX**), while nitrite was produced oxidatively in the absence of glucose.

In an earlier radiotracer study, Sudhakar-Barik and Sethunathan [**47**] showed that the same *Pseudomonas* sp. hydrolysed parathion (**III**) and that the subsequent degradation of *p*-nitrophenol (**XXVII**) to nitrite enhanced bacterial growth. Raymond and Alexander [**48**] isolated a *Flavobacterium* sp. and indicated that the microorganism used *p*-nitrophenol (**XXVII**) as a carbon source and produced nitrite stoichiometrically from the phenol. Cells acclimated to *p*-nitrophenol (**XXVII**) were shown to oxidise *m*-nitrophenol (**XXXII**) to nitroquinone (**XXXIII**) but were unable to utilise the *m*-nitrophenol as a carbon source for growth. *Pseudomonas aeruginosa* also able to

utilise *p*-nitrophenol as the sole carbon source as reported by Daughton and Hsieh **[49]**.

A major trend in microbial degradation research is the isolation of particularly efficient cultures and their subsequent use for detoxification of pesticide wastes and spills. Munnecke and Hsieh **[50]** studied the microbial degradation of parathion **(III)** and *p*-nitrophenol **(XXVII)** in aqueous media, by adapting a mixed culture to grow on parathion, in an attempt to use microbes to detoxify parathion in agricultural wastes. The culture degraded 50 mg of parathion per litre per hour in a chemostat. Furthermore, a pseudomonad, isolated from the mixed culture, showed optimal growth in 0.21 mM *p*-nitrophenol and grew in media containing up to 3.5 mM, metabolising the phenol to nitrite and hydroquinone **(XXXIV)**.

In later experiments the same authors **[49]** isolated *Pseudomonas stutzeri* which hydrolysed the parathion to ionic diethylthiophosphate **(XXXV)** and *p*-nitrophenol **(XXVII)**, and *P. aeurinosa* which was able to utilise the *p*-nitrophenol as a sole carbon source. Dissimilation rates of 8 g parathion per litre per day and 7 g p-nitrophenol per litre per day were attained. Methyl parathion **(IX)** was similarly detoxified.

More recently, attention has been focused on the isolation of enzyme preparations derived from efficient pesticide degrading organisms and the use of these preparations to decontaminate waste water and soil **[51]**. Munnecke **[52]** isolated an enzyme preparation from a mixed bacterial culture that utilised parathion as the sole carbon source. This enzyme hydrolysed parathion **(III)**, methyl parathion **(IX)**, paraoxon **(XXVIII)**, Diazinon **(XXXVII)**, Dursban **(XXXVIII)**, EPN **(XXXIX)**, Cyanophos **(XL)**, fenitrothion **(VI)**, and Triazophos **(XLI)** faster than the currently recommended chemical hydrolysis detoxification procedure with sodium hydroxide. Commercial formulations of parathion **(III)**, Dursban **(XXXVIII)**, Diazinon **(XXXVII)**, Cyanophos **(XL)**, and methyl parathion **(IX)** were enzymatically

hydrolysed to more than 95% in 16 hours when 13-16 mg of enzyme preparation per litre waste water was used. Some loss of enzyme activity resulted from the formulation chemicals.

Immobilizing the parathion hydrolase enzyme by binding to porous silica, to aid in its handling and recovery, was also investigated **[53]**. When waste water was passed through a fluidized-bed reactor at rates of 96 L hr^{-1} and parathion concentrations of 10-250 mg/L, 95% or more was hydrolysed, resulting in less than 500 ppb parathion in the effluent. The enzyme system was, however, susceptible to deactivation by several factors.

Reports of yeast and fungal degradations of organophosphorus pesticides are not nearly as prevalent as those involving bacteria. Lichtenstein and Schulz **[54]** found that a yeast degraded parathion to aminoparathion **(XXVI)**. Matsumura and Boush **[55]** reported that the fungus *Trichoderma viride* inactivated malathion **(VIII)** through hydrolysis and demethylation. The inability of the fungus to convert malathion to the oxon **(XLII)** led the authors to suggest that the microorganism lacked the required oxidative systems. Later the same authors **[56]** showed that *T. viride* also degraded Diazinon **(XXXVII)**, Dichlorvos **(XLIV)** and parathion **(III)**. Metabolites were not identified but only classified as water soluble or solvent soluble compounds. Studies with carbamate and chlorinated hydrocarbon pesticides assayed with *T. viride* led the researchers to suggest the presence of a powerful oxidative system, a result in direct contrast with their earlier study. Clearly, some ambiguity exists regarding the exact nature of the oxidative system in *T. viride*.

1.4.4 Purpose of the Present Study

The present study sets out to determine the degradation of fenitrothion and its metabolites by the soil fungus, *Trichoderma viride*, in liquid culture. Fenitrothion degradation pathways in fungi have not been extensively studied and therefore a contribution in this area will add to the assessment of the environmental impact of fenitrothion use.

CHAPTER 2 - CHEMISTRY

2.1 INTRODUCTION

As discussed earlier in the introductory chapter, the metabolic pathways of the fungal biodegradation of fenitrothion and other organophosphorus pesticides have not been broadly researched and consequently are not well understood. To advance the understanding in this area, two chemical aspects must be considered: the procurement of necessary materials and the development and use of analytical methods, both of which are discussed in this chapter.

2.2 PREPARATIVE CHEMISTRY

Microbial degradation studies of fenitrothion and related parathion have shown that the nature of metabolites formed is dependent on the degradative pathway employed by the microorganism. Such mechanisms may involve the operation of hydrolytic ([**41**], [**46**], [**47**]), oxidative ([**36**], [**40**]), or reductive ([**41**], [**42**], [**43**], [**44**], [**45**], [**54**], [**55**]) enzyme systems as discussed earlier. Consequently, any metabolic study must consider these possibilities and so the following potential metabolites were prepared, or commercially obtained and purified: fenitrothion (**VI**), fenitrooxon (**XIII**), 3-methyl-4-nitrophenol (**XI**), S-methylfenitrothion (**XIV**), aminofenitrothion (**XXII**), 4-amino-3-methylphenol (**XXIII**), and *p*-nitrophenol (**XXVII**).

The thin layer chromatography (TLC) analysis of technical grade fenitrothion showed that the preparation was not of sufficient purity, but contained traces of

3-methyl-4-nitrophenol (**XI**), S-methyl fenitrothion (**XIV**), and fenitrooxon (**XIII**). Previously in this laboratory, fenitrothion had been purified according to the method of Kovacicova *et al.* **[57]** involving counter-current steam distillation. A simpler method was sought and it became apparent that an effective purification could be accomplished by column chromatography.

Fenitrooxon (**XIII**) was synthesized by a method suggested through a personal communication (1977) with the Research Department, Pesticides Division, Sumitomo Chemical Co., Ltd., and involved the condensation of 3-methyl-4-nitrophenol (**XI**) and dimethylphosphite.

S-methylfenitrothion (**XIV**) was synthesized by a method suggested by Kovacicova *et al.* **[58]** which consisted of refluxing fenitrothion with potassium hydrogen sulphide and subsequent treatment of the intermediate phosphorothioic acid potassium salt with dimethyl sulphate.

Aminofenitrothion (**XXII**) was synthesized by a method suggested through personal communication (1977) with the Sumitomo Chemical Company, Ltd., and involved reduction of fenitrothion with zinc in hydrogen chloride solution.

3-Methyl-4-nitrophenol (**XI**) could be effectively purified by recrystallization from hot water. 4-Amino-3-methylphenol (**XXIII**) and *p*-nitrophenol (**XXVII**) are commercially available.

2.3 ANALYTICAL METHODS

2.3.1 Introduction

In order to evaluate fungal cultures for potential pesticide metabolites, analytical techniques are required to efficiently extract, clean-up, and quantify relevant materials contained in the complex culture medium. The general nature of such methods is similar for most studies of this kind as described in the literature, but such procedures are necessarily specific for a particular microorganism-test compound system because of the natural metabolites and growth conditions inherent in such a system. Procedures in the literature must therefore be modified and/or new methods must be developed. Consequently, the study of analytical techniques for the analysis of fenitrothion and its metabolites comprises a substantial portion of the present study.

2.3.2 Gas Chromatography

Preliminary gas chromatography (GC) investigations involved selection of a suitable column packing for resolution of fenitrothion and its hydrolysis product, 3-methyl-4-nitrophenol. Performance of 5% SE-30, 5% DEXSIL, and mixed phase 3% OV-101/3% OV-17 stationary phases on Gas Chrom Q (80-100 mesh) were evaluated. The best resolution was obtained using the OV-101/OV-17 system, so this packing was used throughout the entire project.

Consideration was also given to the nature of the solvent used for sample injecton. Ethyl acetate was selected from a number of organic solvents because of its reduced tailing, as well as the high solubility of the test compounds in this solvent. Carbon

disulphide was used occasionally for injection of samples, but, because of its low polarity, was limited to use with samples analysed only for fenitrothion or fenitrooxon.

Appropriate internal standards were selected from a series of aliphatic dicarboxylic acid esters. This permitted fine adjustment of relative retention times by either changing the length of the carbon chain of the acid or of the ester alkoxy moiety by one or two carbon atoms at a time. It was found that diethyl sebacate was a good reference for 3-methyl-4-nitrophenol while dibutyl sebacate could be used as the internal standard for fenitrothion.

The use of internal standards allows for quantitative determination of the compounds of interest by determining a peak height ratio through measurement of the peak heights of the test compound and the appropriate internal standard [59]. As peak width increases, the uncertainty in peak height also increases so that operating parameters must be adjusted to give the sharpest peak possible. Calibration curves may be constructed by plotting these peak height ratios against test compound concentrations. Good linearity was shown by fenitrothion calibration curves at concentrations ranging from 0-4 mg/mL.

A somewhat lower correlation coefficient was observed for 3-methyl-4-nitrophenol concentrations less than 1 mg/mL. This was attributable to tailing of the 3-methyl-4-nitrophenol peak causing peak broadening. At a 3-methyl-4-nitrophenol concentration of less than 0.2 mg/mL, peak height measurements were not a good indicator of concentration because of the unsymmetrical nature of the peak. In order to reduce tailing caused by the phenol, two methods of derivatisation were investigated. Methylation of 3-methyl-4-nitrophenol, using the reagent Methelute (trimethylanilinium hydroxide in methanol, Pierce Chem. Co.), increased peak symmetry and sharpness and caused shorter retention times. However, when a mixture of fenitrothion and 3-methyl-4-nitrophenol was methylated, the previously sharp fenitrothion peak

degenerated to a number of other peaks, possibly due to transesterifications caused by the methylating reagent. Clearly, methylation with Methelute is unacceptable as a method for the derivatisation of 3-methyl-4-nitrophenol in the presence of fenitrothion.

A significant decrease in the extent of phenol peak tailing at shorter retention time was also observed when the 3-methyl-4-nitrophenol was silylated using the reagent TRI-SIL-Z (N-trimethylsilylimidazole in pyridine, Pierce Chem. Co.). This derivatisation method also resulted in fenitrothion decomposition, so silylation was not an acceptable method either. In working with pure silylated 3-methyl-4-nitrophenol solutions, it was observed that the derivatised 3-methyl-4-nitrophenol hydrolysed after two days, reverting back to underivatised material. It is important, therefore, to analyse silylated phenol residues in one working session.

Good linearity was shown by calibration curves over the concentration range 0-1 mg/mL for derivatised 3-methyl-4-nitrophenol solutions. Since silylation and methylation were not possible without destruction of the parent compound, samples with low concentrations of 3-methyl-4-nitrophenol, which would result in less than adequate calibration curves, were made up to smaller volumes prior to analysis which increased the effective concentration. This occasionally required that instrument sensitivity (attenuation) be changed during a chromatogram because of the disproportionate quantities of parent and hydrolysis compounds present in the extract. This procedure was found to adequately solve calibration problems at low 3-methyl-4-nitrophenol concentration.

Figures 2-1a and **2-1b** are typical chromatograms resulting from the analysis of 3-methyl-4-nitrophenol, in the presence of fenitrothion and fenitrooxon, respectively, by this method.

