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# The Uptake and Metabolism of Fenitrothion During Forest Tree Seed Germination and Seedling Growth

By

Douglas J. Hallet BSc. MSc.

A Thesis

Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
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Scope and Contents: Metabolic pathways for activation and detoxification of fenitrothion were examined in germinating seeds and seedlings of eastern white pine, white spruce, and yellow birch.

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## RESUMÉ

La germination des graines de pin blanc (Pinus strobus L.), d'épinette (Picea glauca L.) et de bouleau jaune (Betula alleghaniensis L.) s'est faite dans une solution aqueuse contenant 10ppm de fenitrothion ou thinophosphate de 0,0 -dimethyle O-(methyle-3-nitro-4-phenyle).

Dans le pin blanc, le fénitrothion a été détoxifié par la transférase S-alcoyl, une enzyme dont l'activite depend du glutathion, et cette detoxification a donné du desmethyle fénitrothion et du S-méthyle glutathion. Le pesticide a aussi été activé, donnant du fenitro-oxon, un compose encore plus toxique que le fénitrothion. On a aussi démontré que la fonction desmethyle presente dans les semences a été réactivée par alcoylation du fénitrothion pour former du S-méthyle fénitrothion, un inhibiteur de la e-cholinestérase, encore plus puissant que la substance mère.

Un traitement à une concentration de 1000 ppm de fénitrothion a été suivi par une inhibition de la germination des semences de bouleau jaune, mais a été sans effet sur les semences de pin blanc et d'épinette. On attribue la toxicité plus élevée envers le bouleau jaune, qui accummula beaucoup plus de pesticide que les deux espèces de conifères, à une diminution sensible du glutathion dans les tissus.

Les propriétés mutagènes du fénitrothion et de sept composés derivés ont été etudiées par alcoylation de l'ADN chez une espèce "indicatrice" de Salmonella typhimurium sensible aux substitutions de bases paires causées par des agents alcoylènes. Aucune propriété mutagène n'a été mise en évidence pour le fénitrothion ou ses derivés.

## Abstract

Fenitrothion (O,O-dimethyl-O-(4-nitro-m-tolyl) phosphorothioate) was absorbed by germinating seeds of eastern white pine; Pinus strobus L. (Moench) Voss, white spruce; Picea glauca L., and yellow birch; Betula alleghaniensis L., from an aqueous solution containing 10 ppm of the pesticide. Metabolism of the pesticide was examined in young seedlings with $OC^{14}H_3$ labelled fenitrothion. Fenitrothion was shown to be detoxified by dealkylation in white pine via a glutathione dependent S-alkyl transferase enzyme to form desmethyl fenitrothion and S-methyl glutathione. Dealkylation was also found in white spruce and yellow birch seedlings. The pesticide was activated to form fenitro-oxon, which is more toxic than the parent compound. The desmethyl form was shown to be reactivated via alkylation by fenitrothion to form S-methyl fenitrothion which is a more potent cholinesterase inhibitor than the parent compound.

Treatment of the seeds at higher concentrations of 1000 ppm fenitrothion inhibited germination of yellow birch seeds but not white pine or spruce. Severe depletion of tissue glutathione levels was shown to be related to toxicity in yellow birch which absorbed much higher levels of pesticide than the two coniferous species.

Fenitrothion and seven derivatives of the compound were tested for mutagenicity via alkylation of replicating DNA in an indicator strain of Salmonella typhimurium, sensitive to base-pair substitutions caused by alkylating agents. No mutagenic activity was evident for fenitrothion or its derivatives in this system.

# Introduction

## A. Review of the Literature:

In recent years, with the increased awareness of the interdependence of organisms and the fragility of the ecological balance within each habitat, many of the more persistent organochlorine insecticides have been replaced by organophosphorus compounds. The former class of compounds persist in the natural environment and accumulate in terrestrial and aquatic biota over long periods of time. As one proceeds up the food chain, residues are concentrated and symptoms of acute toxicity and teratogenicity are expressed in the higher organisms of the food web (Woodwell et al, 1971 and Matsumura, 1975). Organophosphorus insecticides are more susceptible to both physical and biological degradation, and are readily removed from the environment (Bull, 1972). However, they are neurotoxic, and the mode of action of this class of compounds is via inhibition of acetylcholinesterase enzymes in mammals and birds.

Fenitrothion is a broad spectrum organophosphorus insecticide. The low mammalian toxicity of this pesticide was first reported by Drabek and Pelikan in 1956 (Drabek and Pelikan, 1956). The synthesis of fenitrothion was also later described by Nishizawa (1961) by the reaction of o,o-dimethyl phosphorochloridothioate with 3-methyl-4-nitrophenolate. It was examined as an experimental insecticide and later made commercially available by Sumitomo Chemical Co. under the trade name Sumithion. The compound was shown to have a low acute dermal toxicity with an $LD_{50}$ for mice of over 3000 mg/kg (Nishizawa, 1960). It was also introduced independently by Bayer Leverkusen (Folithion) and by American Cyanamid Company (Accothion). The acute oral $LD_{50}$ of the technical product for

rats was 250 to 500 mg/kg, and for mice 870 mg/kg (Schrader, 1961). This mammalian toxicity is much less than that of other organophosphorus insecticides and it is for this reason that the pesticide is used extensively in Europe, China, Japan, and South America to prevent destruction by defoliators, stem borers, and weevils (NRCC No 14104, 1975).

Infestations of the lepidopterus defoliator spruce budworm Choristoneura fumiferana Clem. in Eastern Canadian forests has caused severe damage to economically important stands of Canadian white spruce and balsam fir. Fenitrothion has been registered in Canada and utilized for the control of spruce budworm since 1969 when DDT was banned for this purpose. It is applied to the forests as an aerial spray. The registered rate is 2 to 6 oz/acre, but not to exceed 6 oz/acre annually. This dosage was considered adequate to prevent defoliation by lepidopterous insects (Fettes, 1968). Each year increasingly larger areas of Canadian forests have been sprayed and in 1973 fenitrothion was applied to over 16 million acres of Eastern Canada (NRCC No 14104, 1975). The pesticide is also registered in Canada for agricultural use on a trial basis at 28.8 oz/acre on apples and 15 oz/acre on potatoes (Compendium of Registered Uses of Pesticides in Canada, Plant Products Division, Agriculture Canada, 1973).

Insect outbreaks usually reach epidemic proportions on a cyclical basis. There is a short time period (between the second and third instar larvae), when the pesticide application is most effective for controlling the outbreak. Rapid and massive spray operations have been considered necessary to meet this requirement. Currently, therefore, fenitrothion is applied to Canadian forests from large, multi-engined aircraft

travelling at speeds of about 250 mph (Randall, 1974).

Low dosage rates of fenitrothion (4 oz/acre) were shown to be 80 to 90 percent effective against spruce budworm when sprayed in small droplets (165 to 200 um in diameter) containing a high concentration of active ingredient. The small droplet size increases insect mortality, but it also increases losses due to atmospheric drift (Randall, 1974), although overlap of spray flight paths has been minimized with electronic navigation and guidance systems. The drift of pesticide sprayed from the high speed aircraft may expose localized areas to multi-applications of fenitrothion. Minute droplets of fenitrothion 20 to 30 um in size, were found up to 3 miles from the source of emission (Randall, 1974). Small amounts of fenitrothion have also been recovered in local towns downwind of large scale spray operations in New Brunswick (Yule, Cole, and Hoffman, 1971). They were not considered to constitute a health hazard to residents by the investigators because of the low levels involved. Recently Randall (1977) theoretically demonstrated that with a droplet size ranging from 55 to 120 μ, 1 ounce of fenitrothion per acre would kill 95 percent of 5th instar spruce budworm larvae on contact.

In Canadian forests, the persistence of fenitrothion was studied on balsam fir and mixed spruce foliage (Yule and Duffy, 1972). Fenitrothion was recovered from the foliage at levels of 3 to 4 ppm, one day after an aerial application at a rate of 4 oz/acre. A large part of this spray disappeared rapidly; 50 percent within 2 days, but from 15 to 30 percent persisted for up to 2 weeks. Very little fenitrothion applied by this aerial spray was shown to penetrate the forest canopy and in the top 6 inches of forest soil a maximum of 0.04 ppm of fenitrothion was recovered up to 2 weeks after spraying.

This work represented the only residue data of fenitrothion in a Canadian forest ecosystem. It was therefore determined that as a prerequisite for more detailed environmental impact studies, refined methodology be developed that would enable the absorption and metabolism of fenitrothion to be followed in forest seeds and seedlings under controlled laboratory conditions. Basic information derived from this study would then be applicable to future large scale field studies on the potential environmental impact of the pesticide.

Recently Yule reported that up to 1 ppm of fenitrothion does persist in coniferous foliage throughout the year when subjected to repeated annual applications of the pesticide at a dosage rate of 3 to 5 oz/acre for 5 years (Yule, 1974). McLeod (1975) has also found a possible residual effect of fenitrothion on Swaine jack pine sawfly Neodiprion swainei Midd., 42 days after aerial application of the pesticide to Quebec forests. These two recent pieces of evidence contradict the earlier indications that residual fenitrothion and toxic effects to the ecosystem due to fenitrothion would be negligible 2 weeks following application to forests (Yule and Duffy, 1972).

(i) Mode of toxicity:

Fenitrothion is a desirable insecticide in view of its selectively high toxicity to insects such as spruce budworm and low toxicity to mammals, at operational levels. The mode of toxicity of organophosphorus insecticides to both mammals and insects is via cholinesterase inhibition. This enzyme inhibition causes rapid accumulation of acetycholine in nervous synapses, increasing nervous activity, resulting in the nervous disorientation and eventual death of treated insects.

Nicotinic or muscarinic effects begin in mammals with eventual loss of function of the central nervous system (Brady and Sternberg, 1967).

Several theories have been presented to account for this selective toxicity of fenitrothion. Hollingworth, (1967, a) studied the difference between inhibition of fly head and mammalian cholinesterase by fenitrothion and methyl parathion. Fenitrothion is structurally similar to methyl parathion except for a methyl group in the 3 position of the phenyl ring of fenitrothion. The addition of an alkyl group in this position increased the inhibitory potential of the molecule to fly head cholinesterase while decreasing the inhibition of mammalian cholinesterase. The 3-methyl effect is primarily due to increased affinity for fly head acetylcholinesterase and decreased affinity to mammalian acetylcholinesterase enzymes. Affinity with the enzyme should be dependent largely on the steric features of the enzyme molecule. Hollingworth suggested that there is interaction of the pesticide at both an esteratic and anionic site of the enzyme. The distance between the anionic and esteratic site of the fly head acetylcholinesterase (5.0 to 5.5 Ångstroms), accommodates interaction of the phosphoryl group at the esteratic site and the 3-alkyl group at the anionic site. This would aid initial binding to the enzyme owing to Van der Waals or lipophilic interaction of the 3-alkyl group with the anionic site. This distance between the phosphorus atom and 3-alkyl group is 5.2 to 6.5 Ångstroms. The distance between sites on the mammalian enzyme is too short to accommodate this (4.3 to 4.7 Ångstroms for bovine acetylcholinesterase), thus hindering the enzyme-inhibitor complex formation. Prior studies have not been concerned with the possibility that fenitrothion may be toxic to the host plant

species. Acetylcholinesterase has recently been shown to occur in mung bean roots and to participate in phytochrome mediated responses (Jaffe, 1973). It is therefore possible that fenitrothion could alter plant growth through acetylcholinesterase inhibition.

There are four main factors related to the toxicity of an organophosphorus pesticide to any living species, be it arthropod, mammal, or plant. These involve the rate at which the compound can penetrate the organism, the rate at which the compound can be activated to an acutely toxic form, the rate of enzyme inhibition of the active forms, and the rate at which the organism can detoxify the compound in either its parent or active forms into non-toxic, polar, water soluble compounds which can be eliminated from the animal by normal excretion.

Fenitrothion is a very lipophilic molecule and as such has a great affinity for biological material. It was shown to penetrate the cuticle of insects such as the cockroach and the gut lining of mammals rapidly (Miyamoto et al 1963). Penetration into plants is also rapid. Miyamoto and Sato (1965) found that 50 percent of a 25 percent aqueous emulsion of fenitrothion sprayed on rice plants at the preheading stage of growth penetrated into the inner tissues within 24 hours.

Phosphorothioates themselves, such as fenitrothion are only weak inhibitors of cholinesterase and must be converted to more active forms in order to inhibit the enzyme. The normal route of activation is via oxidative desulfuration of the molecule to give the oxygen analogue fenitro-oxon (Miyamoto et al, 1964). Conversion to fenitro-oxon decreased the oral $LD_{50}^{*}$ of the pesticide to the white mouse from 1250 mg/kg to 120 mg/kg (Hollingworth, 1967, a), and also decreased the

* $LD_{50}$ is the dose which kills half of the animals under the conditions of the test

$I_{50}$* for fly head cholinesterase from 1.47 X $10^{-6}$ (Kovacicova et al, 1973) to 5.6 X $10^{-8}$ (Hollingworth, 1967, a). In vertebrates this oxidative reaction occurs in the liver microsomes in the presence of $NADPH_2$ and oxygen (Brodie et al, 1958). In insects the fat body takes on this function acting not only as a depot for metabolic products, but also as the site of active metabolic processes (Kilby, 1964). In plants, the oxons are formed by microsomal oxidase enzymes such as lipoxidase enzymes present in wheat germ (Rowlands, 1968, Little and O'Brien, 1968). Oxidative desulfuration activity is linked to electron transport (cytochrome P 450) and with the normal aerobic peroxidation of unsaturated fats in the embryo (Gardner and Inglett, 1968).