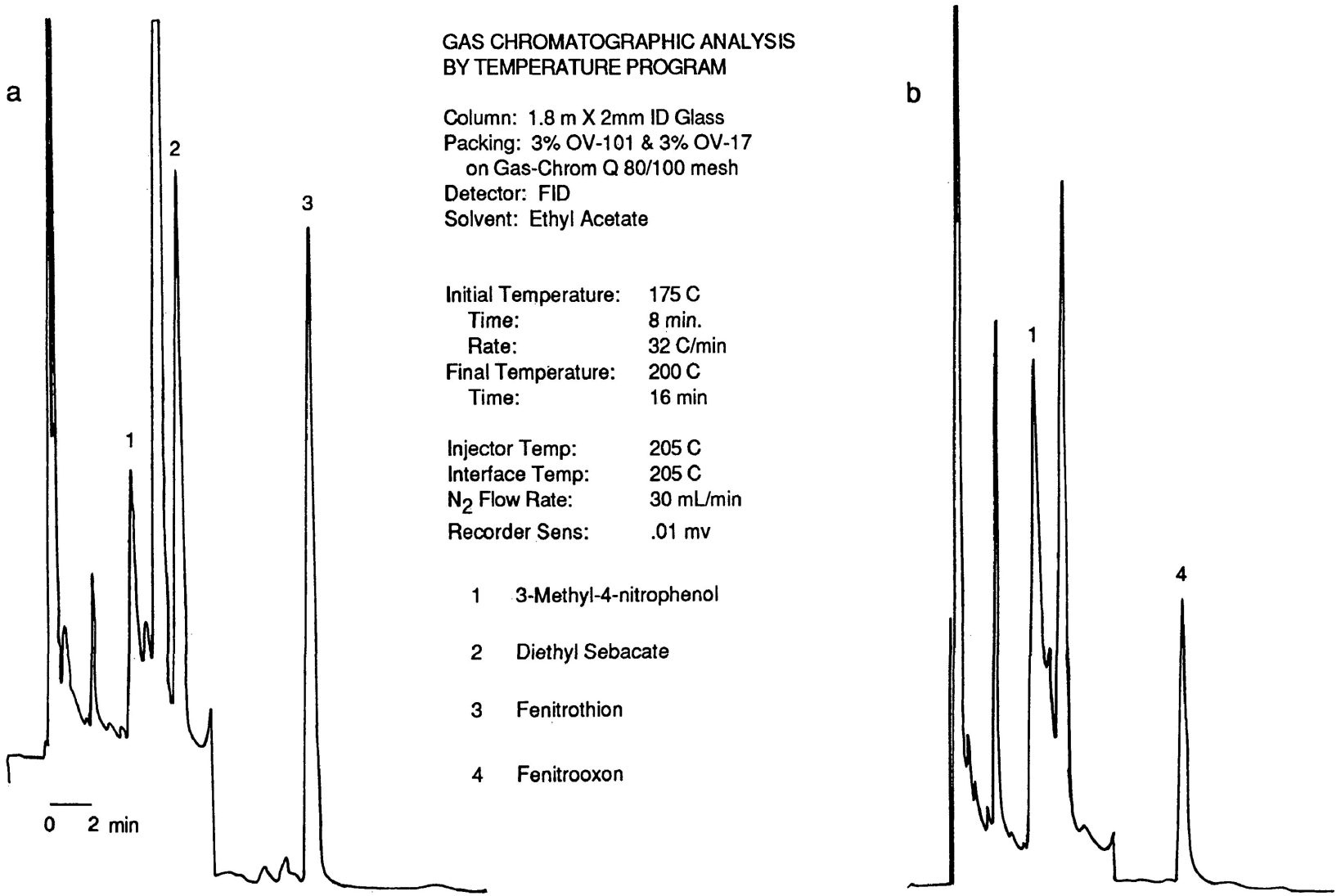


FIGURE 2-1 **CHROMATOGRAMS OF TEST CULTURE EXTRACTS PRIOR TO ION-EXCHANGE RESIN CLEAN-UP**

2.3.3 Extractions

The polarity of phenolic metabolites of methoxychlor led Baarschers *et al.* **[60]** to question the use of non-polar extraction solvents for bacterial and fungal degradation studies on methoxychlor, because these metabolites may not be effectively extracted, if present. Fenitrothion shows the least polarity, relative to its metabolites, when examined by thin layer chromatography on silica gel (page 59). An extraction solvent was therefore sought that had high polarity, but would also result in high recovery of the least polar compound.

Typically, the partitioning of a solute between two immiscible phases is expressed by the partition coefficient, K, where

$$K = p / q$$

and p = fractional amount in non-polar phase

q = fractional amount in polar phase

where $p + q = 1$.

The suitability of ethyl acetate as extraction solvent was shown, however, by the more conveniently described **p**-value **[61]**. The **p**-value is defined as the fraction of the total solute that distributes itself in the non-polar organic phase of an equivolume solvent pair. Here

$$\mathbf{p} = A_a / A_b$$

where A_a = amount of solute in non-polar phase after phase equilibration

A_b = amount of solute in non-polar phase before phase equilibration

when the volumes of the non-polar and polar (aqueous) phases are equal.

The **p**-value is easily determined experimentally by shaking a stock organic solution

with water and then comparing the solute concentration in organic solvent before and after equilibration of the phases (see page 64).

Table 2-1 results indicate that 100% of the fenitrothion solute is found in the ethyl acetate after one equilibration. Clearly, this result shows that ethyl acetate is a particularly efficient solvent for the extraction of fenitrothion and therefore should also be suitable for the extraction of its more polar derivatives. It is, however, important that the ethyl acetate solvent be free of ethanol and acetic acid to preclude potential interferences on subsequent analysis, such as transesterification of the phosphate ester function of fenitrothion. Purified ethyl acetate was therefore used for all fungal culture extractions in this study.

TABLE 2-1 **p-VALUE STUDY**

Sample	Vol. water used	mg. recovered from 1 mL	Mean	p-value
1a	-	3.90		
1b	-	4.06	3.98	1.00
2a	5 mL	4.06		
2b	5	4.13	4.09	1.03
3a	10	3.85		
3b	10	3.93	3.89	0.98
4a	20	4.08		
4b	20	3.88	3.98	1.00

mean **p**-value 1.00

2.3.4 Effect of pH on Extraction Efficiency

Because of the acidic nature of potential phenolic fenitrothion derivatives, the pH of the aqueous fungal cultures on extraction was of concern; too high a pH could result in phenolic metabolites remaining in the aqueous phase as phenolate salts. Experiments were therefore conducted with fungal extracts of control cultures spiked with the test compounds to determine extraction efficiency at various pH levels (see page 65). After a fungal growth period of 14 days, the pH of the cultures was approximately 6.5. Recovery experiments at pH=6.5 and pH=3 indicated that fenitrothion and fenitrooxon recoveries were unaffected by changing the extraction pH in this range. However, direct extraction of cultures at pH=6.5 gave incomplete recovery of the nitrophenol (see **Table 2-2**). The enhanced recovery obtained at pH=3 is in agreement with a two step recovery; acidification of the aqueous solution remaining after extraction at pH=6.5 resulted in the recovery of an additional 33.8% 3-methyl-4-nitrophenol on further extraction. Therefore, the effect of direct acidification of the cultures to pH 3 and lower prior to extraction was also investigated. Adjustment to pH=3 gave almost quantitative recovery, but at pH<3 resulted in the precipitation of much culture medium material and led to severe emulsion problems during subsequent extraction with ethyl acetate. **Table 2-2** summarizes the results of triplicate analyses at several pH values. These experiments show that pH does play a role in the extraction efficiency of 3-methyl-4-nitrophenol and it follows that this compound and other possible acidic metabolites are most effectively recovered at pH=3.

TABLE 2-2 **EFFECT OF pH ON 3-Methyl-4-nitrophenol RECOVERY**

pH	% phenol recovery
8	4.5
6.5	68.9
3	93.8

2.3.5 Ion-Exchange Resin Clean-up of Fungal Extracts

The treatment of liquid cultures before and during harvesting leads to various kinds of fungal extracts, which can be described as follows. Test culture extracts result from liquid fungal cultures to which test compounds were added at the beginning of, or during, the growth period. Fungal cultures grown in the absence of test compounds gave rise to blank extracts on workup, unless test compounds were added just prior to harvesting, in which case control culture extracts were generated. Control extracts permit determination of test compound recovery efficiency.

GC analysis of blank fungal culture extracts indicated that a small peak attributable to the medium itself was coincident with the peak ascribed to 3-methyl-4-nitrophenol, although it would be possible to correct for this impurity by comparison of gas chromatograms of test culture extracts with those of appropriate blank culture extracts. However, since such a method could add appreciable errors to the analytical process, it was found desirable to remove such impurities (ie. clean-up) from these extracts prior to GC analysis.

The acidity of the 3-methyl-4-nitrophenol, discussed above, led to the investigation of ion-exchange resins for this purpose. This type of clean-up was dependent on the interfering material having a non-acidic nature.

A resin that was particularly effective for extract clean-up was Amberlyst A-21 ion-exchange resin, a weakly anionic material that can be used with organic solvents. Acidic compounds are retained by the resin while neutral and basic compounds are eluted with the solvent used to transfer the residue to the clean-up column. The resin is supplied in hydrated form and so must be preconditioned for use with organic solvents. Details of this procedure are given below (page 65). It was found that both fenitrothion and fenitrooxon pass through the resin with ethyl acetate eluent while 3-methyl-4-nitrophenol is retained by the resin. The adsorption of the phenol is observed as a bright yellow band, indicative of a phenolate salt, that penetrates to about 50% of the bed volume. The adsorbed material can be recovered by elution of the column with acidified methanol. This decolourises the resin slightly and causes the beads to swell by about 30% as observed by the increase in the resin bed volume. By varying several parameters (e.g. resin bed volumes, eluent flow rates, eluent volumes, resin capacity and normality of the methanolic acid) the conditions for an efficient clean-up procedure could be optimised. Spiking of triplicate blank fungal cultures with fenitrothion, fenitrooxon and 3-methyl-4-nitrophenol, their subsequent equilibration, extraction and clean-up as described above (see also section 4.7, page 67), gave the recoveries presented in **Table 2-3** as determined by GC analysis. The recovery efficiencies are quite good, considering the extent of material handling involved, and consequently this method of separating fenitrothion and fenitrooxon from 3-methyl-4-nitrophenol was employed where necessary.

This method permits GC analysis for fenitrothion or fenitrooxon in one aliquot of an

extract, and then allows for extract clean-up of the remaining portion to isolate the phenol which can then be derivatised for analysis by GC. The hydrolysis problems encountered when the 3-methyl-4-nitrophenol is derivatised in the presence of fenitrothion or the oxon (see above, page 25) are thereby avoided.

TABLE 2-3 TEST COMPOUND RECOVERY FROM CONTROL CULTURES WITH RESIN CLEAN-UP

compound	mg. added	mg.recovered	%recovery
fenitrothion	12.07	10.35	85.8
fenitrooxon	12.09	10.16	84.0
3-methyl-4-nitrophenol	3.08	2.51	81.5

Comparisons of gas chromatograms of cleaned-up 3-methyl-4-nitrophenol-containing fungal extracts before and after derivatisation indicated that the interfering compound, whose retention time was similar to that of 3-methyl-4-nitrophenol, was no longer present, which confirmed that the objective of the clean-up study had been achieved.

A point should be made regarding the less than 100% recovery of test compounds. As suggested earlier, material transfers can partially account for this. It is also possible that some of the materials may be retained through irreversible adsorption related more to the pore sizes of the resin rather than to its anionic character. That this may be a factor is consistent with the observation that additional volumes of acidic methanol eluent do not result in higher material recoveries.

A more qualitative observation can be made with respect to resin capacity. It was pointed out previously that the retention of 3-methyl-4-nitrophenol by the resin could be observed during elution of the column as a yellow band. After 4 mL of ethyl acetate had passed through the column, this band appeared at a level half the height of the 1.5 cm resin bed. One mg quantities of 3-methyl-4-nitrophenol were used in these experiments and it is therefore suggested that using 1.5 cm resin for each mg of 3-methyl-4-nitrophenol under the described experimental conditions (page 65 section 4.5) affords a good margin of safety in preventing accidental elution of the phenol with the ethyl acetate eluent.

Figures 2-2, 2-3, and **2-4** show the chromatograms of fenitrothion and fenitrooxon test cultures prior to clean-up and 3-methyl-4-nitrophenol analysis after A-21 clean-up, respectively. Each figure is accompanied by the chromatogram of a similar quantity of test compound standard as well as the associated blank culture.