Fenitro-oxon was found in the leaf blade and sheath of rice plants 24 hours after the initial application (Miyamoto and Sato, 1965). Sundaram and Sundaram (1969) determined the persistence of $^{32}P$ labelled fenitrothion in the cocoa tree. They found that fenitrothion was absorbed rapidly when applied to leaves and was completely metabolized within the first five days after application. Fenitro-oxon was first detected in the plant 5 days after application but had completely disappeared by the eleventh day.

Kovacicova et al. (1971) showed that commercial preparations of fenitrothion contained some S-methyl isomer which was reported to inhibit cholinesterase more strongly than fenitrothion itself. During the course of this study the in-vitro toxicity of the S-methyl isomer of fenitrothion was further elucidated by Kovacicova et al, (1973). S-methyl fenitrothion was found to have two to three orders of magnitude

* $I_{50}$ is the molar concentration of an enzyme inhibitor which halves the rate at which the enzyme converts its substrate.

greater anticholinesterase activity than the parent compound. Cholinesterase preparations used in this work were from horse and human blood serum and fly heads. It was later shown by Greenhalgh et al, (1975) that technical grade fenitrothion obtained from different chemical suppliers contained from 0.43 to 4.43 percent S-methyl fenitrothion. It was considered to be formed by isomerization during long periods of storage. Myatt et al, (1975) found that when comparing the inhibitory effect of S-methyl fenitrothion and fenitro-oxon on cholinesterases that there was no difference for inhibition of acetylcholinesterase from bovine erythrocytes or electric eels. There was a two and three times enhancement of inhibition by S-methyl fenitrothion for guinea pig and human plasma or pseudocholinesterases respectively.

The major detoxification mechanism for organophosphorus insecticides in plants, mammals and insects is via enzymatic hydrolysis. With fenitrothion this involves cleavage of the P-O-aryl bond to give water soluble metabolites including; dimethyl phosphorothioic acid (from fenitrothion), dimethyl phosphoric acid (from fenitro-oxon), and 3-methyl, 4-nitrophenol. The major route of detoxification of fenitrothion in mammals occurs through dealkylation of the O-methyl group to form desmethyl fenitrothion and desmethyl fenitro-oxon only when the insecticide is applied at high dosage rates (Hollingworth, 1969, 1973).

O-dealkylation of organophosphorus insecticides to the corresponding mono acid is recognized as one of the main mechanisms for detoxification in animals, and has been implicated in the development of resistance by certain insects to organophosphorus insecticides. Bull, (1972), indicated three possible biochemical mechanisms by which O-dealkylation

could take place. The first involves an oxidation process mediated by NADPH requiring microsomal enzymes. Donninger et al (1966) showed that chlorfenvinphos was desethylated by microsomal preparations of rabbit liver, requiring NADPH and oxygen to give desethyl chlorfenvinphos and acetaldehyde. Hollingworth (1969) showed that dealkylation of fentrothion did not require microsomal oxidase enzymes or require an energy source $NADPH_2$.

The second mechanism involves a glutathione dependent alkyl transferase enzyme. Fukami and Shishido (1963) showed that methyl parathion could be demethylated by the supernatant fraction of rat liver microsomal preparations. Hutson et al (1967) using pig liver supernatant demonstrated that the reaction involved alkylation of the reactive SH of glutathione by isolating S-methyl glutathione and desmethyl gardona. Miyamoto et al (1968) has shown that both fenitrothion and fenitro-oxon were dealkylated by rat liver homogenate in-vitro. Hollingworth et al (1973) examined the glutathione transferase enzymes in liver homogenates and found that dealkylation of methyl phosphate esters such as fenitrothion is favoured over dearylation. The reverse is true for ethyl phosphate esters such as parathion.

The third mechanism involves hydrolytic cleavage of the alkyl carbon-oxygen bond to give the O-dealkylated product and an alchol. Nolan and O'Brien (1970) found ethanol as a major metabolite from the in vivo dealkylation of paraoxon by certain strains of house flies.

Glutathione transferase systems have also been shown to be present in plants. They have been shown to be responsible for the detoxi-

fication of triazine herbicides such as atrazine. An atrazine-glutathione conjugate has been shown to form in corn (Shimabukuro et al, 1970) and sorghum leaf sections (Lamoureux et al, 1970).

Miyamoto and Sato (1965) found that 80 percent of the total $^{32}P$ activity attributed to labelled fenitrothion applied to rice plants at the preheading stage was metabolized into water soluble metabolites after 1 week. These resulted from the hydrolysis of fenitrothion and fenitro-oxon to form dimethyl phosphorothioic acid, phosphorothioic acid, and phosphoric acid. Desmethyl fenitrothion represented about 10 percent of the water soluble metabolites one day after application. The metabolites were found in rice leaf blades and also in rice grains. Some fenitro-oxon was found in the leaves and sheaths but not in the rice grains.

Another detoxification mechanism for fenitrothion, and fenitro-oxon has been reported for the reduction of the nitro group to form the respective amino derivatives. This mechanism of detoxification is of major importance to anaerobic bacteria and ruminant animals, and is only of minor importance in other animals and plants (Bull, 1972). The toxicity of amino fenitrothion is unknown, however reduction of the nitro group of parathion and of para-oxon, which have structures similar to fenitrothion was shown to lower the acute oral $LD_{50}$ to rats by 10 to 20 times respectively and also lowered the topical toxicity to flies 1,000 fold (Ahmed et al, 1958). Bacterial metabolism of fenitrothion has been found to be an important mechanism in polluted river water and in agricultural and forest soils. Bacteria such as Bacillus subtilis were

shown to degrade fenitrothion into amino fenitrothion, desmethyl fenitrothion, and dimethyl phosphorothioic acid (Yasuno et al, 1965, Miyamoto et al, 1966). Fungi and yeast did not metabolize the pesticide. Zitko and Cunningham (1974) showed that river water, especially polluted water, degraded fenitrothion rapidly (half-life, 50 hours) and yielded primarily aminofenitrothion. In agricultural soils, some organophosphorus insecticides alter $CO_2$ evolution rates and nitrate production rates. Chlorinated insecticides have little effect on soil microflora (Bartha et al. 1967, Martin et al, 1957). Treatment of forest soils with massive doses of DDT and fenitrothion did not alter species composition of fungi, bacteria, and actinomycetes. Population numbers of aerobic and anaerobic microbes were not changed. After a 12 month incubation period less than 0.4 percent of the applied fenitrothion remained in softwood or hardwood mull (Salonius, 1971). The microflora of the forest floor, which are important for active plant growth, were capable of degrading the low levels of fenitrothion residues to which they were exposed and were not significantly affected by the pesticide.

(ii) Physical-chemical conversions:

Physical factors and chemical conversions which either activate or detoxify the pesticide readily occur in the natural environment. These processes are important and likely account for the rapid initial losses of up to 50 percent of fenitrothion applied to agricultural crops or forests. This phenomenon occured in spruce and balsam fir foliage within 1 day after aerial application (Yule and Duffy, 1972). Attempts were made to control these processes in the laboratory studies to reduce variability in the results.

Ohkawa et al, (1974) has shown that fenitrothion undergoes rapid photodecomposition on irradiation with u.v. light or sunlight in solution and when applied to plant leaves, to yield carboxyfenitrothion as the main product together with traces of fenitro-oxon, carboxyfenitro-oxon, 3-methyl-4-nitrophenol, 3-carboxy-4-nitrophenol, and S-methyl fenitrothion. Since the S-methyl isomer of fenitrothion was shown to have the highest potential as an anticholinesterase inhibitor of any fenitrothion derivative (Kovacicova et al, 1973) and was unknown as a biological metabolite prior to this work, it will be considered in detail.

S-alkyl isomers of organophosphorus insecticides are readily formed by a number of reactions. Irradiation of parathion with u.v. light produced both paraoxon and S-ethyl parathion (Cook and Pugh, 1957). Joiner and Baetcke (1974) isolated ten more products on irradiation of parathion. Solar radiation of the pesticide on cotton leaves sprayed in the field produced both S-ethyl and S-phenyl parathion (Joiner and Baetcke, 1973).

Isomerization on heating is the main mechanism of forming S-alkyl isomers of phosphorothioate esters. Jaglan and Gunther (1970) showed that methyl parathion would yield 50 percent of the S-methyl isomer after 7 hours at 125$^{o}$C. However, such extreme temperatures were not approached in the germination procedures undertaken for the present study.

A third method for the formation of S-methyl isomers employs the action of alkylating agents. Kovacicova et al, (1973) used dimethyl sulphate to alkylate the potassium salt of desmethyl fenitrothion in order to prepare S-methyl fenitrothion.

Methyl phosphate esters themselves are also alkylating agents. Eto et al, (1968) reported that when fenitrothion was left in dimethyl formamide for 30 days, half of the parent compound disappeared and both desmethyl fenitrothion, and S-methyl fenitrothion were produced. They showed that a mixture of fenitrothion and desmethyl fenitrothion in acetone produced 16 percent S-methyl fenitrothion after 14 hours at $60^{o}C$.

Alkylating agents can act as mutagens (Ames, 1972). Organophosphate insecticides such as dichlorvos have been shown to alkylate nucleic acids in vitro and some micro-organisms in vivo (Bedford and Robinson, 1972). Genetic damage and mutagenicity to plants and animals caused by long term subacute exposure to organophosphates is open to speculation due to the expense and time involved for adequate testing. (Plapp, 1976).

Fenitrothion is soluble in water at low concentrations. It is relatively stable in pure neutral or slightly alkaline water (pH 7 to 9) at $20^{o}C$ for up to 45 days. As alkalinity increases the hydrolysis rate increases (Zitko and Cunningham, 1974). It appears that removal of fenitrothion from natural precipitation and water bodies is partially by microbial metabolism detoxifying the pesticide to form aminofenitrothion and also by photodegradation (Ohkawa et al, 1974).

(iii) Methodology for Residue Analysis:

Analytical methods for fenitrothion residues have been primarily concerned with the parent compound. In the previous review it was pointed out that some metabolites of fenitrothion such as fenitro-oxon

and S-methyl fenitrothion have high anticholinesterase activity. Previous methodology was therefore critically evaluated and further adapted to recover these more actively toxic compounds from the germinating seeds and growing seedlings.

Gas chromatography is generally the method of choice for the quantitation of fenitrothion and its metabolites. Electron capture (EC) detectors were initially used for fenitrothion (Dawson et al, 1964, Kahazawa and Kowhara, 1966). Selective phosphorus detectors (the alkalai flame ionization detector (AFID), and the flame photometric detector (FPD), have been more recently developed. They have proven to be very sensitive, specific, and therefore useful for organophosphorus residue analysis. Bowman and Beroza (1969) employed the FPD whereas Miyamoto and Sato (1969) used the AFID in their studies. The sensitivity of the AFID is generally greater than the FPD by a factor of 10 to 30.

The use of the more sensitive and specific phosphorus detectors reduced the need for the extensive clean up of sample extracts necessary with the EC detectors. Oils and fats can be removed by hexane/acetonitrile partitioning. The p-values in this system at 25°C of fenitrothion, fenitro-oxon, and 3-methyl-4-nitrophenol were 0.035, 0.007, and 0.012 respectively (Bowman and Beroza, 1969). Final cleanup of the sample may then be carried out by column chromatography. Good recoveries (97 percent) were obtained for fenitrothion on either activated Florisil (Beckman and Garber, 1969) or carbon (Watts et al, 1969). Bowman and Beroza (1969) reported 96 percent recovery of fenitrothion, 80 percent recovery of fenitro-oxon, and 96 percent recovery of 3-methyl-

4-nitrophenol on deactivated silica gel (containing 20 percent by weight water). They also reported that active (dried at 110°C overnight), neutral alumina decomposed the fenitrothion. Prior to this study S-methyl fenitrothion had not been implicated as a naturally occurring metabolite of fenitrothion. Sensitive analytical techniques for the quantitation from tissue extracts of this metabolite and also desmethyl fenitrothion were not previously available and had to be developed in the course of the present study.

B. Purpose of the Study:

The essential purpose of this thesis was to follow the absorption and to discover metabolic routes of fenitrothion in different species of forest seeds and seedlings under controlled laboratory conditions. A thorough understanding of the absorption, and potential toxic metabolites and derivatives of the pesticide, which may occur in forest trees, could then be applied by future research teams when examining the impact of this pesticide on the millions of acres of Canadian forests plants, and animals affected by the pesticide. Such studies would entail large scale, extensive field surveys to be carried on a variety of Canadian terrains, watersheds, and under various weather conditions.