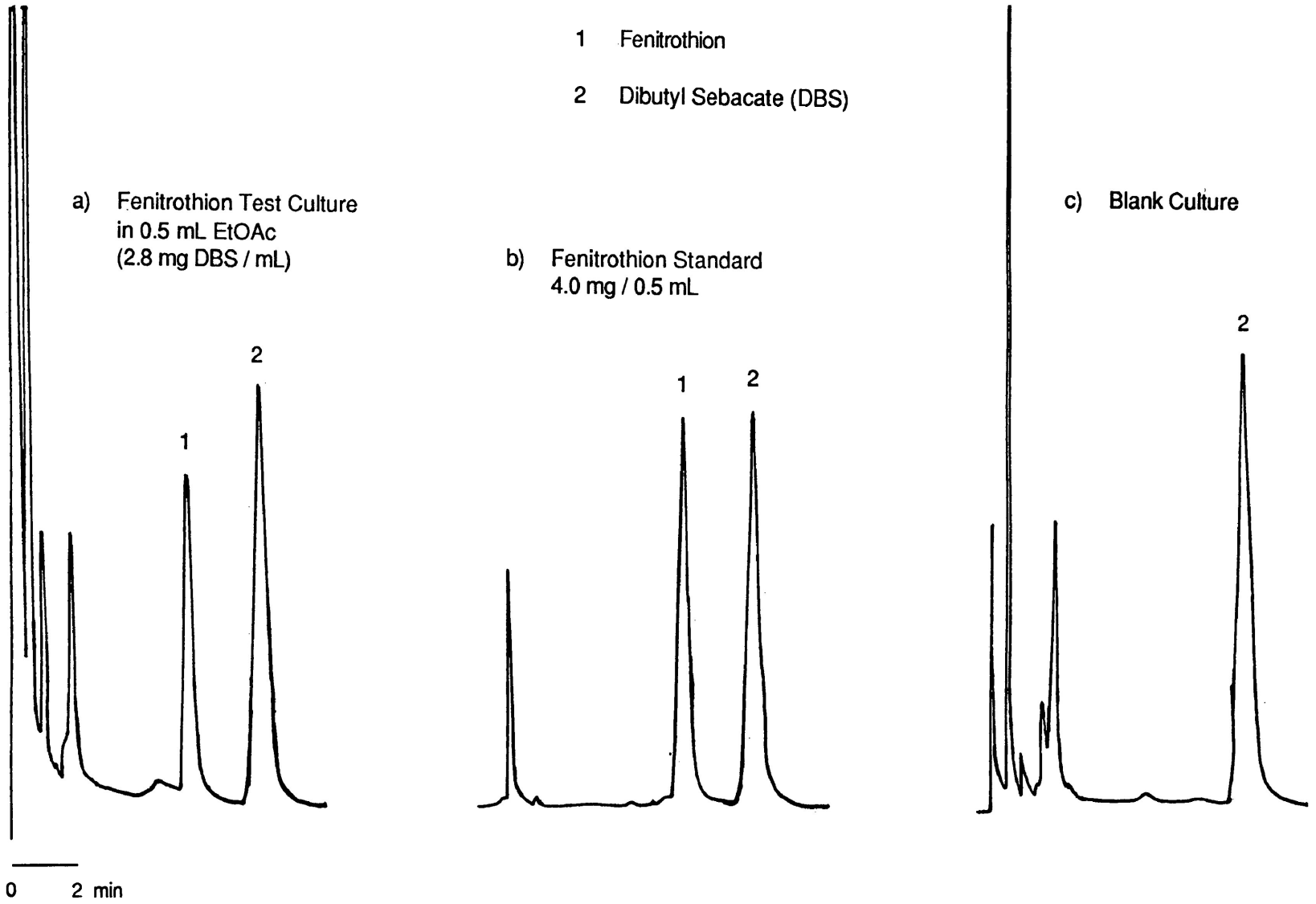


FIGURE 2-2 GC CHROMATOGRAMS OF FENITROTHION ANALYSES

Prior to A-21 clean-up, isothermal @ 210 C, other conditions see FIGURE 2-1

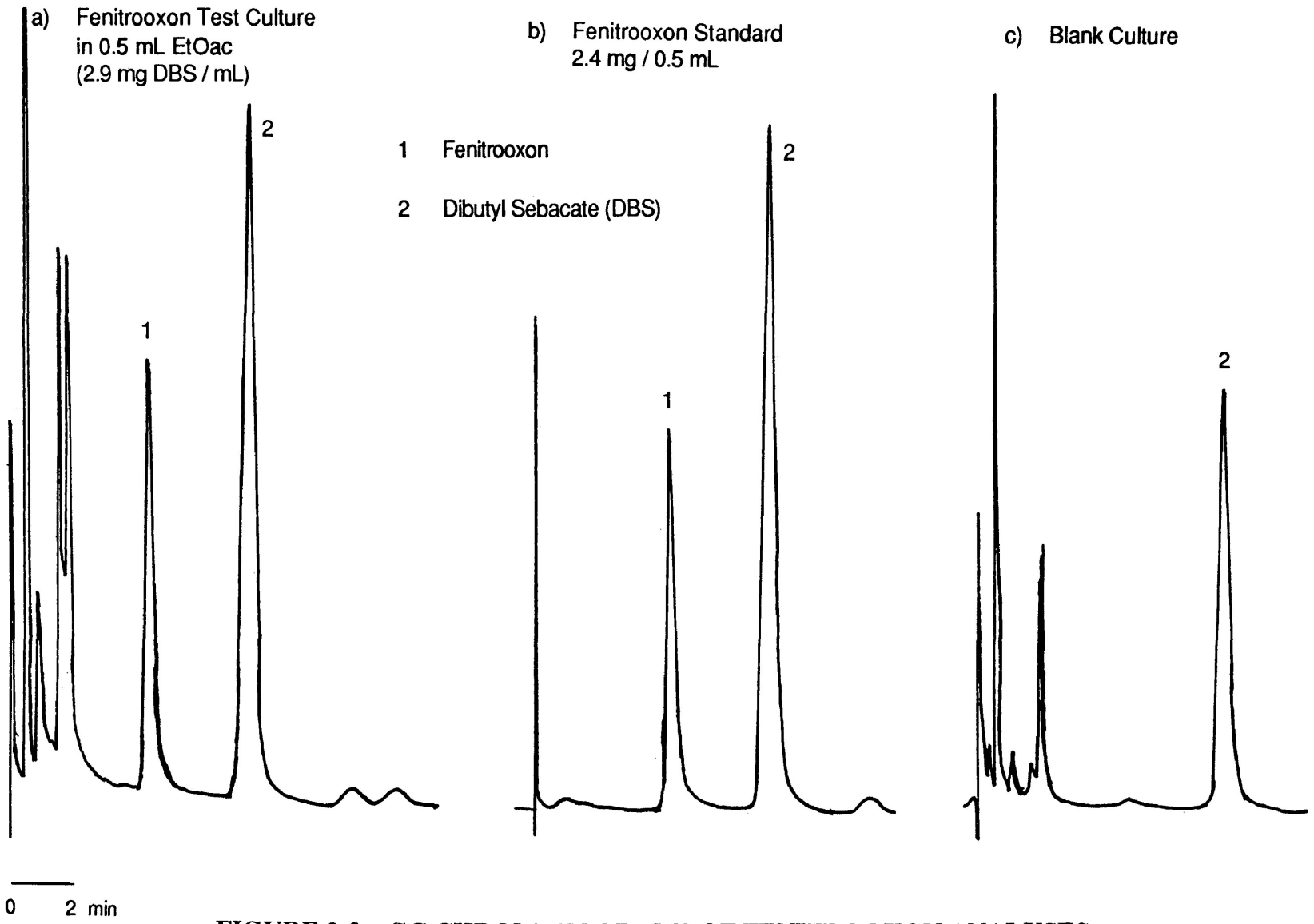


FIGURE 2-3 GC CHROMATOGRAMS OF FENITROOXON ANALYSES

Prior to A-21 clean-up, isothermal @ 210 C, other conditions see FIGURE 2-1

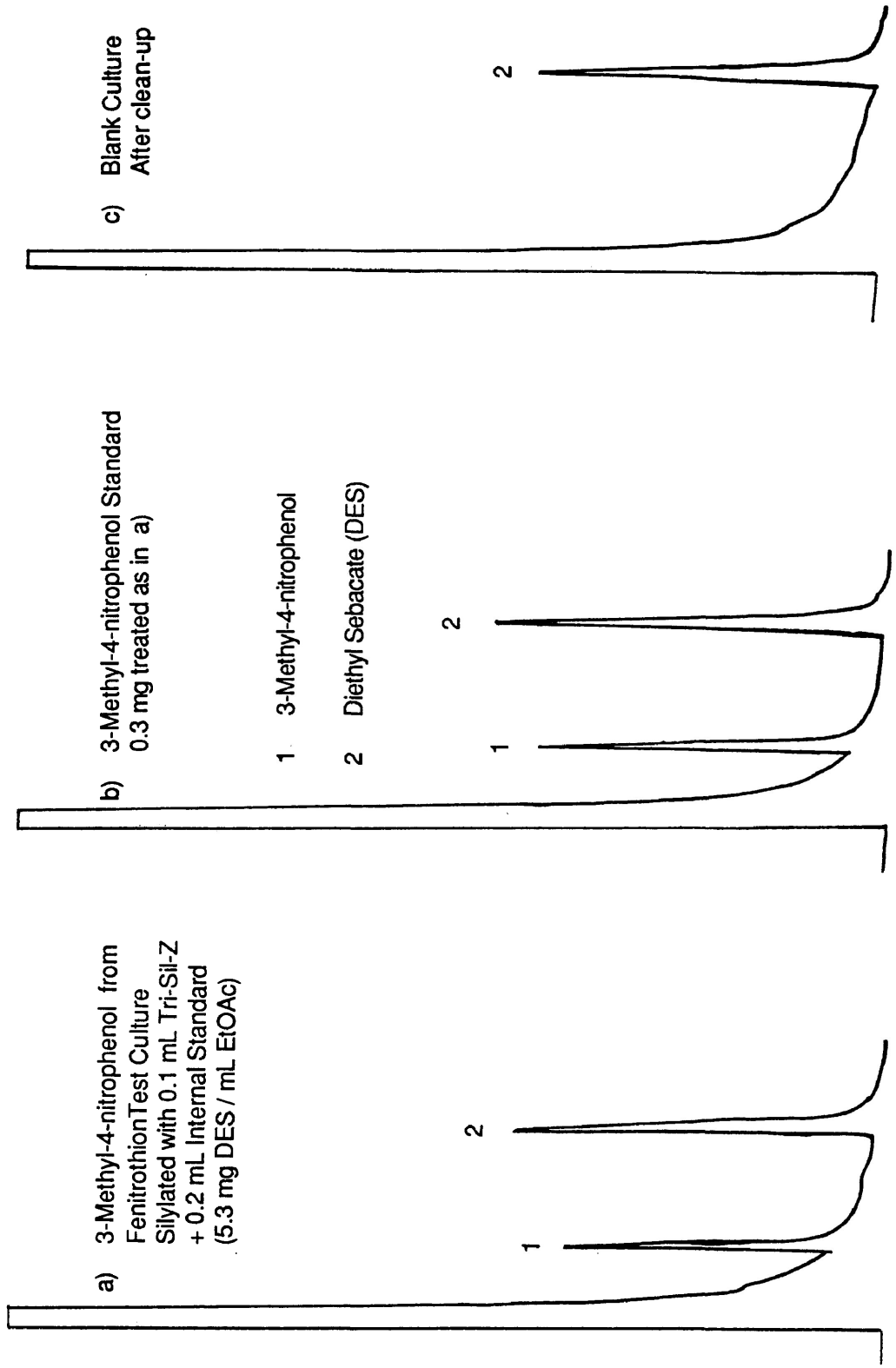


FIGURE 2-4 CHROMATOGRAMS OF 3-METHYL-4-NITROPHENOL ANALYSES

Silylated, after A-21 clean-up, isothermal @ 180 C, other conditions see FIGURE 2-1

CHAPTER 3 - METABOLISM STUDIES

3.1 INTRODUCTION

A soil fungus, *Trichoderma viride* Pers. ex Fr., of the order Moniliales, was selected as a test organism for the present study because it is relatively insensitive to toxic effects of fenitrothion. Also, it is endemic to forest areas populated with balsam fir and white spruce. These areas are currently highly stressed in eastern and central Canada due to severe spruce budworm infestations. One method of controlling this destructive pest has involved the aerial spraying of fenitrothion. As a result of the actual spraying or loss of tree biomass, pesticide residues fall to the forest floor, and consequently can accumulate in the environment of soil microorganisms.