Germinating seeds and young seedlings from three species of Canadian forest trees were chosen, rather than whole plants, for study, because at this stage rapid growth and metabolism takes place. Also, these stages of plant growth could be examined under controlled environmental and experimental conditions, so that valid reproducible experimental results would be obtained. Two coniferous species of Canadian forest trees were chosen; white pine, Pinus strobus, because its size and rapid growth rate was suitable for pilot metabolic studies, and white spruce,

Picea glauca, because it is an economically important Canadian tree species protected by fenitrothion application from spruce budworm infestations. One deciduous tree species was also examined; yellow birch, Betula alleghaniensis, for contrast as a representative hardwood in the Canadian forest ecosystem sprayed with fenitrothion.

The first major problem to solve was to develop accurate techniques to quantitate residues of fenitrothion in the forest seeds and seedling parts. This was necessary to determine whether the pesticide could penetrate the outer, more inert tissues of the seeds into the viable and dynamically growing tissues of the developing embryos and later seedlings where the pesticide might be metabolised. The second problem was then to adapt the existing methodology for the determination of known plant metabolites such as fenitro-oxon and desmethyl fenitrothion. The third problem was to determine the structure of any unknown metabolites, synthesize pure standards, and develop residue methodology for accurate quantitation. The possibility of forming the S-methyl isomer by metabolic pathways had not been considered previously in any insect, animal, or plant species. However, since the isomer is such a potent cholinesterase inhibitor, the conversion of fenitrothion to the more active isomer was examined in detail during the germination and early seedling growth of forest seeds under controlled conditions. S-methyl fenitrothion and other impurities were removed from fenitrothion used in all studies so that the absorption and metabolism of fenitrothion could be clearly studied without any added variations. The fourth problem was to confirm the biochemical pathways for the formation of any previously

unknown metabolites. Resolution of the final problem required that some assessment be made of the residues absorbed and metabolites formed in the seeds and seedlings. Potentially toxic compounds could then be monitored on a large scale or in model systems if environmental stress due to applications of fenitrothion became evident.

## MATERIALS AND METHODS

1. Forest Tree Seeds:

The seeds of white pine (Pinus strobus), white spruce (Picea glauca), and yellow birch (Betula alleghaniensis), were extracted from fallen cones collected during the fall of 1972 in unsprayed regions of Petawawa forest (PFES), Ontario. Seeds were surface sterilized by washing them for 15 minutes in a 1 percent solution of sodium hypochlorite. This minimized any absorption or metabolism of the pesticide by bacteria such as that reported by Miyamoto et al, (1966). The seeds were further rinsed for another 15 minutes in continuously running distilled water to remove all residual sodium hypochlorite. It was found that residual sodium hypochlorite would oxidize the pesticide. Viability of birch seeds was ascertained by floating the seeds on a solution of 80 percent ethanol. Seeds which floated contained embryos insufficiently developed for germination and were discarded. All seeds were stored dry at $5^{o}C$ until use to maintain high viability (Wang, 1973).

2. Treatment of Seeds with Fenitrothion:

Surface sterilized seeds were arranged into groups of 30. Population homogeneity was optimized by selecting seed groups with air dried weights to within ± 5 mg. Groups of 10 white pine seeds were first stratified or prechilled in 15 mls of a $0.4 \times 10^{-4}M$ or 10 ppm solution of pure fenitrothion in distilled water (pH 6.8) in 9 cm diameter petri plates at $10^{o}C$ for 21 days in the dark. White spruce and yellow birch seeds were similarly treated and then stratified at $5^{o}C$ for 28 days. The 10 ppm solution

of fenitrothion approximates a field concentration of 4 oz/acre aerial spray. A second set of seeds were treated in a $0.4 \times 10^{-2}$ or 1000 ppm emulsion of fenitrothion in distilled water. This concentration was for better illustration of the affects of the pesticide under extreme conditions. The seeds were then germinated on moist filter paper in sterile 9 cm petri plates. Germination was also carried out in darkness with a diurnal temperature variation of $20^{o}C$ nights (8h) and $30^{o}C$ days (16h). (U.S.D.A., 1949)

Following germination, pine seeds were dissected into 3 parts: the embryo, fleshy endosperm*, and seed coat. Samples of duplicate groups of 30 seeds were taken during germination and early seedling growth at 4 days (when the radicle has just emerged from the seed coat), 8 days (when the hypocotyl has emerged), 12 days (when the hypocotyl has developed), and 14 days (when the colyledons have emerged from the seed coat). Birch and spruce seeds were not dissected due to their minute size. They were sampled in duplicate groups of 30 at 4, 10, 16, and 21 days of germination and growth which corresponded to comparable stages in morphological development as the sampling times of pine. Samples were

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* Fleshy endosperm is used here to denote the female gametophyte of the germinating seed as referred to by Durzan et al (1972). This is also referred to as the perisperm.

frozen in liquid nitrogen and stored at -70°C until the pesticide was extracted.

3. Treatment of White Pine Seedlings with Radioactive Fenitrothion:

Surface sterilized white pine seeds were stratified and germinated for 12 days, as before, except that no fenitrothion was added to the growth medium. The germinating seeds were then immersed in a 0.4 x $10^{-2}$M emulsion of $OC^{14}H_3$ radioactively labelled fenitrothion in distilled water for 24 hours. Although conifer seedlings are sensitive to high levels of tritium (Durzan et al, 1971) the effect of radioactivity on the seedlings was not considered in this experiment due to the low specific activity of $C^{14}$ used and since long term growth effects on the seedlings were not being considered but rather the transfer of the $C^{14}H_3$ group as a confirmation of the metabolic formation of desmethyl and S-methyl fenitrothion. Following exposure to $OC^{14}H_3$ labelled fenitrothion the seeds were then transferred and germinated for an additional 48 hours on filter paper.

4. Growth Studies:

In parallel experiments, control and fenitrothion treated seeds were planted in flats containing loam soil and put in a controlled environment room with a monitored diurnal light and temperature regime. A 16 h day (10.1 lux light intensity) and 27°C temperature and an 8 h dark period at 20°C was maintained. The flats were watered daily to field capacity during the early stages of seedling growth and later three times weekly.

5. Reagents and Solvents:

Silica gel, 60 to 100 mesh (grade 950), was obtained from Grace Co. Ltd. It was activated by heating overnight at 200$^{o}$ C to remove water. It was then cooled in a vacuum dessicator and stored in a reagent jar to prevent extraneous water uptake.

All solvents used were nanograded and glass distilled.

Methyl iodide was obtained from BDH Chemicals, Poole, England. Diazold was obtained from Aldrich Chemicals, Poole, England.

Aquasolve Scintillation Fluid was obtained from New England Nuclear Limited, U.S.A.

6. Preparation of Fenitrothion and Metabolites:

Fenitrothion, (O, O-dimethyl-O-(4-nitro-m-tolyl) phosphorothioate) technical grade, was obtained from Sumitomo Chemical Co. Japan. It was purified by a modification of a method of Kovacicova et al (1971). Ten grams of fenitrothion were dissolved in 25 ml of a 5 percent by weight solution of KOH in methanol. This was allowed to stand at room temperature for 2 hours. Since the hydrolysis rates of fenitro-oxon and S-methyl fenitrothion are much faster than the parent fenitrothion they were selectively removed leaving fenitrothion and various water soluble hydrolysis products. These hydrolysis products were then removed by taking the solution up in 100 ml of benzene, and washing this with 4 separate aliquots of an aqueous solution containing 1 percent ammonium hydroxide. This was followed by 4 washings with distilled water (pH 6.8). Residual water was removed from the organic phase

by filtering through anhydrous sodium sulphate. The solvent was removed on a rotary vacuum evaporator at 35°C. Final traces of solvent were removed under a stream of dry nitrogen. This yielded 7 grams of 99.5 percent pure fenitrothion which did not contain any impurities detectable by gas or thin layer chromatography.

Fenitro-oxon (O, O-dimethyl-O-(4-nitro-m-tolyl) phosphate) was obtained from Sumitomo Chemical Co. Japan. It was also synthesized by slow addition of O.O-dimethyl phosphorochloridate in acetone to the sodium salt of 3-methyl-4-nitrophenol which had been prepared in benzene using sodium hydride. The yield was 92 percent. Further purification from residual fenitrothion was accomplished by column separation on silica gel (see Section 10).

S-methyl fenitrothion (S-methyl, O-methyl, O-(4-nitro-m-tolyl) phosphorothioate was prepared by the method of J. Kovacicova et al, (1973). The method entails dealkylation of fenitrothion with potassium hydrosulfide and subsequent remethylation of the desmethyl product with dimethyl sulphate.

Desmethyl fenitrothion (O-methyl-O-(4-nitro-m-tolyl) hydrogen phosphorothioate) was prepared via dealkylation of fenitrothion with potassium hydrosulfide. Potassium hydrosulfide was obtained by dissolving 17 g of KOH in 15 ml of methanol and then saturating this solution with $H_2S$. To this solution 10 grams of pure fenitrothion were added dropwise and the mixture was refluxed for 5 hours. After refluxing, the methanol was removed by rotary evaporation and the residual oily crystals were washed 4 times with benzene to remove any unreacted fenitrothion. This yielded 5 grams of desmethyl fenitrothion.

The hydrolysis product 3-methyl-4-nitrophenol was obtained from Aldrich Chemical Co. Amino fenitrothion was prepared by the reduction of fenitrothion with chromous chloride in acetone giving a 93 percent yield (Forbes et al, 1975). Bis-fenitrothion and bis S-methyl fenitrothion were obtained from V. Batora (Research Institute of Agrochemical Tech., Bratislava, Czechoslovakia). Trimethyl phosphate and triethyl phosphate were obtained from Aldrich Chemical Co. Ltd. Radioactive $C^{14}$ labelled in the $OCH_3$ position (Specific Activity 4uC/mM) was obtained from Dr. J.R. Duffy, University of Prince Edward Island.

7. Biochemical Compounds:

Glutathione (reduced form) was obtained from Fischer Scientific Co. Ltd. Cysteine, glycine, and L-glutamine were obtained as salts of the amino acids from BDH Chemical Co.

$S\text{-}CH_3$ glutathione was prepared as the sodium salt by methylation of reduced glutathione (Hutson et al, 1972). Reduced Glutathione (1.33 mMole) and 0.5 mMole NaOH were dissolved together in 25 ml of water. Methyl iodide (2 Mole) was added to the solution. Methanol was added to bring the mixture into one phase. The solution was allowed to stand for one hour and then the methanol and methyl iodide were removed by rotary evaporation. The pH was adjusted to 3.0 by addition of 2 N HCl. The product yielded one spot with TLC having an Rf characteristic of S-methyl glutathione. Its structure was confirmed by MS.

8. Extraction of Pesticide Residues:

a. Plant Material: Thirty seeds or seed parts were homogenized for 1 minute in a 125 ml Erlenmeyer flask containing 50 ml of acetonitrile with a Polytron (Brinkman Co.) sonicator. The homogenate was filtered under suction through celite to remove seed debris. Fifty ml of acetonitrile was added to the flask and was sonicated once again to remove any residual pesticide on the sonicator and on the walls of the flask. This wash was also filtered through the same celite. Oils and fats were removed by partitioning the acetonitrile with 50 ml of hexane 3 times (Getz, 1962). The hexane extract was then flash evaporated almost to dryness and redissolved in 50 ml of benzene for further separation of residues.

b. Seedling Growth Medium: The aqueous growth media were concentrated to 2 ml by rotary evaporation and then extracted by partitioning with 2 ml of benzene, three times.

9. Derivatization of Water Soluble Residues:

Water soluble metabolites were separated by washing the benzene extract once with an equal volume of water. The water was then removed by flash evaporation and the residues were redissolved in 0.5 ml of methanol. An ethereal solution of pure diazomethane was added until the solution was bright yellow. The mixture was stoppered and allowed to stand at 20$^{o}$C for 30 minutes (Shafik and Enos, 1973). It was then evaporated under a stream of nitrogen to the original 0.5 ml volume removing residual diazomethane. This procedure allowed detection and quantitation of desmethyl fenitrothion by gas chromatography. Derivatiz-

ation of desmethyl fenitrothion produced a 44 percent yield of fenitrothion and 56 percent of the S-methyl isomer. This ratio was relatively constant throughout the range from 0.05 ppm, 0.5 ppm and 5 ppm. Both derivatives were quantified in determining levels of desmethyl fenitrothion for accuracy. The overall recovery of extraction and derivatization of seed samples spiked with desmethyl fenitrothion at the 0.05 ppm level was 78 ± 3 percent.