In previous investigations in this laboratory **[62]**, four species of fungi were used to determine the toxic effects, in terms of growth inhibition, of fenitrothion and some of its common metabolites. Results of this work indicated that *T. viride* was moderately sensitive to toxic effects of fenitrothion, as shown by the $^{*}EC_{50}$ value of 7 ppm. Fenitrooxon, S-methylfenitrothion, and 3-methyl-4-nitrophenol were found even less fungitoxic: EC_{50} values of 350, 90, and 23 ppm, respectively, were recorded. **Figure 3-1** illustrates the dosage response relationships for various concentrations of fenitrothion and derivatives assayed with *T. viride* . S-methylfenitrothion and 3-methyl-4-nitrophenol were used to show that the inhibitory effects observed on plate cultures were fungistatic rather than fungicidal.

$^{*}EC_{50}$ is defined as the effective concentration causing 50 % inhibition of growth

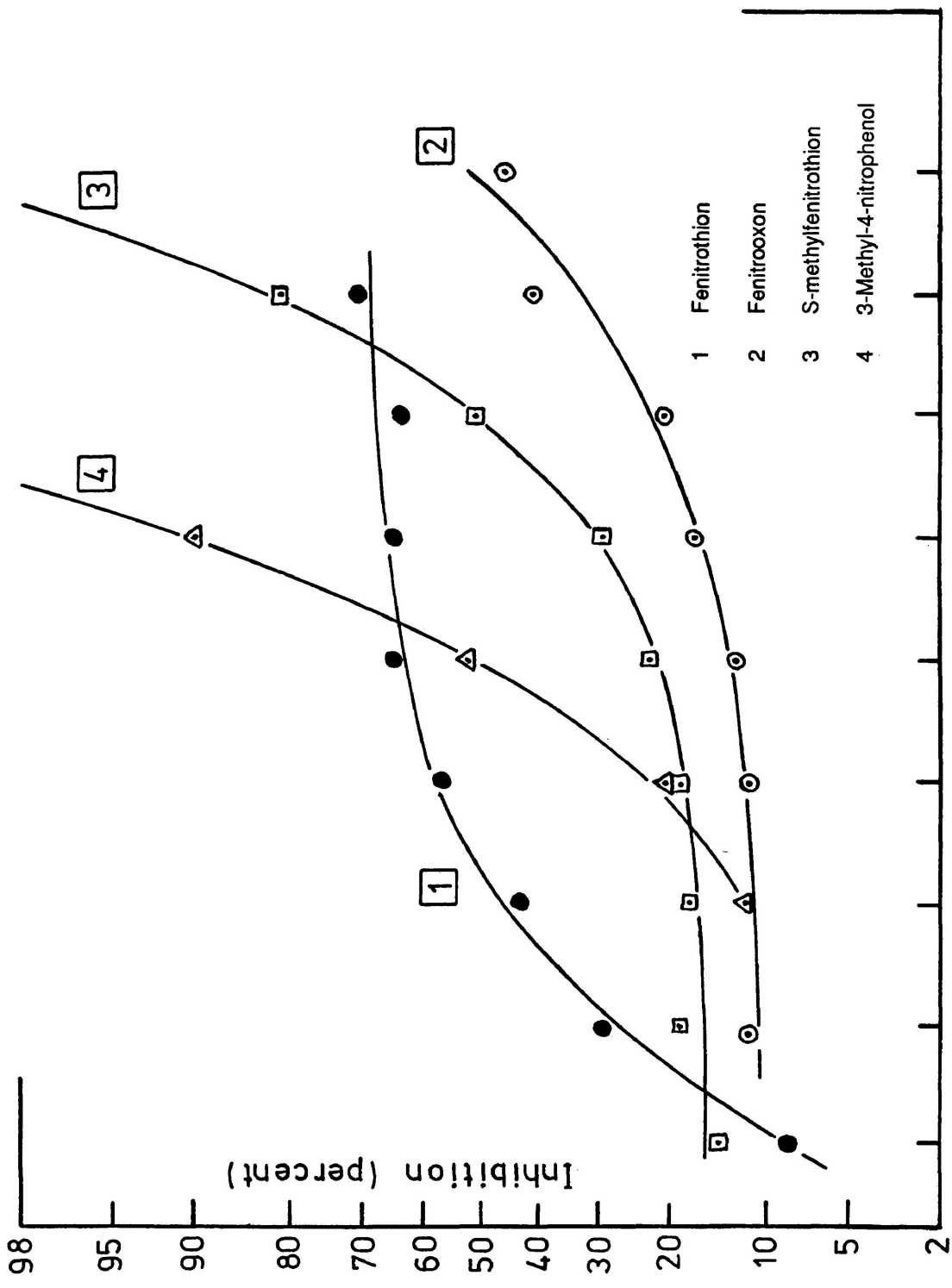


FIGURE 3-1 DOSAGE RESPONSE CURVES OF FENITROTHION AND SOME DERIVATIVES ASSAYED WITH *Trichoderma viride*

T. viride was also shown to hydrolyse fenitrothion to 3-methyl-4-nitrophenol [Baarschers, unpublished results], and to do so more efficiently than three other fungal species tested.

These observations support the choice of *T. viride* as a test microorganism for further investigating the biodegradation of fenitrothion. A pure isolate of the fungus was obtained from Dr. J. P. Kutney, Department of Chemistry, University of British Columbia, Vancouver.

3.2 CULTURE MAINTENANCE AND GROWTH CONDITIONS

Trichoderma viride was maintained in the dark by serial and sterile subculturing on nutrient agar in plastic Petri dishes. Typically, plates could be kept for about a month before the agar medium became desiccated and subculturing was again necessary.

Liquid shake cultures were used for pesticide degradation studies. A standard size innoculum of *T. viride* , taken from the actively growing front of a plate culture, was transferred under sterile conditions to a 500 mL Erlenmeyer flask containing 200 mL of the nutrient medium. Appropriate concentrations of pesticide or its suspected metabolite were added in 0.5 mL acetone carrier to the test culture flasks. The temperature of the medium upon addition of the test compound was such that no hydrolysis or other reaction due to heat would be expected, although the solution was still warm enough to permit the evaporation of most of the acetone carrier. The effect, if any, of remaining acetone on culture growth was compensated for by addition of pure acetone to flasks representing blank samples. Flasks were closed with a foam plug and then kept on a rotary shaker (12 cm stroke, 90 rpm) at a temperature of 20-23 C. Typically, the

incubation period lasted 14 days. Cultures were then harvested by filtration and solvent extraction of the filtrate as described in section 4.7.

In order to establish a rational protocol for biodegradation experiments, some attention was paid to growth rates in relation to minor variations in the medium, primarily in terms of glucose concentration. Liquid cultures were grown as outlined above, but with a number of different dextrose concentrations, varying from 0.5, 1.0, 1.5, and 2.0 times that described **[62]**. Also, 0.5 mL acetone, normally used as test compound carrier, was added to each culture. Growth was assessed by dry weight determinations **[62]** of triplicate cultures harvested at two day intervals. **Figure 3-2** depicts the resulting growth curves obtained in this manner.

Regardless of the dextrose concentration, **Figure 3-2** shows that the maximum quantity of biomass is quickly achieved, and then decreases with time as the mycelia mature and degenerate. In the case of the normal dextrose concentration **[62]**, maximum growth is achieved within 3 days, and then biomass begins to decline, levelling off to between 0.4 and 0.5 g of dry mycelium after 7 days. Dextrose appears to be a growth limiting factor under these conditions, with mycelial growth being directly proportional to dextrose concentration. For example, a 50% increase in the normal dextrose concentration corresponds to approximately 1.5 times the normal mycelial growth. The normal (*ie.* 1.0 times) dextrose concentration was selected. because the amount of biomass generated at this dextrose concentration was found to be the most manageable.

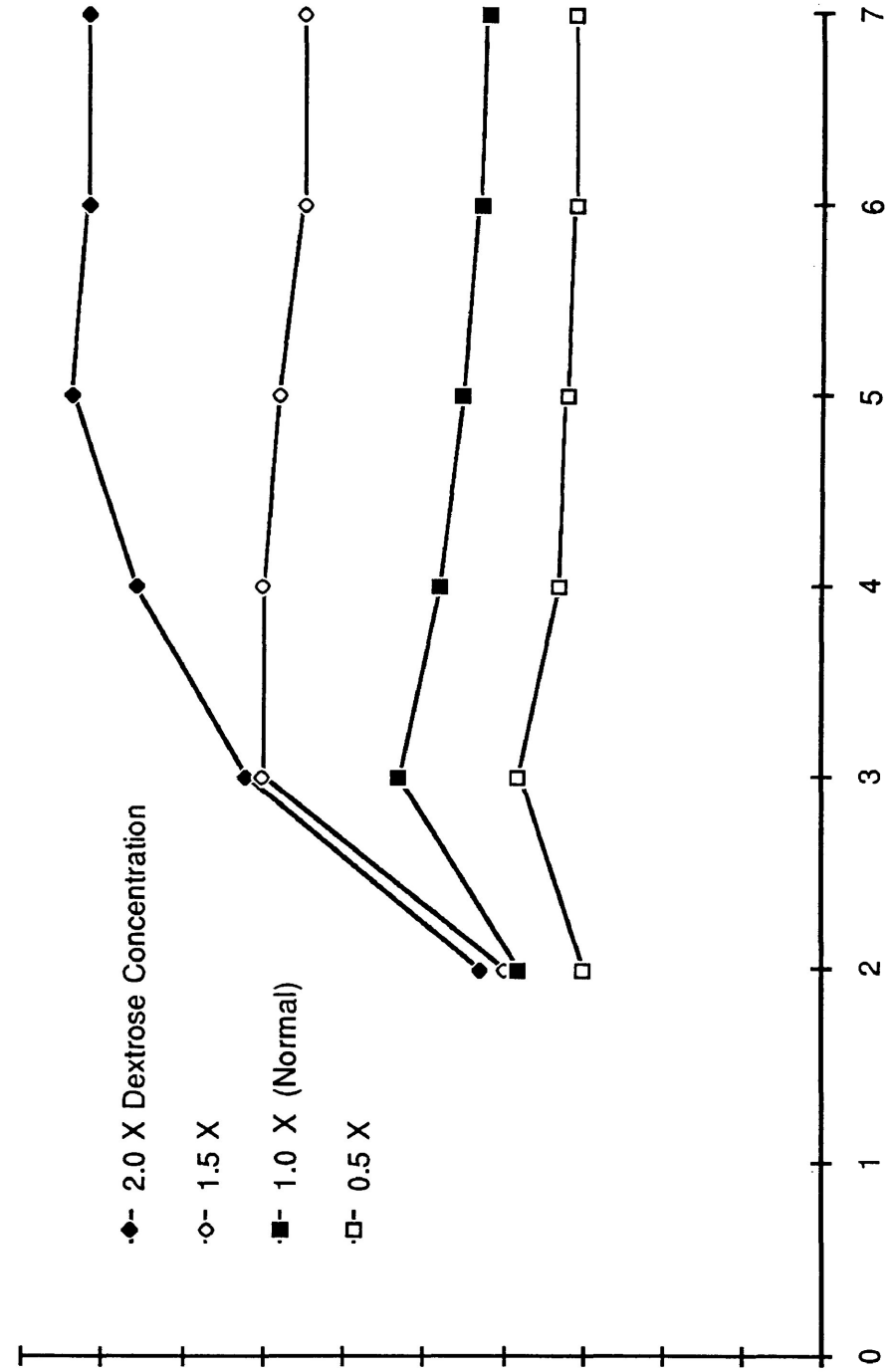


FIGURE 3-2 EFFECT OF DEXTROSE CONCENTRATION ON RATE OF MYCELIAL GROWTH

3.3 *T. viride* INTERACTIONS WITH TEST COMPOUNDS

3.3.1 Qualitative Investigations of Interactions

Toxicity data from dosage response relationships describing the effect of fenitrothion and derivatives on *Trichoderma viride* (see section 3.1) permitted the selection of test compound concentration ranges in which the growth of the test organism was not excessively inhibited. Preliminary experiments involving the interaction of fenitrothion with pure *T. viride* cultures were therefore carried out using 20 ppm of the pesticide. While this concentration was expected to cause approximately 60% inhibition (see section 3.1), this ensured that an excess of fenitrothion would be present, so that some unchanged pesticide would be recovered on extraction of the cultures. In addition, significant mycelial growth was observed in 200mL liquid cultures at this concentration.