10. Column Separation of Benzene Soluble Residues:

A slurry was made containing 20 ml of hexane to 20 gm of silica gel. This was poured into a 25 x 450 mm glass column to a depth of 65 mm. Anhydrous sodium sulphate was added on top of the adsorbent to a depth of 10 mm. The sample was added to the column in 50 ml of benzene and elutions of 200 ml benzene, and 100 ml acetone: benzene, 1:3, were made. Fenitrothion and amino-fenitrothion were recovered in the benzene fraction. Fenitro-oxon and S-methyl fenitrothion were recovered in the acetone-benzene fraction. The overall residue recoveries for extraction and column clean up of seed and growth medium samples spiked at the 0.05 ppm level were 90 to 95 percent for fenitrothion, amino-fenitrothion, fenitro-oxon, and S-methyl fenitrothion.

11. Gas Chromatography:

A Pye (Model 104) gas chromatograph fitted with an alkalai flame ionization detector (AFID), having a cesium bromide annulus was used. A glass column, 0.9 mx 4 mm ID, was packed with 100/120 mesh Gas Chrom Q coated with 3 percent OV-17 (Applied Science Labs, State College, Pa.). With a column flow of 40 ml/min nitrogen, and a column temperature

of 202°C, the retention time of fenitrothion was 3.4 min. and S-methyl fenitrothion 6.6 min. Better separation was necessary to quantitate fenitro-oxon residues. Therefore, a glass column 1.8 mx 4 mm ID packed with 4 percent SE30/6 percent QF-1 on Chromsorb W was used. With a column flow of 40 ml/min and column temperature of 205°C, the retention time of fenitro-oxon was 6.4 min and 3-methyl-4-nitrophenol was 1.3 min. Duplicate determinations on all samples were carried out, bracketed by injections of sample standards. It was necessary to thoroughly condition the columns with S-methyl fenitrothion or fenitro-oxon throughout the experiment in order to obtain reproducible results. On-column injection was used and the injector block was unheated. No on-column isomerization of fenitrothion to S-methyl fenitrothion was detected under these conditions employed for analysis.

12. Thin Layer Chromatography:

Baker-flex silica gel precoated sheets 250 microns thick were used to determine the toxic residues. The plates were developed with a solvent system of ethyl acetate/cyclohexane 1:3. Compounds which were strong cholinesterase inhibitors were visualized with the enzyme inhibition technique described by Mendoza (1972) using extract of steer liver homogenate as the spray reagent. Fenitrothion has an Rf of 0.58, S-methyl fenitrothion 0.15, and fenitro-oxon 0.09. Desmethyl fenitrothion due to its acidic nature was chromatographed on cellulose plates, which were developed with a solvent system of 2, propanol: water: ammonium hydroxide, 75:24:1. These plates were sprayed with 2, 6-dibromo-N-chloro-p-benzoquinone-imine in glacial acetic acid to visualize desmethyl fenitro-

thion. This chromagenic reagent is specific for the P=S bonds of phosphorothionates (Menn et al, 1957).

13. Extraction of Glutathione Conjugates:

Glutathione conjugates were extracted from seedlings treated with radioactive fenitrothion by sonication in 50 ml of 80 percent ice cold methanol for 1 minute. This was similar to the method used by Lamoureux (1970) for extracting glutathione-atrazine conjugates. The homogenate was filtered through celite under suction and the flask and sonicator rinsed for 1 minute in cold methanol. The filtrate was evaporated almost to dryness and taken up in 1.0 ml of distilled water. The water was partitioned 3 times with chloroform to remove non-conjugated pesticide residues. Both water and chloroform extracts were then concentrated, and examined by thin layer chromatography. Water soluble glutathione conjugates were chromographed on cellulose plates. Two solvent systems were used namely (a) butanol: acetic acid: water: (11:4:5) (Morello et al, 1968), and (b) phenol: water: (4:1), (Johnson et al, 1966). Plates were developed by spraying with a 1 percent solution of ninhydrin dissolved in acetone. Portions of plates were left undeveloped, and areas with Rf's similar to S-methyl glutathione were scraped off the plates and dissolved in methanol. Aliquots were then hydrolysed in 6 N HCl for 20 h at 110$^{o}$C and rechromatographed. Similar aliquots were concentrated to dryness and submitted for analysis by direct probe mass spectroscopy. All ninhydrin positive spots were scraped from the plate, dissolved in Aquasolve scintillation fluid and counted on a Picker Nuclear Ansitron liquid scintillation counter.

14. Quantification of Free SH Groups in Seeds:

Seeds which had been stratified and germinated with and without the fenitrothion treatment for 12 days were examined for free SH groups indicating levels of reduced glutathione present. Seeds were placed in 50 ml of 80 percent ice cold methanol (Lamoureux et al, 1970) containing 5 percent sulfosalicylic acid (Spragg and Yemm, 1958) and were sonicated with a Polytron (Brinkman) sonicator for 1 minute. The homogenate was then filtered and the residue re-extracted as before. This was also filtered and the combined filtrates were flash evaporated to 2 ml.

Free SH groups were measured colorimetrically using the nitroprusside reaction of Grunert and Phillips, (1951). Five ml of saturated NaCl was added to the extract. After mixing, a 0.4 ml aliquot was placed in a cuvette along with 0.2 ml of 0.067 M sodium nitroprusside and 0.2 ml of a cyanide/carbonate solution (1.5 M $NaCO_3$ and 0.067 M KCN). The reaction was measured colorimetrically at maximum transmission of 520 mu using a Unicam SP-800 double beam UV Spectrophotometer. The amount of free SH groups was calculated from a standard curve (See Appendix I). Response is linear through a range of 1 to 100 µg of glutathione.

15. Determination of Mutagenicity of Fenitrothion and Metabolites Using Ames Enteric Bacteria:

A mutant culture His G-46; TA 1535 of Salmonella typhimurium Li-2 was obtained from Dr. B. Ames, Department of Biochemistry, University of California, Berkeley, California.

The method of Ames (1971) was used to test the ability of fenitrothion and its derivatives to cause mutations in bacterial DNA. A mutant culture of Salmonella typhimurium Li-2 was used: His G-46; TA 1535, a histidine requiring mutant susceptible to base-pair substitutions caused by alkylating agents. Bacteria were plated on sterile plastic petri plates containing minimal agar medium (Vogel-Bonner 1956; 1.5 percent agar, 2.0 percent glucose). A mixture of 0.1 ml of a nutrient broth culture of bacteria ($3 \times 10^8$ bacteria) and 2 ml of liquified ($45^oc$) 0.6 percent agar containing 0.5 percent NaCl, 0.1 uMole histidine, and 0.1 uMole biotin was prepared for each plate, poured onto the agar, and spread by rotating the plate slowly. Pesticides and fenitrothion derivatives were first dissolved in spectrophotometric grade sterile dimethyl sulfoxide. This solvent dissolved all of the compounds (as many were not water soluble) and was not toxic to the bacteria. Dilutions were made in DMSO and 1.0 ml aliquots were spread over the surface of the plate. Duplicate plates were made in each case. In a second experiment the pesticide was added to the plate along with the "S-9" liver homogenate of Graner et al, (1972). The S-9 mixture contained: 0.3 ml of S-9 liver homogenate, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mMTPN, and 100 mM sodium phosphate (pH 7.4). The homogenate allows biological activation of potential alkylating agents.

16. Nuclear Magnetic Resonance Spectrometry:

NMR spectra were performed on a Varian T-60 Spectrometer. Optimal spectra were obtained with 10 percent solutions of compounds dissolved in deuterated DMSO. $\tau$ values were calculated using

tetramethylsilane (TMS) as the internal standard. Relative peak areas were integrated for the diagnostic doublet peaks for $SCH_3$ protons and $OCH_3$ protons to obtain the percentage conversion of desmethyl fenitrothion to S-methyl fenitrothion.

17. Mass Spectrometry:

Mass Spectra were obtained on a Finnigan 3100 Mass spectrometer interfaced with a D6000 data acquisition system. Spectra of fenitrothion and metabolites were obtained by GC/MS whereas spectra of S-methyl glutathione and amino acids were obtained by direct probe analysis.

18. Liquid Scintillation Counting:

Samples and standards were dissolved in Aquasolve scintillation fluid and counted using a Picker Ansitron liquid scintillation counter. A quench correction curve was obtained from a series of standards containing a fixed amount of $OC^{14}H_3$ labelled fenitrothion and varying amounts of unlabelled sample calculating the counting efficiencies and plotting against the count rate of a known standard. The counting efficiencies of $OC^{14}H_3$ labelled samples were then obtained from this quench correction curve and the disintegration rate in DPM for each sample obtained from its count rate in CPM. Each sample was taken as a 2 cm X 2 cm region of silica gel of uniform thickness scraped from a TLC plate, placed in 10 mls of aquasolve and counted for 20 minutes to ensure accuracy in determining relative levels of the $C^{14}$ labelled metabolic products produced by the $OC^{14}H_3$ labelled fenitrothion.

## RESULTS

### 1. Absorption and Metabolism of Fenitrothion (10 ppm) by *Pinus strobus* Seeds and Seedlings.

The amount of pesticide absorbed by the seeds increased during germination (Figure 1). The accumulation of fenitrothion in the three seed parts can be correlated with duration of exposure to the pesticide. A large amount of fenitrothion, 160 ppm, was initially absorbed from the 10 ppm aqueous solution in the seed coat after 4 days of germination. The seed coat presents the largest absorbing surface exposed to fenitrothion. The concentration in the seed coat fell slowly during the course of germination to 60 ppm after 14 days of germination. The concentration of fenitrothion absorbed by the endosperm rose from 70 ppm at 4 days, to 180 ppm after 14 days of germination. This increase was parallelled by the concentration in the embryo which rose from 24 ppm at 4 days to 120 ppm after 14 days of germination. Some fenitrothion may be absorbed into the seed through the micropyle and, after the seed coat splits, a small surface of the endosperm and embryo is exposed to the pesticide. From the data, it seems reasonable to assume that fenitrothion was absorbed and transported from the seed coat to the endosperm and from there into the embryo (Figure 1).

When the residues of fenitrothion were confirmed by TLC using microsomal oxidase enzyme inhibition (Mendoza, 1972) the presence of two other strong enzyme inhibitors were shown to be present (Figure 2). Residues

with an RF similar to fenitro-oxon were detected in the growing embryos of the treated seeds. The structure of fenitro-oxon was confirmed by GC/MS and residues were quantitated by GC (Figure 3). Very low concentrations of fenitro-oxon were found in the seed coat and endosperm (0.2 ppm) over the 14 days germination period indicating a lack of oxidative metabolism of the pesticide by these tissues.

It would appear however, that seeds of Pinus strobus contain oxidative desulfurase enzymes in the growing embryos which can oxidize fenitrothion to fenitro-oxon. The concentration of fenitro-oxon rose in the growing embryos to 1.2 ppm at 12 days and 2.5 ppm after 14 days of germination and seedling growth.

S-methyl fenitrothion was also first detected in the growing embryos of treated seeds by TLC using the enzyme inhibition technique (Figure 2). However, the existence of this isomer was unknown at that time. The compound was as sensitive to the enzyme inhibition technique as fenitro-oxon but had an RF intermediate between fenitro-oxon and fenitrothion. The molecular weight by GC/MS was similar to fenitrothion but the structure of the molecule was confirmed by GC and TLC to be the S-methyl isomer. Attempts to synthesize the molecule by u.v. irradiation and heat isomerization gave low yields of the compound which were contaminated with fenitro-oxon. Finally when a pure standard of S-methyl fenitrothion was obtained it was possible to quantify this compound and follow the formation of the molecule in white pine seedlings using gas chromatography

(Figure 4). The appearance of the S-methyl isomer paralled the formation of fenitro-oxon as a metabolite. Little S-methyl fenitrothion was found in the seed coat or endosperm during the course of seedling germination and growth. However, amounts in the range of 0.5 ppm were found in the embryos at 8 days of germination and these increased to 2.4 ppm by 14 days. No enzyme pathways for the formation of this compound had previously been considered.

Fenitrothion had been reported to be dealkylated in rice plants (Miyamoto and Sato, 1963, 1965). Was then desmethyl fenitrothion formed in white pine during early seedling growth? After many different attempts to synthesize pure desmethyl fenitrothion a pure standard was obtained. The compound showed one spot detectable by TLC. Rf 0.90. However, this technique was much too insensitive for detection of residues of desmethyl fenitrothion in the white pine seedlings. Since desmethyl fenitrothion is ionic it cannot be chromatographed by GC without having first been derivatized. This reaction was carried out using diazomethane giving a mixture of fenitrothion and its S-methyl isomer. The latter compound was used for quantification. Figure 5 shows the concentration of desmethyl fenitrothion found in the germinating seeds. Dealkylation activity was primarily found in the embryo of the growing seeds. The embryo contained 0.5 ppm of the desmethyl derivative after 4 days germination and this concentration rose rapidly between 8 and 14 days germination to 36 ppm. The metabolite was found in low concentrations (1.0 ppm) in both the seed coat and endosperm throughout germination.