To obtain some preliminary indication of the presence of possible fenitrothion metabolites, fungal cultures, to which were added 20 ppm fenitrothion (4 mg/200 mL culture medium), were grown as above, then harvested by filtration. This was followed by solvent extraction of the filtrate and analysis of the residues by thin layer chromatography. Fenitrothion, 3-methyl-4-nitrophenol, and fenitrooxon were identified on the basis of their predetermined R_f values and colour reaction with potassium hydroxide (see section 4.1). 3-Methyl-4-nitrophenol, the hydrolysis product of fenitrothion and fenitrooxon, gives a bright yellow spot (the potassium phenolate salt) on treatment with base, while amino derivatives produce brown spots following this treatment. Pooling of samples to increase concentrations and reanalysis by TLC did not indicate the presence of any other recognizable metabolite: aminofenitrothion and

fenitrooxon are the major reactions effected by *T. viride*. This rapid initial metabolite identification gave a good starting point for the refinement of extraction and analysis procedures as described in chapter 2.

3.3.2 Fungal Hydrolysis of Fenitrothion

Since *T. viride* was qualitatively found to effect the hydrolysis of fenitrothion, subsequent experiments were designed to quantify the hydrolysis and consequently determine the efficiency of the reaction.

Triplicate fenitrothion-spiked liquid test cultures, as well as blank cultures, were prepared and grown as outlined above. After 14 days incubation, all cultures were harvested by filtration and extraction of the filtrate which was concentrated and the residues analysed by gas chromatography for recovery of parent material and 3-methyl-4-nitrophenol hydrolysis product. The extent of enzymatic hydrolysis can be described as mg of fenitrothion hydrolysed per gram dry weight of mycelium. This may serve as an indicator of the hydrolytic capacity of a given quantity of biomass, and can be used to normalize this capacity, thereby permitting comparisons with other organisms. More applicable to this study, however, is the quantity of hydrolysis product relative to the amount of parent material initially present. This is represented as a percentage. **Table 3-1** summarizes the results of a number of these hydrolysis experiments with fenitrothion.

Experiments 1, 2a, 3, and 4a show that an average of 55.1% of initially added fenitrothion was recovered, while 9.2% of parent material was recovered as the hydrolysis product 3-methyl-4-nitrophenol. Attempts to account for the remaining 35.8% of material must begin by considering earlier qualitative investigations (section

TABLE 3-1 **FENITROTHION RECOVERY FROM TEST CULTURES**

Exp. no.	%rec'd(ee) [a]	mg hydrolysed [b]	% hydrolysed(ee) [c]	bal. [d]
1	60.3 (80.0) [e]	0.83	10.4 (56.2)	29.3
2a	54.4 (93.2)	0.54	6.7 (93.6)	38.9
2b	90.8 (93.2)	--	0.6 (93.6)	8.6
3	59.1 (85.0)	0.64	8.0 (75.7)	32.9
4a	46.4 (85.5)	0.94	11.6 (81.5)	42.0
4b	94.9 (85.5)	--	1.1 (81.5)	4.0
means (1,2a,3,4a)	55.1 (85.9)	0.74	9.2 (76.8)	35.8
5	85.0 (85.0)	0.74	9.3 (75.7)	5.7

[a] % fenitrothion/oxon recovered based on extraction efficiency (ee, as % recovery from controls);

[b] milligrams of fenitrothion/oxon hydrolysed per gram dry weight mycelium;

[c] % fenitrothion/oxon hydrolysed (ee, extraction efficiency of 3-methyl-4-nitrophenol as % recovered from controls);

[d] % fenitrothion/oxon unaccounted for;

[e] all values result from triplicate cultures and analyses.

3.3.1) which indicated the presence of trace quantities of fenitrooxon in test culture extracts. This oxidation product was indeed identified in these experiments, and was considered to have arisen through interaction with the culture medium, since the fenitrothion spike did not contain any fenitrooxon as an impurity to begin with. Quantitative determination of fenitrooxon was, however, not possible because of the low concentration of this material. Obviously, the formation of some fenitrooxon accounts for some of the amount missing in the material balance, but only to a minor extent. Further, exhaustive extraction of the mycelia remaining after filtration of the liquid cultures yielded traces of fenitrothion and 3-methyl-4-nitrophenol, although chromatograms of this type of extract were particularly complex as a result of the release of a large variety of cellular material. Notwithstanding these two identified sources of loss, more than 30% of the original compound was not recoverable.

For further attempts to account for a major part missing in the material balance, it was important to determine at which point in the incubation term the hydrolysis of fenitrothion occurred. To this end, fenitrothion was added to cultures which had already grown a period of seven days (see section 4.9), and an additional 10 days incubation were then allowed after addition of the test compound. Subsequent extraction and analysis (as above) indicated that the extent of hydrolysis of initially added parent material was virtually the same as the mean of experiments 1, 2a, 3,and 4a. However, parent material was recovered in higher yield (85.0% compared with 55.1%), and the quantity of material unaccounted for was significantly less (5.7% as compared to the earlier35.8%). It is clear from the growth curves (**Figure 3-2**, page 42) that in this experiment fenitrothion was added after the actual growth of the fungal culture was essentially complete. Comparison of the two sets of experimental results suggests that the substantial loss of material occurs during the first few days of incubation, *ie.* the

period of exponential growth of the cultures.

3.3.3 Fungal Hydrolysis of Fenitrooxon

In the qualitative experiment (section 3.3.1) and the fenitrothion experiment (section 3.3.2), fenitrooxon was found to be the only other identifiable fenitrothion metabolite, in addition to 3-methyl-4-nitrophenol. Although the oxon was found in very small quantities, it was of interest to determine the interaction of *T. viride* with this more toxic fenitrothion derivative. The investigation was conducted in the same manner as the fenitrothion hydrolysis (see above). **Table 3-2** summarizes results of several experiments on the interaction of fenitrooxon with *T. viride.*

The results of experiments 6, 7, 8, 9a, 10a, and 11a indicate that an average of 34.9% of the original fenitrooxon was recovered, considerably less than the 55.1% mean fenitrothion recovery (section 3.3.2). Initially (experiments 6, 7, and 8) 3-methyl-4-nitrophenol was not quantified because of interferences in the analysis caused by the production of some fungal metabolites apparently stimulated by the presence of the oxon. Development and application of the resin cleanup described in section 2.3.5 allowed for the measurement of this hydrolysis product in later experiments (9a, 10a, and 11a). **Table 3-2** shows that, on average, more than three and a half times as much 3-methyl-4-nitrophenol was recovered from the fenitrooxon incubation than from the corresponding fenitrothion experiments. An average of 33.0% of originally added fenitrooxon was hydrolysed in comparison to the 9.2% hydrolysis observed with fenitrothion. This means that fenitrooxon is more readily hydrolysed by the fungus than is fenitrothion. This is not surprising in view of the fast chemical hydrolysis of fenitrooxon relative to fenitrothion as reported by Zitko and Cunningham

TABLE 3-2 **FENITROOXON RECOVERY FROM TEST CULTURES**

Exp. No.	%rec'd(ee) [a]	mg hydrolysed [b]	% hydrolysed(ee) [c]	bal. [d]
6	29.9 (82.6) [e]			
7	43.1 (84.8)		[f]	
8	26.3 (86.6)			
9a	33.1 (96.5)	2.26	33.3 (80.3)	33.6
10a	47.9 (84.0)	2.05	31.9 (80.3)	20.2
11a	29.0 (89.0)	2.46	33.8 (81.0)	37.2
means	34.9 (87.3)	2.26	33.0 (80.5)	30.3
9b	87.4 (96.5)		5.0 (80.3)	7.6
10b	90.7 (84.0)		6.0 (80.3)	3.3
11b	88.3 (89.0)		3.0 (81.0)	8.7
means	88.8 (87.3)		4.7 (80.5)	6.5

[a - e] see notes **Table 3-1**

[f] 3-methyl-4-nitrophenol not measured

[19], although the difference by enzymatic hydrolysis was found to be not nearly as great. Therefore, if fenitrooxon is indeed formed from the fenitrothion during incubation, either biochemically or by air oxidation, it would only be present in trace amounts (as was indeed found) since the faster hydrolysis of the oxon prevents it from accumulating in such cultures.

Another observation results from the comparison of **Tables 3-1** and **3-2** in that the balance of parent material unaccounted for is similar in both experiments. Fenitrothion hydrolysis by *T. viride* leaves 35.8% of the initially added material unrecovered while an average of 30.8% of the fenitrooxon is unaccounted for. Exhaustive mycelial extraction of hydrolysis cultures did not yield additional parent material or metabolites in either case. Since both sets of experiments were conducted under the same conditions, nutrient requirements and utilisation in both cultures would be similar. It is therefore possible that the missing material in both cases can be accounted for by assuming further fungal metabolism of 3-methyl-4-nitrophenol, the common hydrolysis product of both parent compounds. Metabolism and utilisation of 3-methyl-4-nitrophenol as a carbon and energy source has been exhibited by bacteria ([47], [48], [49]). In this context it is noted that experiments involving delayed addition of fenitrooxon to established cultures led to increased recovery of the starting material (*ie.* 67 %) as compared to similar experiments with fenitrothion (34 %). The results of these delayed addition experiments with the oxon are summarized in **Table 3-3.**

The increased recovery of both fenitrothion and fenitrooxon when these compounds are added after exponential growth has come to a halt (*ie.* after day 4 or 5) could be explained by postulating that the common hydrolysis product, 3-methyl-4-nitro- phenol, is further metabolised by the fungus, but only during that first growth period. In other words, during the exponential growth period the phosphate esters (thion and/or oxon)

undergo hydrolysis, and the resulting 3-methyl-4-nitrophenol is consumed at the same time. After growth has halted, sufficient hydrolase enzyme is still available for further hydrolysis, but the 3-methyl-4-nitrophenol produced in the resting culture (day 5-14) is no longer utilised. During the experiments 12 -14 (**Table 3-3**) 3-methyl-4-nitrophenol was not measured due to the interference discussed above. When the clean-up procedure was developed, it was decided that supportive evidence for the above hypothesis could be more efficiently obtained by delayed addition experiments with the nitrophenol itself (section 3.3.4).

TABLE 3-3 RECOVERY OF FENITROOXON AFTER ADDITION TO MATURE CULTURES

Exp. No.	% rec'd (ee) [a]
12	61.0 (82.6)[e f]
13	83.0 (84.8)
14	57.7 (86.6)
means	67.2 (84.7)

[a e] see notes **Table 3-1**

[f] nitrophenol not measured

3.3.4 Fungal Metabolism of 3-Methyl-4-nitrophenol

The hypothesis of nitrophenol metabolism (section 3.3.3) required delayed addition experiments in the manner described above. The clean-up and derivatisation methods developed allowed these experiments to be carried out with relatively low concentrations (*ie.* 5 ppm) and the results (**Table 3-4**) seem to confirm that hypothesis.