Although the analytical procedure could also detect amino-fenitrothion, no trace of residues of this metabolite were detectable. The main hydrolysis product; 3-methyl-4-nitrophenol, was not evident in detectable amounts, either by thin layer chromatography or gas chromatography. Methods of detection are 100 times less sensitive to this metabolite than for fenitrothion, since the alkalai flame ionization GC detector used was selective for phosphorous and TLC was specific for cholinesterase inhibitors.

The water moistening the seeds was monitored for the metabolites throughout the experiment. None were detected.

2. Absorption and Metabolism of Fenitrothion (10 ppm) by Pinus strobus, Picea glauca, and Betula alleghaniensis.

White spruce is not only an economically important tree in Eastern Canadian forests but is a major host species (second to balsam fir) for the spruce budworm. It is therefore a key species protected from insect defoliation by fenitrothion treatments. Both white spruce and white pine are gymnosperms. Angiosperms such as yellow birch are also prevalent in the sprayed forests. A study was undertaken to compare the absorption and metabolism of fenitrothion in the two gymnosperms and the one angiosperm.

The amount of fenitrothion absorbed by white pine, spruce, and birch seeds is shown in Figure 6. By 4 days of germination, yellow birch had absorbed 160 ppm of fenitrothion. This was 1.5 times more than that absorbed by pine and double that found in spruce. This level fell rapidly in the birch throughout the course of germination. The concentration of fenitrothion decreased slightly in the spruce but increased in the pine.

Fenitro-oxon was evident as a metabolite in the seeds and seedlings of all three forest species (Figure 7). The concentration reached a high of 11 ppm after 4 days of germination of the birch seeds. The maximum in spruce (7.5 ppm) was not reached until 10 days of germination. The concentration was much lower in pine (1.4 ppm), and this maximum was not reached until 14 days of germination and seedling growth.

The highest concentration of S-methyl fenitrothion was found in spruce (Figure 8). The level rose rapidly in spruce to a maximum of 8.0 ppm by 16 days of growth. The highest concentration found in the birch was 5.0 ppm at 10 days of germination. The concentrations found in pine were comparatively much lower.

Dealkylation of fenitrothion occured in all three species of tree seedlings, as evidenced by the production of desmethyl fenitrothion (Figure 9). The birch seeds developed the highest concentrations reaching 15 ppm by 4 days of germination increasing to 37 ppm by 16 days of growth. Lower levels were found in spruce which developed a maximum concentration of 9.3 ppm by 4 days of germination. This species showed a gradual decline in production of desmethyl fenitrothion reaching 5 ppm by 21 days of germination. The concentration of desmethyl fenitrothion was lowest in pine, and slowly increased to a maximum of 11.2 ppm by 14 days growth of the young seedlings.

3. Metabolism of 1,000 ppm Fenitrothion by Betula alleghaniensis

Pomber and Wienberger (1974) showed that a 1,000 ppm emulsion of fenitrothion did not significantly affect the growth of white pine, or white spruce, but did severely affect the growth of yellow birch. In this study yellow birch seeds treated with 1,000 ppm fenitrothion produced only a short radicle and hypocotyl by 10 days of germination, following this, they stopped growing and underwent rapid senesence. These seeds treated at the 1,000 ppm level during stratification and germination absorbed a maximum of 5,000 ppm fenitrothion after 4 days of germination, (Figure 10). The concentration was thirty-two times higher than the maximum concentration absorbed by yellow birch seeds treated with 10 ppm fenitrothion. A much lower amount of the absorbed fenitrothion was metabolized to fenitro-oxon, 32 ppm, and to S-methyl fenitrothion, 16.7 ppm. These levels are only three times higher than those found with the 10 ppm treatment. The amount of desmethyl fenitrothion was only five-fold higher than the concentration found in seeds treated at the 10 ppm level, reaching a maximum of 155 ppm.

4. Dealkylation of Fenitrothion by Germinating Seedlings.

Fenitrothion has been shown to be dealkylated via an alkyl transferase enzyme pathway using glutathione (a tripeptide containing a reactive sulfhydryl group) as substrate to form desmethyl fenitrothion, and S-methyl glutathione in mammalian liver and kidney (Hollingworth, 1969. Desmethyl fenitrothion was found in white pine embryos, and white spruce and yellow birch during germination (Results, Section 1 and 2). It was suspected to be an intermediate in forming the toxic S-methyl

isomer. In order to investigate whether this pathway existed seedlings were treated with $OC^{14}H_3$ labelled fenitrothion between 12 and 15 days of seedling growth when the highest concentrations of desmethyl and S-methyl fenitrothion were found to be produced. Extraction of glutathione conjugates from the seeds after treatment yielded a series of ninhydrin positive compounds (Table 1). The major compound had 1500 dpm of radioactivity and an Rf similar to that of S-methyl glutathione. This would indicate that glutathione was an acceptor of methyl groups. When this compound was hydrolysed and rechromatographed, ninhydrin positive molecules with Rf's similar to glutamine, cysteine, S-methyl cysteine, and glycine, the constituents of S-methyl glutathione, were evident in approximately equal amounts. The structure indicated by GC/MS was that of S-methyl glutathione. This qualitative experiment demonstrated that fenitrothion was dealkylated via a glutathione-dependent alkyl transferase pathway in pine seed embryos.

Thin layer chromatography of the chloroform extract containing pesticide residues showed three spots sensitive to the enzyme inhibition technique (Table 2). A spot identified as fenitrothion had 300,000 dpm of radioactivity. Two further spots with Rf's similar to fenitro-oxon and S-methyl fenitrothion contained 1,000 dpm and 1,500 dpm of radioactivity respectively further demonstrating the ability of the plant to produce these metabolites.

5. Effect of Fenitrothion Treatment on Seedling Glutathione Levels.

Desmethyl fenitrothion was shown to be a major metabolite of fenitrothion formed during germination and early seedling growth of

treated seeds. Glutathione was indicated to be the substrate acceptor of the methyl group on O-dealkylation of fenitrothion in the white pine seedlings forming S-methyl glutathione. Glutathione has been implicated as a regulator of enzymes in mammalian systems and in pea seedlings during germination (Spragg and Yemm, 1958). Glutathione levels were monitored in the three seed species to examine the effect of the pesticide on tissue glutathione levels.

Table 3 shows that treatment with fenitrothion at the 10 ppm level only slightly depleted glutathione levels in all three seed species. Levels of glutathione in these tree seedlings were comparable to those found in germinating peas by Spragg and Yemm (1958) using the same method. Treatment at the 1,000 ppm level depleted the glutathione level slightly in spruce (25 percent), more severely in pine (43 percent), and drastically in birch (75 percent). Glutathione depletion in the yellow birch seedlings treated with 1,000 ppm of fenitrothion may partially account for the phytotoxic response of these seedlings to the high level of pesticide.

6. Confirmation of the Structures of S-methyl Fenitrothion and S-methyl Glutathione by GC/Mass Spectroscopy:

Figure 11 shows the GC/mass spectrum of S-methyl fenitrothion obtained from a purified white pine seed extract. The base peak at m/e 125, is the same as fenitrothion. The major ions are found at 277, 260, 130, 125, and 79 m/e. The fragmentation pattern is shown in Figure 12. The parent or molecular ion is evident at m/e 277. Rupture of the P-O-aryl bond results in the base peak at m/e 125,

and a fragment at m/e 153 resulting from the phenyl moiety. The ion at m/e 125 further breaks down to one at m/e 79. The absence of a peak at m/e 109 corresponding to the $CH_3O\text{-}P\text{-}OCH_3$ moiety found in fenitrothion and fenitro-oxon, indicated the presence of a $CH_3SP$ bond rather than a P=S. The peak at m/e 260 representing the m-17 ion is diagnostic for the methyl group ortho to the nitro group. This ortho rearrangement which is referred to as a "McLafferty rearrangement" (McLafferty, 1973 is shown in Figure 13. The elimination of OH (m-17) is initiated by the abstraction of a hydrogen from the methyl group by an oxygen of the nitro group. Oxidation of the methyl or reduction of the nitro group would prevent formation of the m-17 ion. The structure indicated by this mass spectrum together with the previous GC and TLC evidence confirmed the presence of S-methyl fenitrothion.

The structure of S-methyl glutathione was also confirmed by direct probe mass spectroscopy (Figure 14). The molecular or parent ion is not evident. Electron impact ionization induces very strong fragmentation in the molecular ions of the free amino acids and many of their derivatives rendering the $m^+$ peaks difficult to detect even at low electron energies (Vetter, 1972). The base peak m/e 61 is indicative of the $CH_2\text{-}S\text{-}CH_3$ fragment characteristic of S-methyl cysteine and S-methyl glutathione. The peak at m/e 84 is characteristic of the base peak of glutamic acid which further breaks down to $CH_2SH$ at m/e 56. The peak at m/e 34 is characteristic of the base peak of glycine. The latter are the other two constituent amino acids of glutathione. Coupled with the TLC and hydrolysis evidence described previously this mass spectrum confirmed the structure of S-methyl glutathione.

7. Growth Studies or Pinus strobus Seeds Treated with Fenitrothion

No developmental deviations were apparent in six month old seedlings derived from seeds treated with 10 ppm fenitrothion during stratification and subsequent germination together with a non-treated control, Figure 15.

All treated plants appeared as sturdy as control plants. Studies by Pomber and Weinberger (1974 a, b) showed no developmental deviations in white spruce and yellow birch seeds treated with 10 ppm fenitrothion during stratification and germination. However, treatment of yellow birch seeds with 1,000 ppm fenitrothion during germination caused severe dwarfing of the seedlings after 10 days of germination.

8. Determination of Mutagenicity Using Ames Enteric Bacteria as Indicator.

Fenitrothion and its metabolites: fenitro-oxon, S-methyl fenitrothion, aminofenitrothion, bis fenitrothion, bis S-methyl fenitrothion, and 3-methyl-4-nitrophenol were tested for their potential to be mutagenic via alkylation of nucleic acids causing base pair substitutions. All compounds were tested with and without liver homogenate as a biological activator. All gave a negative response at concentrations of 1,000, 100, 50, and 10 ppm. Trimethyl phosphate and tri-ethyl phosphate gave a negative response in this system although they have shown positive responses to mutagenicity in other bacterial systems and Drosophila, (Dyer and Hanna 1973).

9. Formation of S-methyl Fenitrothion Followed by NMR

Methyl phosphate esters (Hilgetag and Teichmann, 1965) and specifically methyl phosphorothioate esters such as fenitrothion (Eto et al, 1968) were shown to act as alkylating agents. S-methyl fenitrothion could be formed by the alkylation of desmethyl fenitrothion in the S position by fenitrothion. To confirm that this was the main pathway, 10 percent solutions of fenitrothion, potassium desmethyl fenitrothion, and a mixture of both (1:1) in deuterated $d_6$ DMSO were heated at 60°C and the solutions examined by proton NMR at various time intervals (Table 4). After 21 h, no change was observed in the spectrum of potassium desmethyl fenitrothion and the spectrum of fenitrothion indicated about 9 percent decomposition. The mixture of desmethyl fenitrothion and fenitrothion, however, showed extensive reaction had taken place. Approximately 55 to 60 percent of the fenitrothion had disappeared as indicated by reduction of the $CH_3OP$ protons ($\tau$ 6.18). The present of S-methyl fenitrothion was shown by the formation of a doublet ($\tau$ 7.41 J = 16 Hz) corresponding to the $CH_3SP$ protons. The amount present (34 to 37 percent) was deduced by comparison of the peak area of the $CH_3OP$ protons of desmethyl fenitrothion ($\tau$ 6.10) with those of the S-methyl protons ($\tau$ 7.41).

FIGURE 1

Concentration of fenitrothion found in white pine seeds, Pinus strobus, during germination after: (1) stratification in an aqueous solution containing 10ppm, ($0.4 \times 10^{-4}$M) pure fenitrothion for 21 days at 10°C in the dark, and (2) germination in the dark at a 20°C to 30°C diurnal temperature regime until emergence of cotyledons from the seed coat. Each point represents the mean of two determinations on two separate populations of seeds treated similarly with fenitrothion. Vertical lines represent the range.