Thus, it was found that 3-methyl-4-nitrophenol was recovered in progressively greater amounts when the addition of the phenol was increasingly delayed. This indicates that fungal activity, with respect to 3-methyl-4-nitrophenol disappearance, is higher during the first few days of culture growth. This is consistent with the observations concerning recovery balance in the experiments with fenitrothion and fenitrooxon interactions (sections 3.3.2 and 3.3.3). Furthermore, no other metabolites could be found in the culture extracts or mycelium. It is suggested that this observation supports the hypothesis of cometabolism of 3-methyl-4-nitrophenol by the fungus. As suggested earlier (page 49), the missing parent material in the fenitrothion or fenitrooxon experiments can be accounted for if the hydrolysis product is metabolized as it forms. Finally, it was of interest to determine whether the fungus could utilise the 3-methyl-4-nitrophenol as a nutrient. To encourage this possibility, the major carbon and energy source in the culture medium, dextrose, was excluded. The experiment was conducted as usual for a test culture, except that no dextrose was added to the culture medium. Culture growth was measured by dry weight determinations (section 4.12). Results of this one experiment are tabulated in **Table 3-5.**

In dextrose-free medium, 5 ppm 3-methyl-4-nitrophenol appears to have some toxic effect on the fungus, as shown by dry mycelial weights for test cultures containing fungus, 3-methyl-4-nitrophenol and culture medium (71.9 mg), and blank cultures

TABLE 3-4 3-Methyl-4-nitrophenol RECOVERY FROM TEST CULTURES AFTER DELAYED ADDITION

	% 3-methyl-4-nitrophenol recovered [a] addition of nitrophenol to culture at day:				
Exp. No.	0	3	5	7	14 (ee)
15	55.3 [b]	57.0	70.3		102.6 [c]
16	61.3	60.8	71.6	74.1	99.0

[a] no cleanup of extracts, 3-methyl-4-nitrophenol silylated for analysis

[b] values result from triplicate cultures and analyses

[c] extraction efficiency after 2 hours

TABLE 3-5 3-Methyl-4-nitrophenol RECOVERY FROM TEST CULTURES LACKING DEXTROSE NUTRIENT [d]

Culture	% phenol recovered(ee) [a]	mycelial wgt (mg) [b]	unaccounted mg/g [c]
Test	91.7 (99.0)	71.9	1.21
Blank	-- --	90.9	

[a] % 3-methyl-4-nitrophenol recovered based on extraction efficiency (ee, as % recovery from controls)

[b] average dry weight of mycelium

[c] mg unaccounted material expressed in terms of 1 gram biomass

[d] values result from triplicate cultures and analyses

containing only the fungus and culture medium (90.9 mg). Some 3-methyl-4-nitrophenol is unaccounted for (8.3% or 1.21 mg/g biomass), but it is difficult to say if this material was utilised for fungal growth since its presence inhibited mycelial growth by 21% relative to the blank. Conversion of 3-methyl-4-nitrophenol to other derivatives cannot be ruled out since no attempts were made to identify such other metabolites.

As discussed in the introduction, (page 13), the availablity of other nutrients can determine the biodegradation pathway of a given compound. Consequently, degradation in the presence of readily available natural nutrients (*ie.* dextrose) would seem more significant from an environmental viewpoint.

3.3.5 pH Control Experiments

Since both fenitrothion and fenitrooxon can be hydrolysed under alkaline conditions [**19**], it was essential to ascertain that it is indeed the fungus that causes the observed hydrolysis. This required careful monitoring of the pH of cultures during the entire growth period of *T. viride* (see **Figure 3-2**). pH controls were prepared and consisted of culture medium without fungus. They were spiked with the same quantity of test compounds as the test cultures and were treated similarly in all other respects. At periodic intervals, an aliquot of test culture medium was removed from the culture flask and the pH measured. pH controls were then adjusted to this pH with dilute acid or base as required.

Figure 3-3 shows a typical pH profile for the 14 day growth period of test cultures, blank cultures containing only *T.viride* and no pesticide, as well as the stepwise pH adjustments of control flasks in a fenitrothion experiment. Similarly, **Figure 3-4** gives

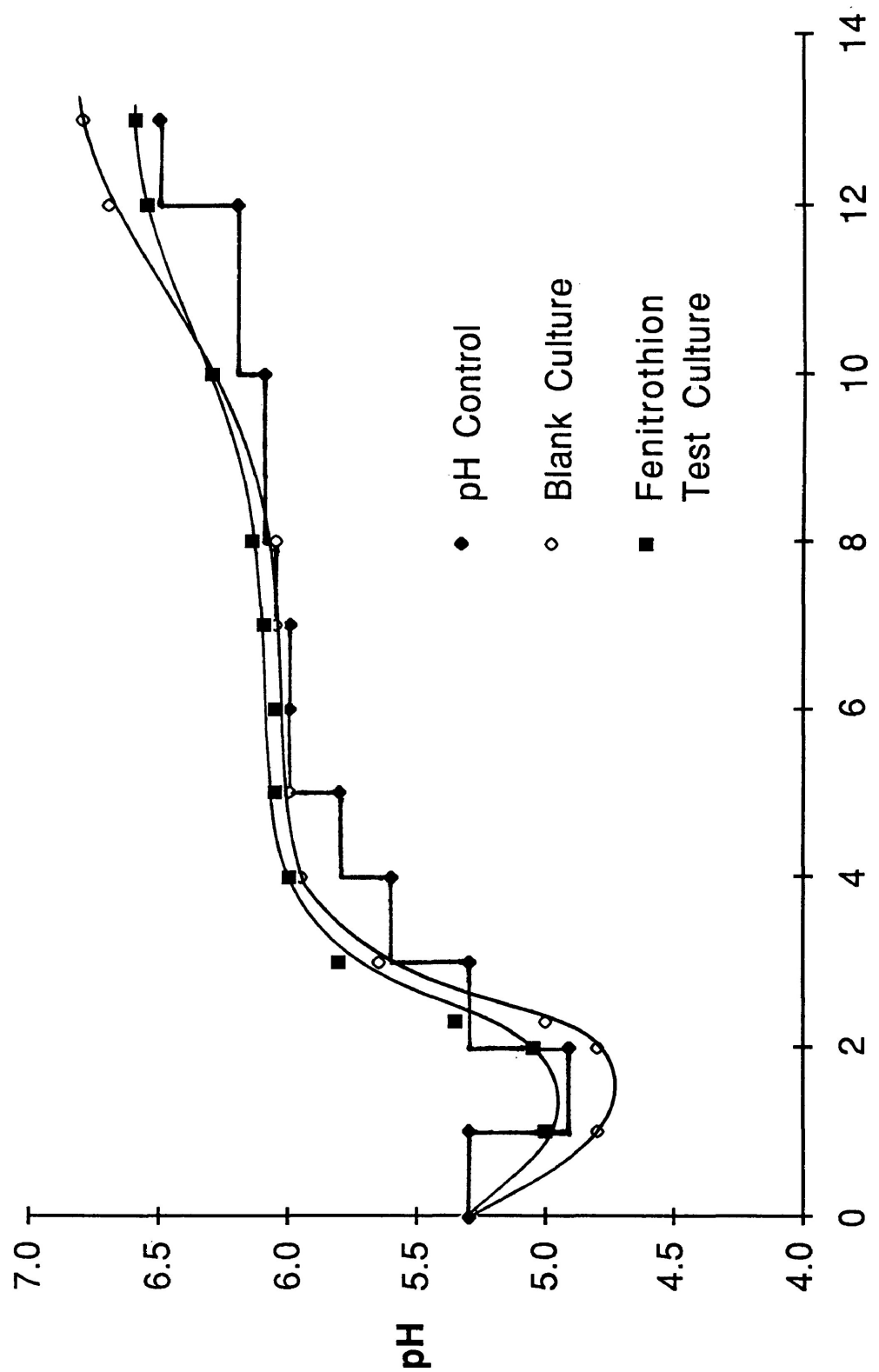


FIGURE 3-3 FENITROTHION pH CONTROL EXPERIMENT

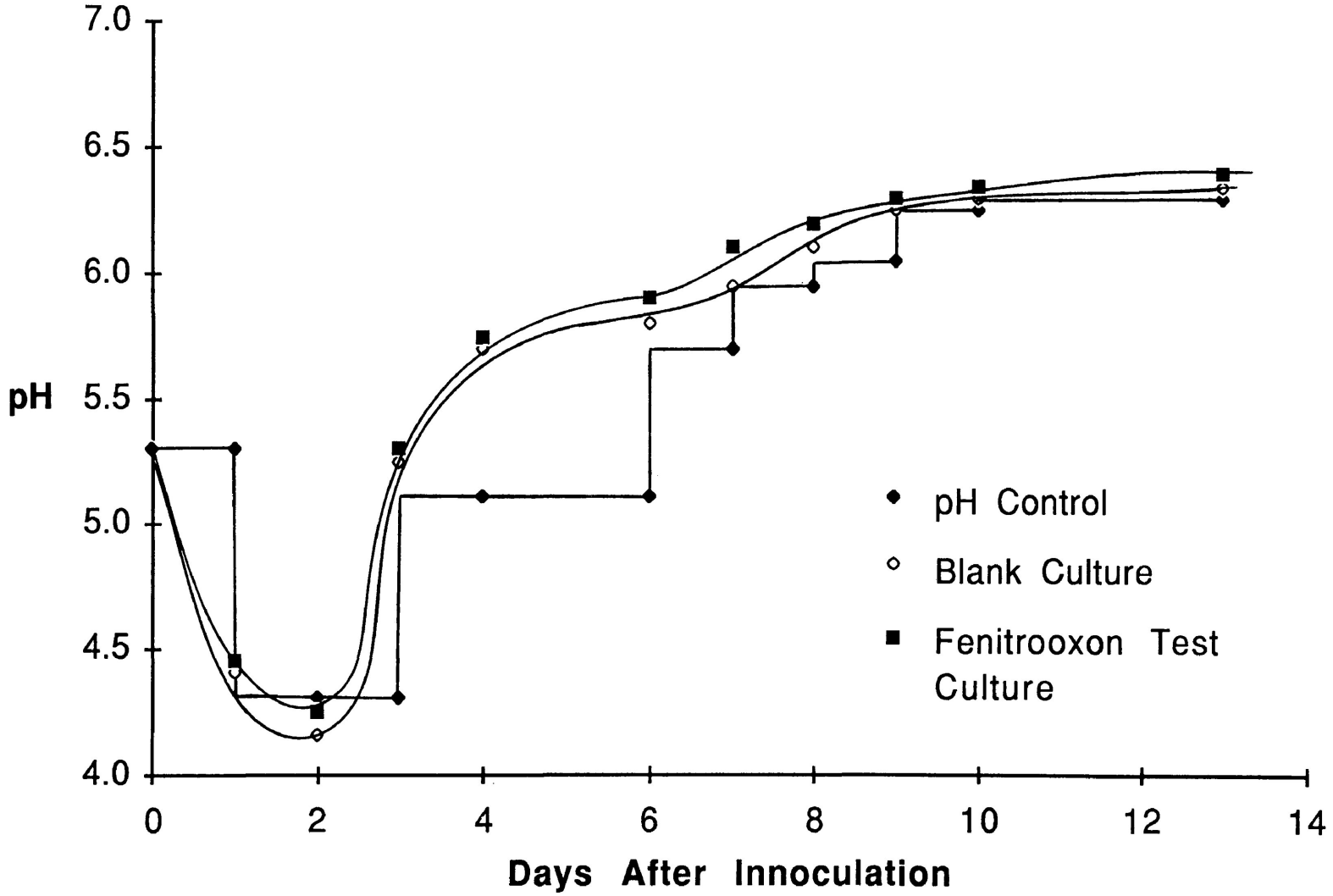


FIGURE 3-4 FENITROOXON pH CONTROL EXPERIMENT

the results from pH monitoring of fenitrooxon tests and controls.

At the end of the monitoring period, cultures and control media were harvested, extracted, cleaned-up and analysed by GC. The results of two experiments examining the effect of pH adjustments on fenitrothion hydrolysis are given in **Table 3-1** as experiments 2b and 4b. These indicate that an average of 0.85% of initially added fenitrothion was hydrolysed under the experimental conditions in the absence of the fungus. This represents less than 10% of the total fenitrothion hydrolysis observed in test cultures. In other words, while some fenitrothion hydrolysis results from interaction with the nutrient medium and associated pH effects, the extent of hydrolysis occurring as a result of actual fungal activity is much more significant.

Results of similar pH control experiments using fenitrooxon as the parent material are given in **Table 3-2**. 3-methyl-4-nitrophenol arising from fenitrooxon hydrolysis averages 33.0% for test cultures, while only 4.7% of added fenitrooxon is hydrolysed to 3-methyl-4-nitrophenol in the pH controlled medium. Even though somewhat more hydrolysis of oxon is evident in the pH controls, fenitrooxon hydrolysis by *T. viride*. is substantially more significant: nutrient medium interaction accounts for only 14% or the total hydrolysis observed in test cultures. In comparison, the hydrolysis of fenitrooxon by the nutrient medium and pH effects is 1.5 times that of the fenitrothion hydrolysis. This is consistent with findings of previous studies (eg. **[19]**) indicating the greater susceptibility of fenitrooxon toward hydrolysis than fenitrothion.