## FENITROTHION IN PINE SEEDS DURING GERMINATION

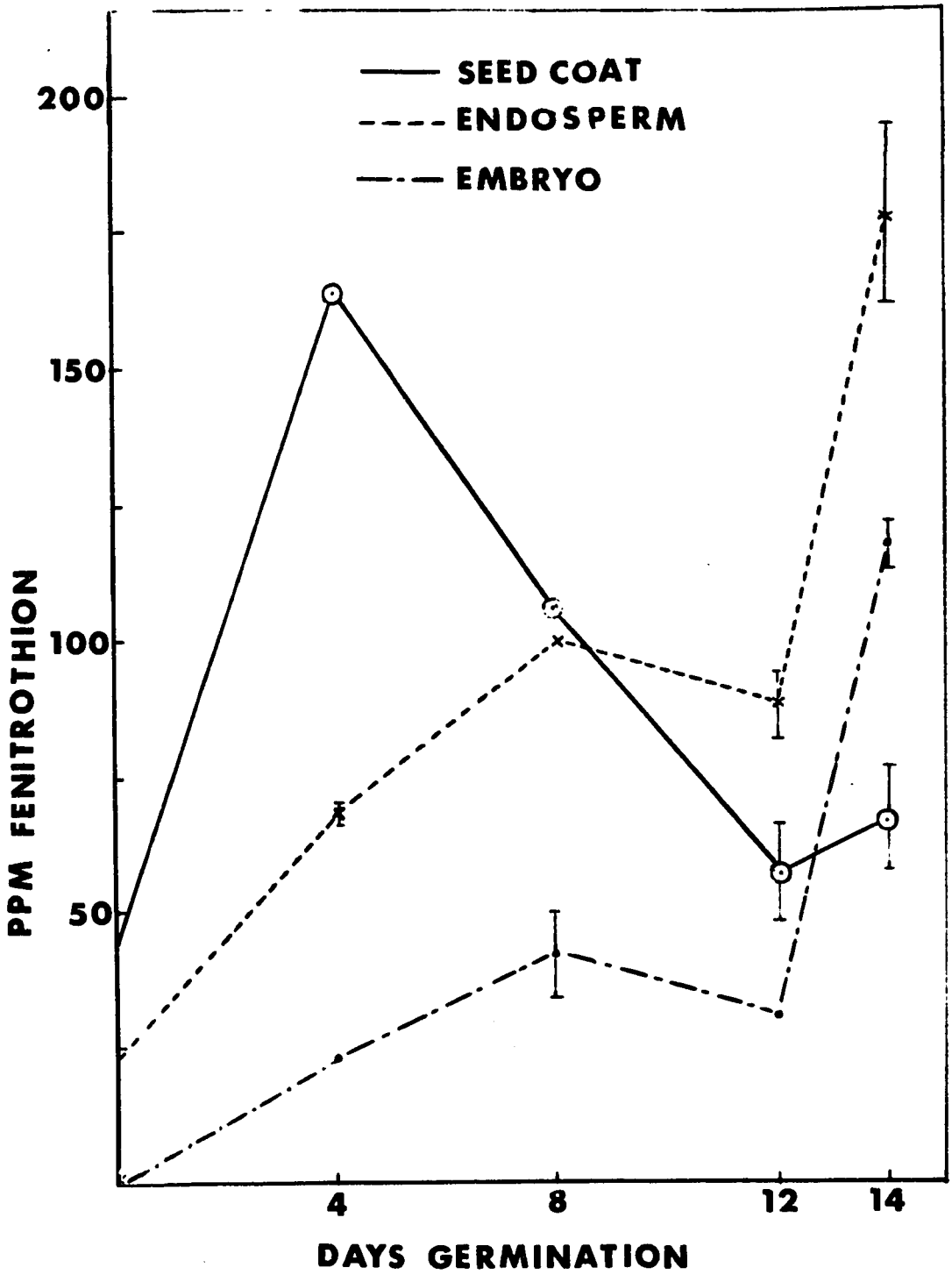


FIGURE 2

Thin layer chromatogram of acetone-benzene and benzene eluant fraction from white pine seeds, Pinus strobus. For treatment see Figure 1.

## THIN LAYER CHROMATOGRAM of Sg8 SEEDS

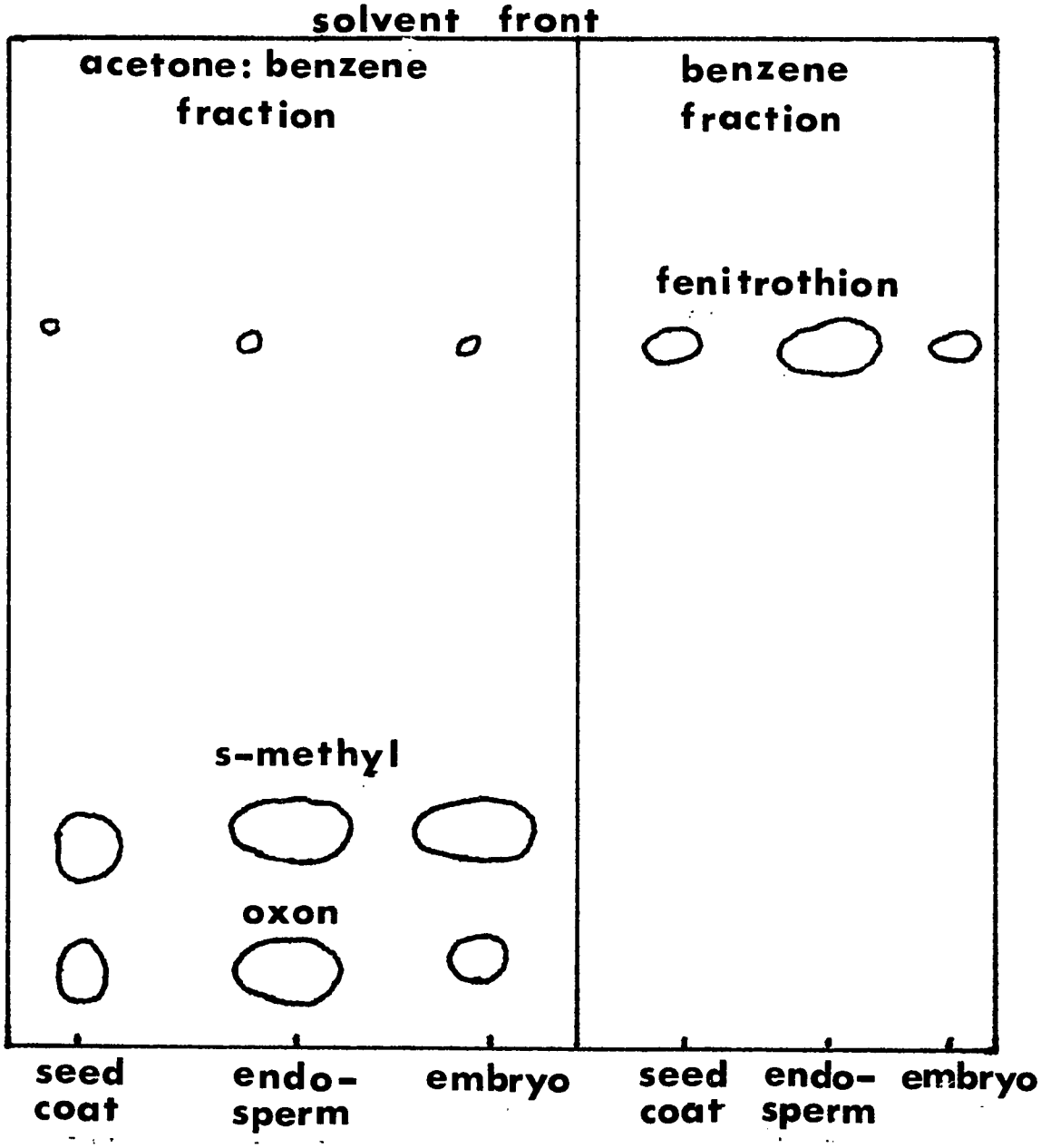


FIGURE 3

Concentration of fenitro-oxon in white pine seeds, Pinus strobus during germination. For treatment see Figure 1.

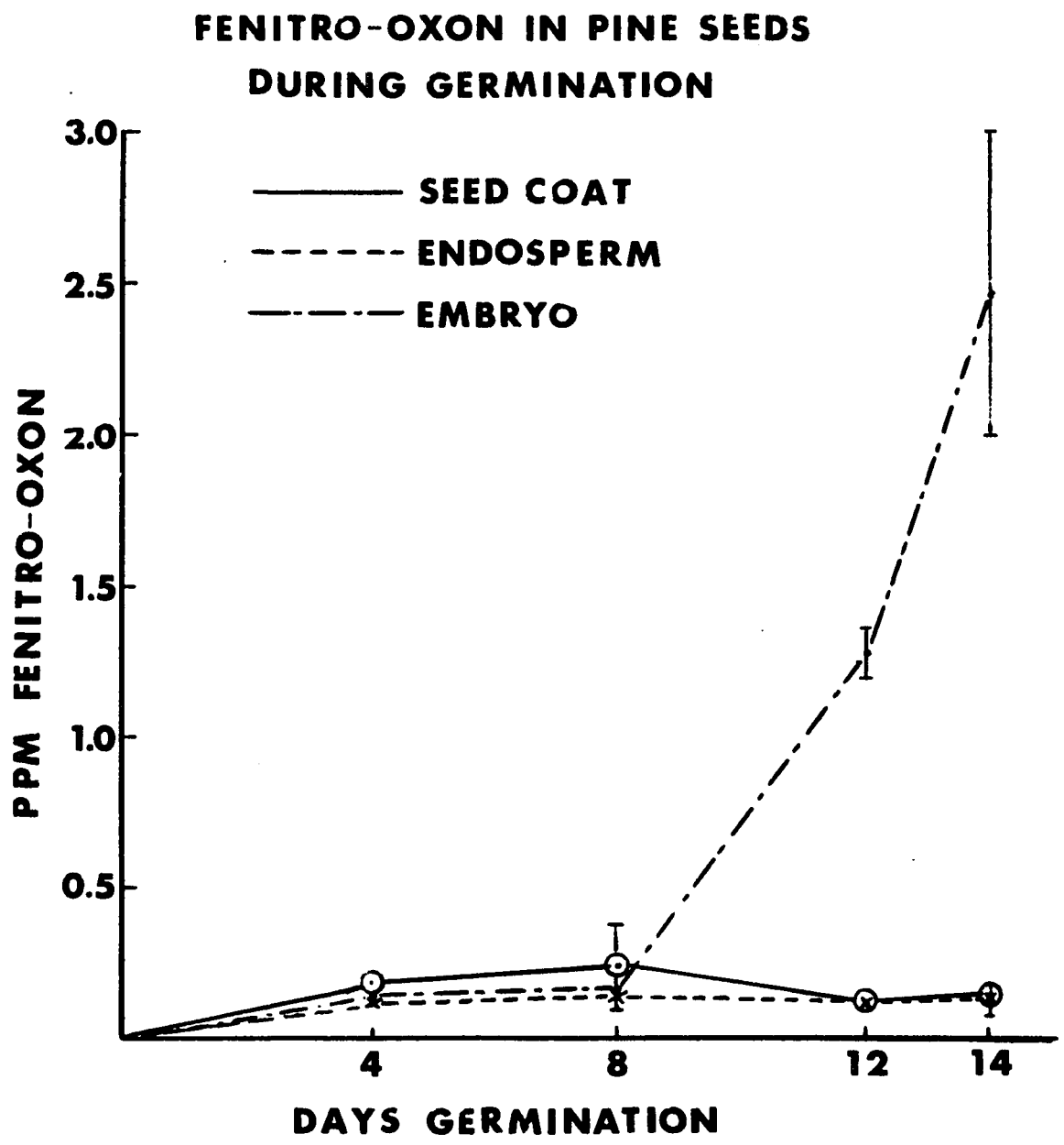

FENITRO-OXON IN PINE SEEDS
DURING GERMINATION
SEED COAT
ENDOSPERM
EMBRYO
PPM FENITRO-OXON
3.0
2.5
2.0
1.5
1.0
0.5
4
8
12
14
DAYS GERMINATION

FIGURE 4

Concentration of S-methyl fenitrothion in white pine seeds *Pinus strobus*, during germination. For treatment see Figure 1.

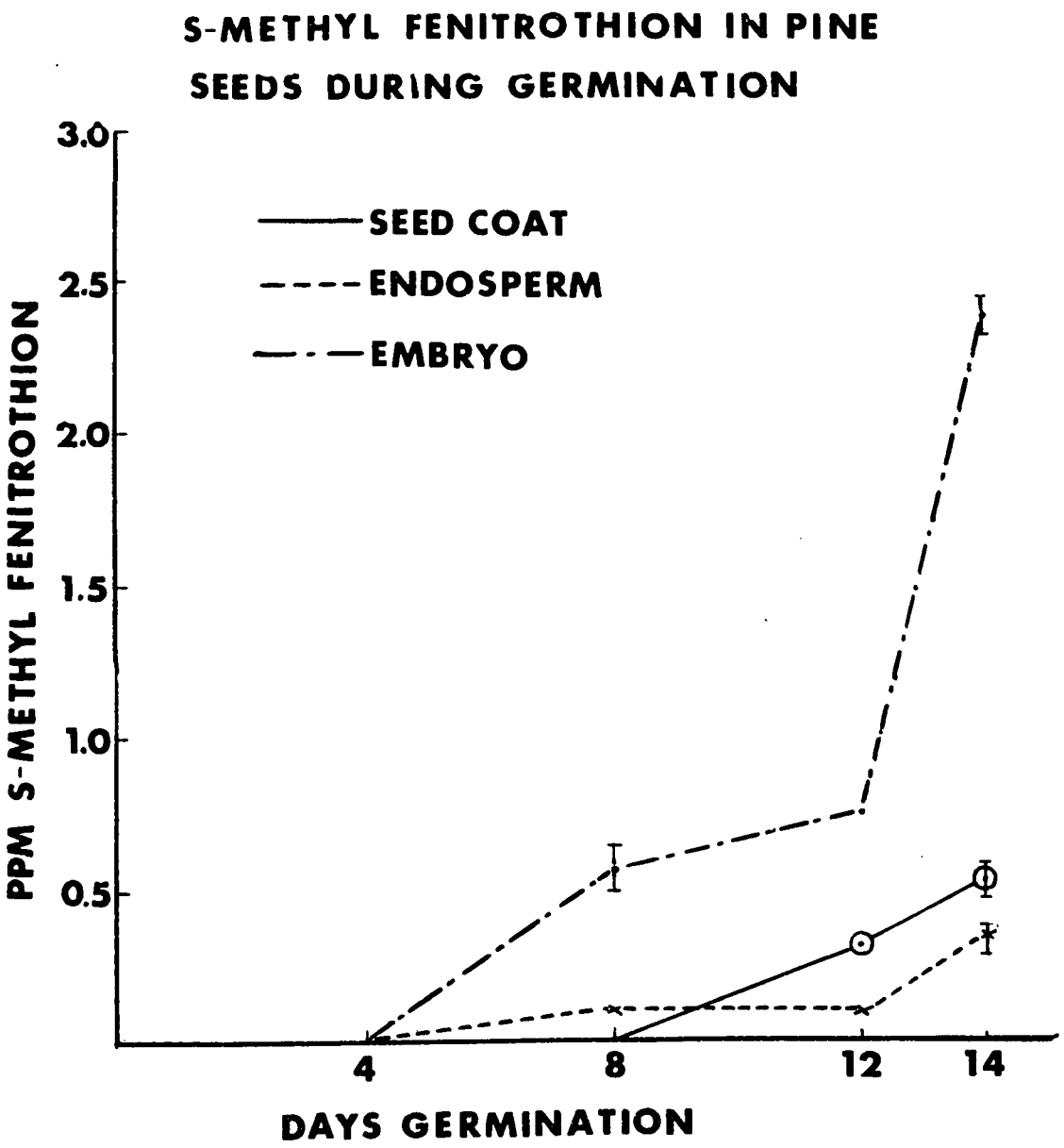

S-METHYL FENITROTHION IN PINE SEEDS DURING GERMINATION
SEED COAT
ENDOSPERM
EMBRYO
PPM S-METHYL FENITROTHION
3.0
2.5
2.0
1.5
1.0
0.5
4
8
12
14
DAYS GERMINATION

FIGURE 5

Concentration of desmethyl fenitrothion in white pine seeds, *Pinus strobus*, during germination. For treatment see Figure 1.

## DESMETHYL FENITROTHION IN PINE SEEDS DURING GERMINATION

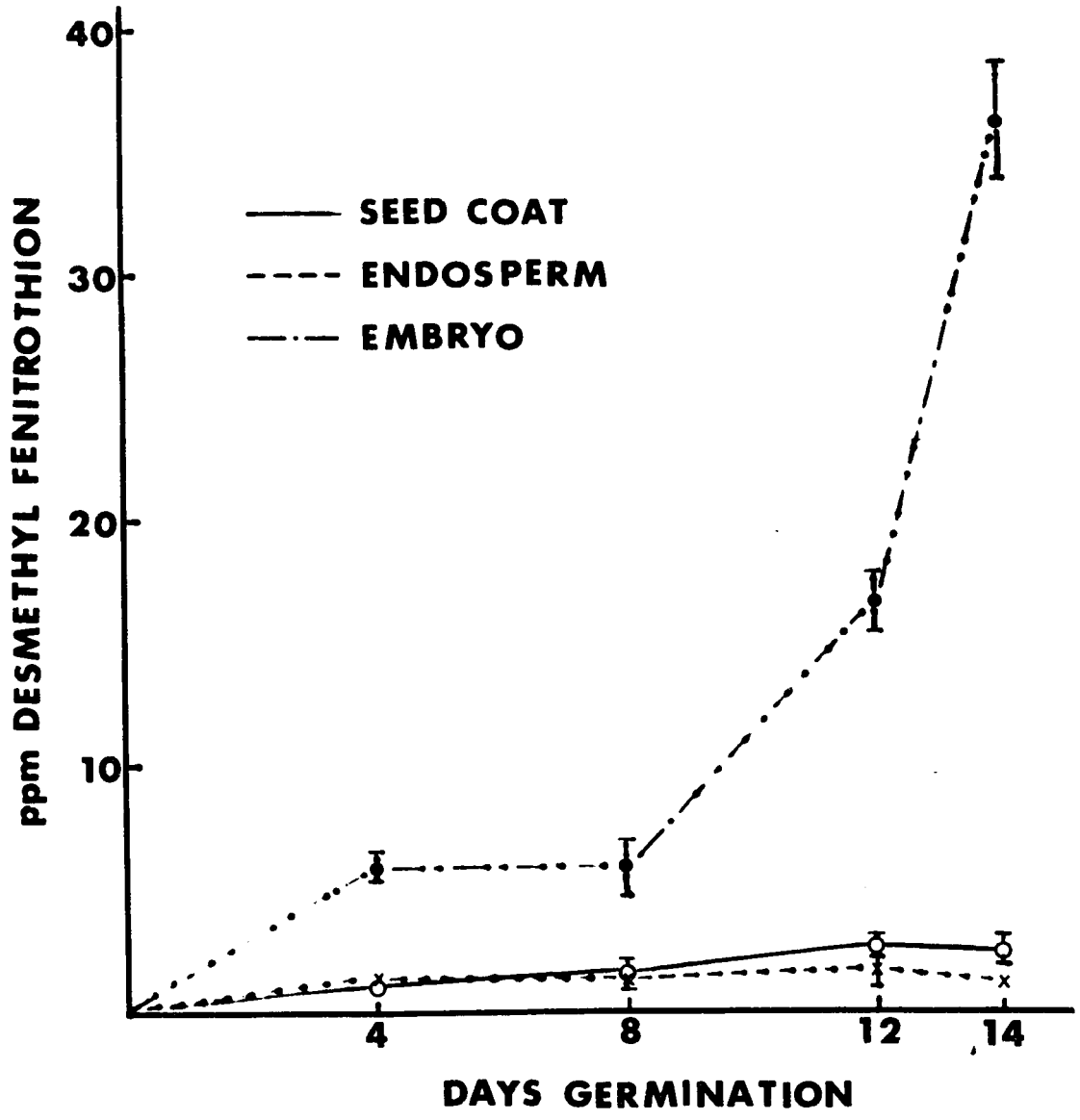


FIGURE 6

Concentration of fenitrothion in whole seeds of white pine, Pinus strobus, white spruce, P. glauca and yellow birch B. alleghaniensis, during germination. Seeds were stratified in an aqueous solution containing 10 ppm ($0.4 \times 10^{-4}$M) of pure fenitrothion for 21 days at $10^{o}$C in the case of white pine, and for 28 days at $5^{o}$C in the case of white spruce, and yellow birch. Seeds were germinated as in Figure 1. Each point represents the mean of two determinations on two seperate populations of each species treated similarly with fenitrothion. Vertical lines represent the range.

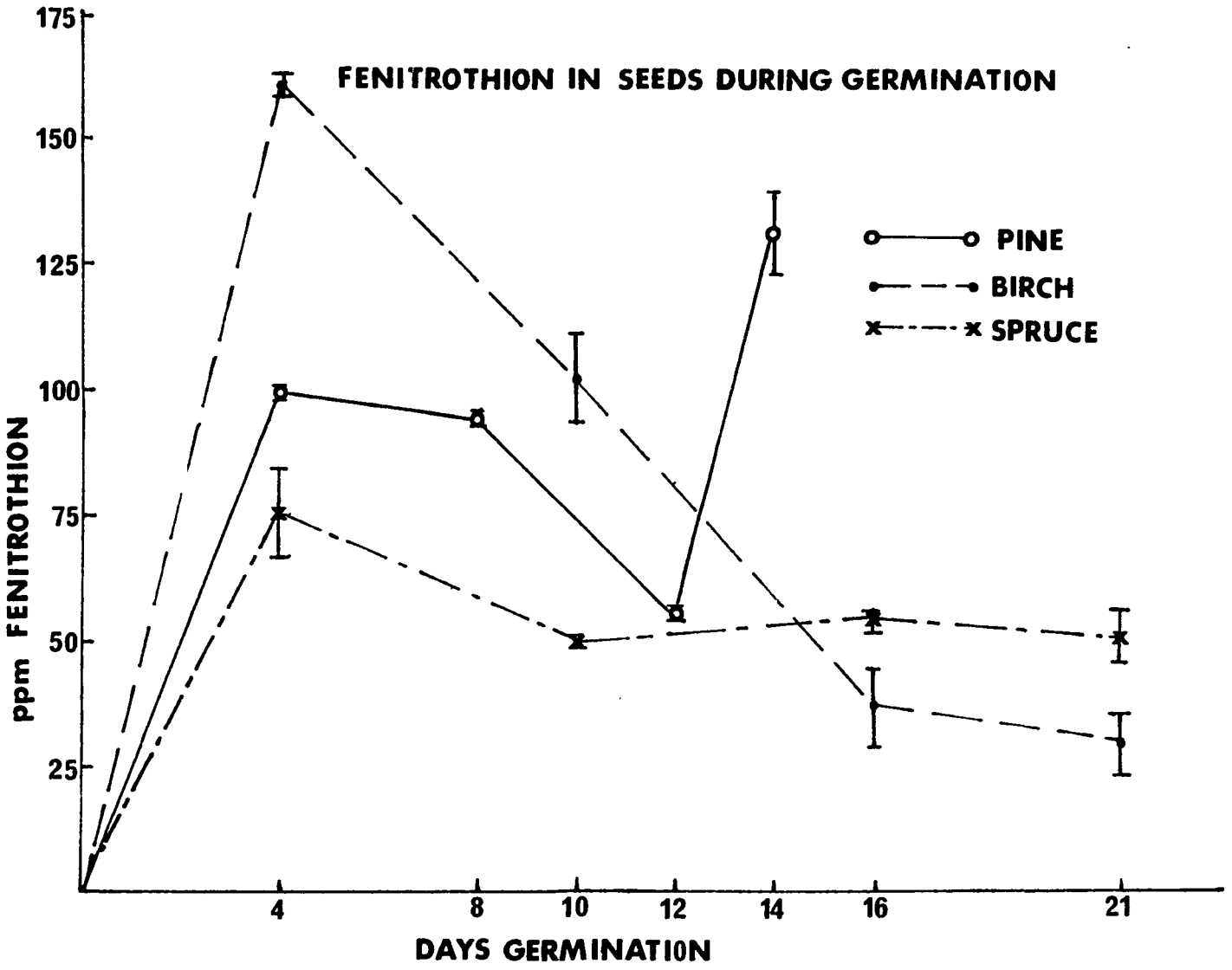
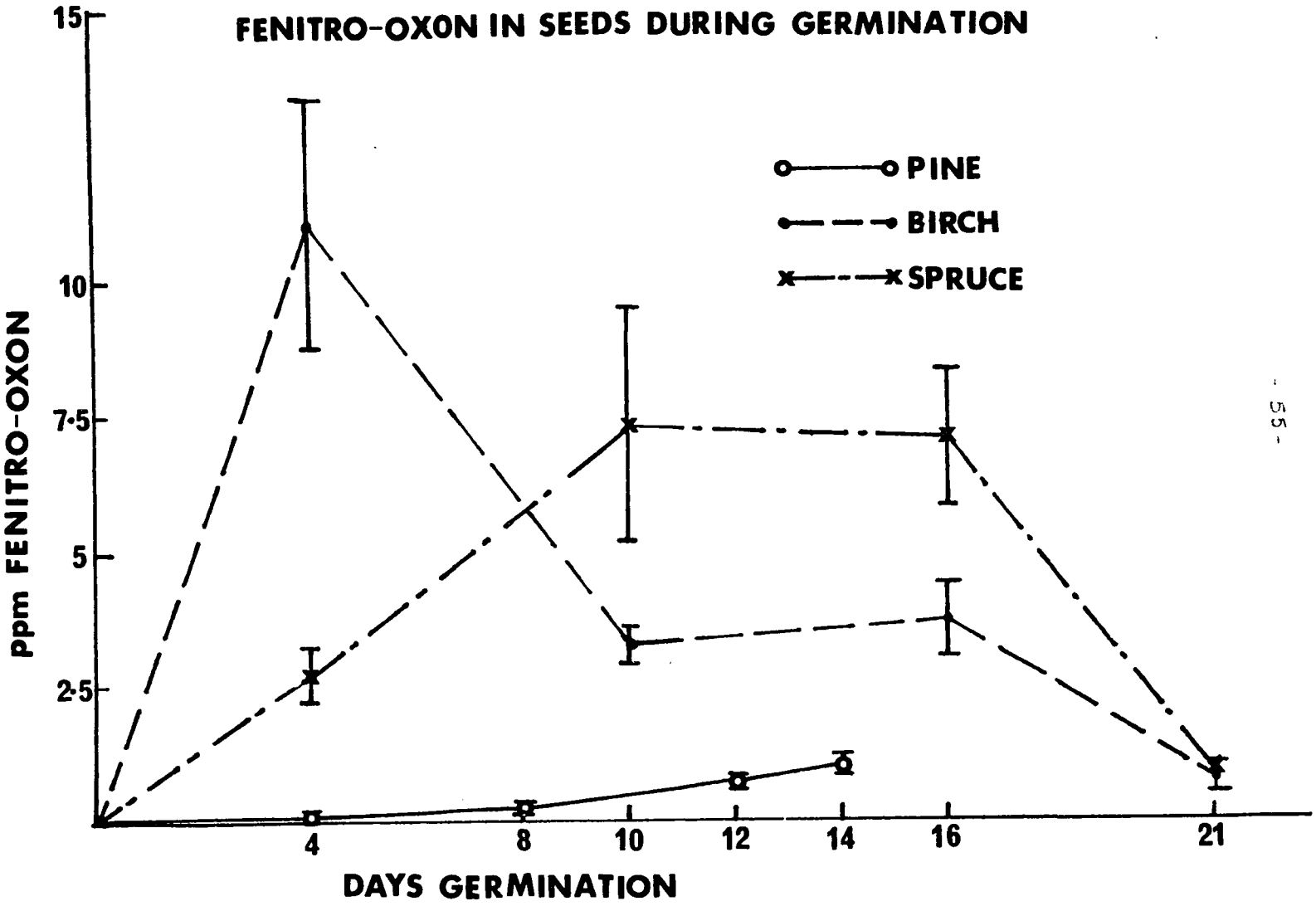