3.3.6 Interactions of Acclimatised Fungal Cultures

During the course of this entire study, a separate culture of T. viride was acclimatised to grow on culture medium containing 100 ppm fenitrothion. The original intent was to use

this adapted culture in a comparison of hydrolytic efficiency with "unaltered" fungus. However, measuring the relative fungitoxicity of the acclimated and original cultures in terms of mycelial growth inhibition by culture plate bioassay as described by Richardson and Miller **[63]** showed only marginal differences. The response of acclimatised *T. viride* toward 100 ppm fenitrothion and 20 ppm 3-methyl-4-nitrophenol was compared with those of unadapted fungus towards these same materials in the same concentrations. A pure culture growing on pesticide free medium served as the control.

The results of such measurements in quadruplicate are summarized in **Table 3-6.**

TABLE 3-6 RELATIVE INHIBITION OF ADAPTED FUNGAL CULTURE

	%inhibition relative to control	
	100 ppm fenitrothion	20 ppm 3-methyl-4-nitrophenol
pure *T.viride*	75 %	55 %
acclimated *T. viride*	67 %	52 %

The rather small differences between the two cultures were taken as an indication that resistance to both compounds had not changed substantially. Although this experiment does not indicate whether or not any particular enzyme system had become activated as a result of acclimatisation, the results did not appear to justify further hydrolysis experiments with the acclimatised cultures.

CHAPTER 4 - EXPERIMENTAL

4.1 INSTRUMENTATION AND ANALYSES

Proton Nuclear Magnetic Resonaance (^{1}HNMR) spectra of samples dissolved in hexadeuteroacetone or deuterochloroform were recorded using a Bruker WP-80 spectrometer. Tetramethylsilane was used as the internal standard at $\int = 0$ ppm

Mass spectra were recorded using a Hitachi-Perkin-Elmer RMU-7 double focusing mass spectrometer. Samples were introduced *via* the direct or liquid inlet depending on the nature of the sample.

A Beckman IR-12 spectrophotometer was used to record infrared spectra prepared either neat or in chloroform solution.

Combustion analyses were carried out with a Perkin-Elmer model 240 elemental analyser.

A Perkin-Elmer model 3920B gas chromatograph equipped with a flame ionization detector and glass columns (1.8 m X 6.4 mm OD) was operated isothermally at temperatures from 160-280 C, depending on the nature of the residue to be analysed. Nitrogen was used as the carrier gas at 30 mL/min. The stationary phase consisted of 3% OV-101 and 3% OV-17 on Gaschrom Q (80-100 mesh). Ethyl acetate and carbon disulphide were used as GC solvents.

Silica gel plates were prepared from microscope slides dipped into a silica

Silica gel plates were prepared from microscope slides dipped into a silica gel/chloroform slurry. Plates were then activated by drying at 110 C for one hour and spotted with pure compounds. The plates were eluted with a 4:1 benzene:ethyl acetate solvent system. Compounds were visualized by spraying the dry plates with 10% ethanolic potassium hydroxide and heating the plates at 110 C for 5 minutes. **Table 4-1** shows R_f values found using this method and comments on the reaction with the visualization reagent.

TABLE 4-1 R_f VALUES [a] OF FENITROTHION DERIVATIVES

STANDARD	R_f	REACTION WITH 10% KOH/ETOH
Fenitrothion	0.77	yellow spot, slowly with heating
Fenitrooxon	.34	yellow spot, quickly with heating
S-methylfenitrothion	.42	yellow spot, quickly with heating
Aminofenitrothion	.69	brown spot, slowly with heating
3-methyl-4-nitrophenol	.62	yellow spot, without heating

[a] conditions as described in the text above

4.2 SYNTHESIS AND PURIFICATION OF COMPOUNDS

4.2.1 Fenitrothion

One gram of technical Sumithion® (fenitrothion), a gift from the Sumitomo Chemical Co. Ltd., was dissolved in benzene (10 mL) and added slowly to the top of a 2 cm ID glass column containing 25 grams of silica gel which had been slurried in benzene. After discarding the first 20 ml of eluate, 5 ml fractions were collected and analysed by TLC. Each fraction showed only one spot, corresponding to fenitrothion, until a total of 175 ml eluate had been collected. At this point there was no evidence of any material present which was sensitive to the TLC test and so the development of the column was stopped. The combined fractions were filtered by gravity through filter paper and then benzene was evaporated *in vacuo*. The material was then dried *in vacuo* for four hours to ensure complete solvent removal. Analysis by MS, ^{1}HNMR, GC and combustion, indicated that the material was fenitrothion of high purity. Calculated for $C_9H_{12}O_5NSP$: C, 38.99 ; H, 4.36 ; N, 5.08 ; Found : C, 39.89 ; H, 4.43 ; N, 5.05 %

4.2.2 Fenitrooxon

1.3 grams of recrystallized 3-methyl-4-nitrophenol (see section 4.2.5) was placed in a 100ml pear-shaped three-necked flask fitted with a thermometer, a dropping funnel, stopper and magnetic stirrer bar. 2.7 ml of carbon tetrachloride was added and then 0.7 ml of dimethyl phosphite (d @ 25 C=0.7255 g/ml) was added dropwise *via* the dropping funnel over a period of 30 minutes. The reaction temperature was maintained at <10 C

during the addition by immersing the reaction flask in an icebath. Stirring continued for an additional 1.5 hours at room temperature (21 C). Solvent was then removed by evaporation *in vacuo* and the residue suspended in 4 ml water and extracted two times with 5 ml benzene. The benzene layer was washed twice with 4 ml of 2% aqueous sodium carbonate and dried overnight with one gram sodium sulphate in a glass stoppered flask. Solvent was removed by flash-evaporation after filtration through scintered glass and then further dried *in vacuo*. A yield of 1.27 grams or 64% was realized. Analysis of the crude product by TLC (silica gel/ether, KOH/heat) showed traces of 3-methyl-4-nitrophenol. To remove this impurity, 0.8 grams of the residue was taken up in 6 ml benzene, transferred to a 15 ml separatory funnel and washed once with 4ml water and then twice with 4 ml 2% aqueous sodium carbonate. The benzene layer was then washed five times with 5 ml portions of water until the bright yellow colour of the aqueous layer (attributable to 3-methyl-4-nitrophenol) was removed. Drying of the organic layer with 1 gram sodium sulphate and then filtering of the solution through fine scintered glass allowed evaporation of solvent under reduced pressure to give a residue which was further dried in vacuum for three hours. Samples submitted for combustion analysis, ^{1}HNMR, MS, and GC showed that the product isolated was fenitrooxon of high purity. Calculated for $C_9H_{12}O_6NP$: C, 41.39 ; H, 4.64 ; N, 5.36 ; Found : C, 41.2 ; H, 4.67 ; N, 5.37 %.

4.2.3 S-Methylfenitrothion

Fenitrothion (27.7 g = .1 mol) was added dropwise to a solution of potassium hydrogen sulphide (5.6 g KOH in 50 mL methanol saturated with H_2S). This mixture

was refluxed for 5 hours and then methanol was removed *in vacuo*. Water (160 mL) was then added, permitting the removal of unreacted fenitrothion from the O-methyl-O-(3-methyl-4-nitrophenyl) phosphorothioic acid potassium salt by 5 washings with 30 mL benzene.

Dimethyl sulphate (12.7 g) was added to the aqueous solution which was stirring at room temperature. After stirring for one hour, crude S-methylfenitrothion was taken up in 100 mL benzene and washed 10 times with 30 mL of 1% NH_4OH until the aqueous layer was no longer yellow. The benzene layer was then washed three times with 30 mL water and dried with anhydrous magnesium sulphate. Benzene was removed *in vacuo* to give 8.8 g of S-methylfenitrothion. The oil gave one spot on TLC analysis and one peak on GC analysis. Calculated for $C_9H_{12}O_5NSP$: C, 38.99 ; H, 4.36 ; N, 5.08 ; Found : C, 38.78 ; H, 4.32 ; N, 5.03 %.

4.2.4 Aminofenitrothion

Technical grade fenitrothion (0.2 g) was dissolved in 30 mL ethanol. 45 mL of 2N HCl and 2.35 g of zinc powder were added and the mixture heated in a water bath at 80 C for 10 minutes. After cooling, zinc was removed by filtration and the filtrate neutralized by addition of sodium bicarbonate solution. Two 20 mL portions of benzene were used to extract the crude product. The extract was dried (anhydrous magnesium sulphate), filtered, and 4 mg of 2,6-di-*t*-butyl-4-methylphenol (BHT) was added prior to removal of 25 mL of benzene *in vacuo* to give a solution volume of 50 mL. The storage flask was flushed with nitrogen and stoppered; this was necessary as the aminofenitrothion is very unstable under oxidizing conditions. TLC analysis gave one

spot. Calculated for $C_9H_{14}O_3NSP$: C, 43.72 ; H, 5.71 ; N, 5.67 ; Found : C, 44.47 ; H, 5.65 ; N, 5.73 %.

4.2.5 3-Methyl-4-nitrophenol

3-Methyl-4-nitrophenol was obtained from the Aldrich Chemical Co. and was purified by recrystallization from boiling water to give a melting point 129-131 C, uncorrected. Calculated for $C_7H_7O_3N$: C, 54.90 ; H, 4.70 ; N, 9.14 ; Found : C, 54.30 ; H, 4.72 ; N, 9.04 %.

4.2.6 4-Amino-3-methylphenol, *p*-Nitrophenol

4-Amino-3-methylphenol, obtained from Chemical Procurement Laboratories, and *p*-Nitrophenol, obtained from British Drug Houses, Ltd. were used as supplied.

4.2.7 Solvents and GC Internal Standards

Pure ethyl acetate was obtained by refluxing a mixture of 3 L ethyl acetate (reagent grade, Caledon Laboratories Ltd.), 300 mL of acetic anhydride and 30 drops concentrated sulphuric acid in a 5 L boiling flask equipped with a calcium chloride drying tube for 4 hours. Following distillation, the distillate was shaken with about 70 g anhydrous potassium carbonate, filtered and then redistilled and the fraction boiling at 77 C was collected. This procedure effectively removed traces of water, ethyl alcohol, acetic acid and suspended solids.

Carbon disulphide was obtained from Matheson Coleman and Bell Manufacturing Chemists in spectrograde quality and used as supplied.

Diethyl sebacate and dibutyl sebacate, used as GC internal standards, are commercially available from the Eastman Kodak Company and were used as supplied. Chromatograms resulting from GC analysis of these standards showed one peak for each compound.

4.3 p-VALUE STUDY

Ethyl acetate and water were equilibrated with respect to each other by shaking equal volumes in a separatory funnel. Fenitrothion (0.19985 g) was then dissolved in 50 mL equilibrated ethyl acetate to give a stock solution (3.997 mg/mL). Five, ten or twenty mL of stock solution was placed in a separatory funnel and shaken well with an equal volume of equilibrated water. One mL of the ethyl acetate phase was then withdrawn, dried by passing through a 3 cm silica gel column in a Pasteur pipette, and collected in a sample vial. The column was washed with an additional 1 mL of dry ethyl acetate which was also added to the sample vial. A stream of dry nitrogen was used to evaporate the solvent in the vial and the residue was made up with internal standard solution to 2.0 mL in preparation for GC analysis. One mL volumes of fenitrothion stock solution were similarly treated to permit analysis of the non-polar solvent phase before equilibration.

4.4 EFFECT OF pH ON EXTRACTION EFFICIENCY

In order to determine the effect of pH on extraction efficiency, blank fungal cultures were grown for a 14 day period as outlined in section 4.6.2. To these cultures was then added 1.0 mg of 3-methyl-4-nitrophenol in 0.5 mL acetone. After a two hour equilibration period (see section 4.7), triplicate cultures were either acidified using 0.5 N HCl (3 mL gave pH 3) or 2 N KOH (15 mL gave pH 8), or left neutral (cultures were pH 6.5 at the end of the growth period). The cultures were then extracted and worked up as per section 4.7 and prepared for GC analysis.

4.5 AMBERLYST A-21 RESIN CLEANUP

Water hydrated A-21 ion-exchange resin was preconditioned by placing 25 cm of resin in a 1.5 cm ID chromatography column and eluting the column with 100 mL methanol at a rate of 2 bed volumes (50 mL) per hour. Methanol was then displaced by ethyl acetate using the same procedure and flow rate. Conditioned resin was stored in ethyl acetate in glass stoppered flasks.

Ion-exchange columns were made by drawing out one end of a KIMAX 10-M glass sealing tube in which was imbedded a medium pore size scintered glass disc. The column (1.15 cm I.D.) was 10 cm in height above the disc and 2.5 cm below, at which point the tube had been drawn out to 0.45 cm ID. Columns were graduated to 1.5 mL above the glass scinter to permit the use of a consistent volume of resin throughout the clean-up. Test runs of ethyl acetate sovent flow through the resin and the scintered glass disc gave flow rates of 3 mL/30 minutes or 4 bed volumes per hour. This rate was constant among the 7 tubes manufactured.

In preparation for an extract cleanup, 1.5 mL of resin was added to the column in ethyl acetate slurry and a filter paper disc placed on top of the resin to prevent disturbing the bed on addition of solvent. At least 5 mL of clean ethyl acetate was then allowed to flow through the bed. The extract was then taken up in 0.5 mL ethyl acetate and transferred by Pasteur pipette to the top of the filter paper disc once the solvent had reached that level. 0.5 mL volumes of clean ethyl acetate were added to the sample vial and subsequently transferred to the column to ensure complete extract transfer. This procedure was repeated until a total of 4 mL ethyl acetate had been added to the column. All ethyl acetate eluate was collected as one fraction in a sample vial for each column.

One mL of 1.5 N HCl in anhydrous methanol was pipetted into the column before the last aliquot of ethyl acetate had drained past the top of the resin bed. This acidic solution was prepared by bubbling hydrogen chloride, generated by the action of suphuric acid on ammonium chloride, through sulphuric acid to remove any water vapour, and into a preweighed bottle containing anhydrous methanol until the solution had gained sufficient weight to give the required normality (5.6 g HCl/100 mL MeOH). Anhydrous solvent was used to avoid hydrolysis of derivatising reagents employed in the subsequent GC analysis. Once the acidic methanol had reached the top of the resin bed, an additional 5 mL was placed on the column, the acidic eluate being collected as one fraction. Progress of elution could be monitored since adsorbed 3-methyl-4-nitrophenol appeared as a yellow band which moved downward with acidified solvent. Ethyl acetate or methanol was removed from the eluates in a stream of dry nitrogen. The cleaned-up residues could then be made up with internal standard solution or derivatised as required for gas chromatographic analysis.

4.6 CULTURE MAINTENANCE AND GROWTH CONDITIONS

Trichoderma viride was maintained in the dark by serial and sterile subculturing on nutrient agar (7 g dextrose, 1 g casamino acids, 0.5 g yeast extract, and 1 g cellulose powder per litre of distilled water) in plastic Petri dishes. After autoclaving (20 minutes @ 250 C), plates were poured (about 30 mL solution per plate), allowed to cool, and then innoculated with a 9 mm diameter innoculum cut from a previous subculture.

For liquid cultures 200 mL of nutrient medium (5 g dextrose, 1 g casamino acids, 0.5 g yeast extract, 1 g KH_2PO_4, 0.5 g $MgSO_4{:}7H_2O$ per litre distilled, deionized water plus 0.2 mL vitamin stock and 2.5 ml mineral stock solutions) was placed in 500 mL flasks and the flasks sealed with foam plugs. Flasks and contents were then autoclaved at 250 C for 20 minutes. Mineral stock (98 mg $FeCl_3{:}6H_2O$, 78.5 mg $CuSO_4{:}5H_2O$, 40.5 mg $MnSO_4{:}4H_2O$, and 88 mg $ZnSO_4.7H_2O$ made up to 250 mL with distilled , deionized water) and vitamin stock (25 ug Biotin, 500 ug Thiamine, 500 ug Pyridoxine, and 25 ug Inositol made up to 100 ml with 40% ethanol and diluted a factor of 1:100 with distilled water before use) were separately prepared and stored at room temperature and under refrigeration, respectively. Liquid cultures were grown on a table shaker (12 cm stroke, 90 rpm) at room temperature (22 C).

4.7 HARVESTING AND WORKUP OF LIQUID CULTURES

After the typical incubation period of 14 days, all culture flasks, except those designated as extraction efficiency controls, were removed from the shaker table. The test compounds in acetone solution were added to controls and the culture flasks then

placed on the shaker for another 1 1/2 hours to permit an equilibration of the test compounds between aqueous solution and mycelium. This allowed a compensation for mycelial adsorption, if any, of the test compounds.

All liquid cultures were individually filtered by suction through filter paper to separate mycelia and the aqueous media. After removal of the agar plug of the original innoculum, the mycelia were washed with 10 mL water and the solution quantitatively transferred to a separatory funnel. Mycelia were then washed with 10 mL ethyl acetate which was transferrred to the separatory funnel, with rinsing of the flask to ensure complete material transfer. The mycelial mat, which was at this point partially dehydrated as a result of the solvent washing, was then either retained for further exhaustive extraction with ethyl acetate or transferred to a drying oven for dry weight determination in the growth studies (see sections 4.11 and 4.12, respectively).

The aqueous phase (pH=6.5) was acidified in the separatory funnel to pH=3 with 3 mL 0.5N HCl and then extracted three times with 50 mL volumes of ethyl acetate. Relatively stable emulsions formed were broken by the addition of about 2 mL ethanol. The organic phases were combined and dried with approximately 5 grams of anhydrous sodium sulphate. Solutions were then filtered by suction through scintered glass into a round bottom flask and ethyl acetate was removed by flash evaporation. Residues were quantitatively transferred by pipette to 10 mL screw cap vials with a minimal amount of ethyl acetate. This solvent was subsequently evaporated with a stream of dry nitrogen and the use of a heated (45 C) sand bath. The vials were sealed with a rubber/teflon septum and cap and stored under refrigeration until further cleanup, if required, and preparation for GC analysis.

4.8 QUALITATIVE IDENTIFICATION OF FENITROTHION METABOLITES

Culture medium was separated from the mycelia by suction through filter paper. The aqueous solutions were then extracted with three 50 mL portions of ethyl acetate and the extracts concentrated by evaporation of solvent under reduced pressure. A small amount of ethyl acetate was added to the extracts which were then analysed by TLC.

4.9 FUNGAL INTERACTIONS WITH TEST COMPOUNDS

Liquid cultures for fenitrothion and fenitrooxon hydrolysis and 3-methyl-4-nitrophenol metabolism experiments were grown as described in section 3.2. Cultures were spiked upon innoculation with either 20 ppm (4 mg/200 mL) fenitrothion or fenitrooxon or with 5 ppm (1 mg/200 mL) 3-methyl-4-nitro- phenol in 0.5 mL acetone depending on the experiment. All cultures were grown and treated in triplicate. Control cultures were equilibrated, after spiking with the test compound, for an additional 2 hours on the rotary shaker before extracton and analysis to determine extraction efficiency. Blank cultures, containing only fungus and culture medium, were grown as well, and used to determine potential interferences created by natural fungal metabolites. To blank and control cultures was added 0.5 mL acetone to compensate for any effects of the acetone carrier of the test cultures. All cultures were harvested after 14 days incubation.

In the case of delayed addition experiments, the pesticide or metabolite in 0.5 mL acetone was added aseptically to the fungal culture by syringe through the foam plug at the appropriate time.

4.10 pH CONTROL EXPERIMENTS

At periodic intervals, cultures, grown under the conditions of section 4.7, were removed from the rotary shaker. To sample a culture, the foam plug was removed briefly from the flask and a wide-mouthed pipette, previously sterilised by autoclaving, was used to withdraw a 5 mL aliquot of culture medium or mycelial suspension after which the foam plug was replaced. The sample was transferred to a test tube which could accomodate a pH electrode and the pH of the solution recorded. pH control cultures were then adjusted to the pH of their corresponding test culture by the injection of an appropriate volume of 0.5 N HCl or KOH, using a sterile syringe, through the foam plug. The volume of acid or base added for adjustment was determined by first correcting the pH of the 5 mL control aliquot, and then converting the volume added to compensate for the larger liquid volume in the culture flasks. This method permitted fine pH adjustments to within .1 pH unit. Material removed for sampling was not be returned to the original culture to maintain aseptic conditions.

At the end of the growth period, test compounds were added to extraction efficiency control cultures in order to determine recovery efficiences. All cultures were extracted, cleaned-up, and analysed by GC as outlined in the experimental sections 4.7 and 4.5.

4.11 EXHAUSTIVE MYCELIAL EXTRACTION

The dehydrated mycelial mat from the harvest and workup of liquid cultures (section 4.7) was placed in a ceramic mortar, frozen with liquid nitrogen, and ground to a fine powder with a pestle. The powder was transferred to a 250 mL beaker equipped with a magnetic stirrer bar and 50 mL ethyl acetate was added. After 4 hours stirring at room

temperature, the suspension was filtered, the residue rinsed with 10 mL ethyl acetate, and the filtrate transferred to a round-bottom flask. Evaporation of solvent under reduced pressure gave residues which were transferred to 10 mL screw cap vials with minimal amounts of ethyl acetate. The solvent was removed with a stream of dry nitrogen, and the vial sealed and stored in preparation for GC analysis (section 2.3.2).

4.12 DRY WEIGHT DETERMINATIONS

Liquid cultures were filtered through preweighed, oven-dried and desiccated filter paper and rinsed with ethyl acetate. The mycelial mat and filter paper were placed in a drying oven at 105 C for 2 days and transferred to a desiccator overnight. Dry mycelial mass was determined by weight difference.

4.13 CULTURE PLATE BIOASSAY

Fungitoxicity testing was carried out in Petri dishes with the medium used to maintain fungal cultures (section 4.6) except that the medium was amended with 100 ppm fenitrothion or 20 ppm 3-methyl-4-nitrophenol. The acclimatised *T. viride* used in this experiment was obtained from the seventeenth subculture of *T.viride* on 100 ppm fenitrothion medium. Innocula were cut from the appropriate fungal culture plate and placed in the centre of a newly prepared amended or unamended agar plate in quadruplicate. When the controls had grown to the full extent of the plate (about 5 days), colony diameters were measured. Degree of mycelial growth inhibition was calculated from the mean differences between the controls and the treated plates, and expressed as a percentage of the former.

CHAPTER 5 - CONCLUSIONS

From the experiments discussed in chapters 2 and 3 some general conclusions can be drawn.

Studies of the extraction of fenitrothion and derivatives from liquid culture indicate that ethyl acetate is an efficient extraction solvent for fenitrothion metabolites, especially the more polar phenolic derivatives. The pH of the aqueous phase is an important consideration and must be adjusted to ensure optimal recovery of acidic (or basic) test compound metabolites. Furthermore, differences in metabolite polarity or acidity can be used to advantage when clean-up of complex residues is required: in this study the acidic 3-methyl-4-nitrophenol is easily separated from fenitrothion and fenitrooxon by using a weakly-basic anion-exchange resin.

Experiments involving fungal interactions with fenitrothion indicate that biodegradation involves oxidative and hydrolytic pathways. The extent of fenitrothion oxidation was not quantifiable because the oxidation product was shown to be hydrolysed more rapidly than the parent material. Whether fenitrothion is first oxidised and then hydrolysed, or if this pathway operates in conjunction with the direct hydrolysis of fenitrothion is unclear.

Fungal metabolism studies with 3-methyl-4-nitrophenol show that a substantial portion of this phenol disappears during the first few days of fungal growth and that progressively higher nitrophenol recoveries are obtained when this material is added after the period of maximum culture growth. This can be explained by cometabolism of the

phenol: organophosphorous hydrolysis products have been reported to be used by microorganisms as carbon and energy sources (eg. **[47] [48] [49]**). The hypothesis of cometabolism can account for the missing material in the fenitrothion and fenitrooxon metabolism experiments. This may be particularly valid if cometabolism of the nitrophenol occurs after fenitrothion hydrolysis rather than through its direct consumption. Clarification of the precise biodegradation pathway might be obtained through further studies involving radio-isotopically-labelled parent and metabolite compounds.

Trichoderma viride may be an excellent microorganism with which to carry out further studies of enzymatically-controlled detoxification of organophosphorous compounds. Significant quantities of biomass are produced in a relatively short time and so this may be an ideal source of the required hydrolase (or oxidase) enzyme: *T. viride* is resistant to the toxicological effects of fenitrothion and is capable of degrading the pesticide.

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