FENITROTHION IN SEEDS DURING GERMINATION
ppm FENITROTHION
175
150
125
100
75
50
25
4
8
10
12
14
16
21
DAYS GERMINATION
PINE
BIRCH
SPRUCE

FIGURE 7

Concentration of fenitro-oxon found in whole seeds of white pine, Pinus strobus, white spruce, Picea glauca and yellow birch, Betula alleghaniensis, during germination. For treatment see Figure 6.


FENITRO-OXON IN SEEDS DURING GERMINATION
PINE
BIRCH
SPRUCE
ppm FENITRO-OXON
15
10
7·5
5
2·5
4
8
10
12
14
16
21
DAYS GERMINATION

- 55 -

FIGURE 8

Concentration of S-methyl fenitrothion found in whole seeds of white pine, Pinus strobus, white spruce Picea glauca, and yellow birch, Betula alleghaniensis, during germination. For treatment see Figure 6.

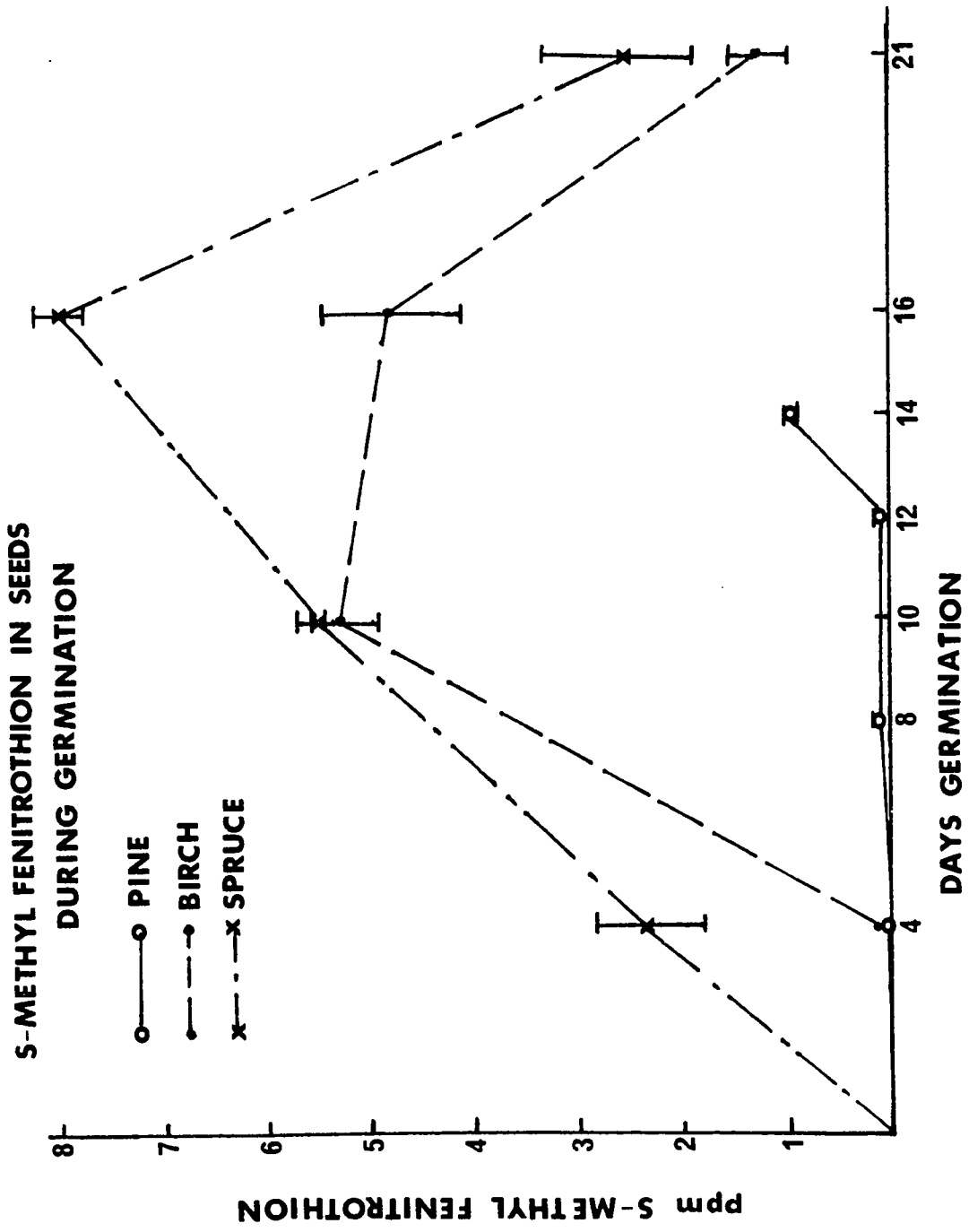

S-METHYL FENITROTHION IN SEEDS
DURING GERMINATION
PINE
BIRCH
SPRUCE
ppm S-METHYL FENITROTHION
DAYS GERMINATION
1
2
3
4
5
6
7
8
4
6
8
10
12
14
16
21

FIGURE 9

Concentration of desmethyl fenitrothion found in whole seeds of white pine, *Pinus strobus*, white spruce *Picea glauca* and yellow birch, *Betula alleghaniensis*. For treatment see Figure 6.

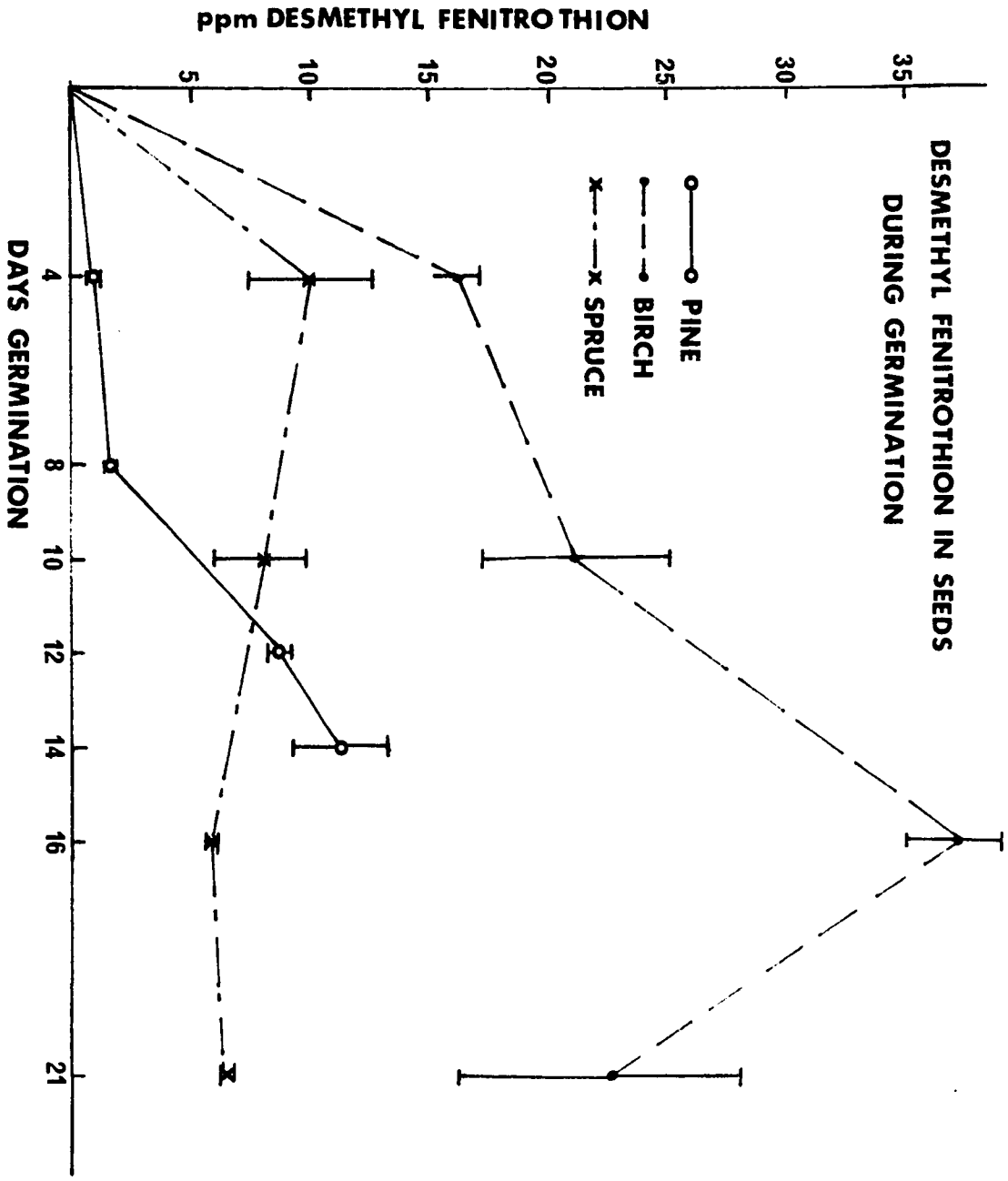
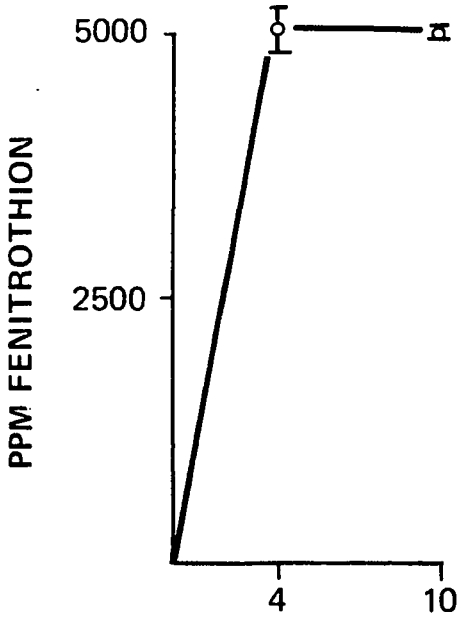

DESMETHYL FENITROTHION IN SEEDS
DURING GERMINATION
ppm DESMETHYL FENITROTHION
5
10
15
20
25
30
35
DAYS GERMINATION
4
8
10
12
14
16
21
PINE
BIRCH
SPRUCE

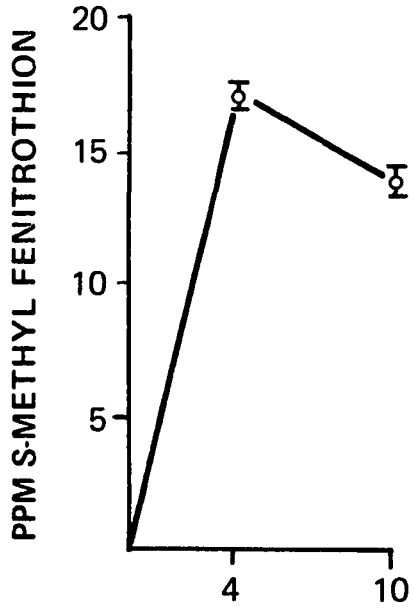
FIGURE 10

Concentration of fenitrothion, fenito-oxon S-methyl fenitrothion, and desmethyl fenitrothion in whole seeds of yellow birch, Betula alleghaniensis during germination. Seeds were treated with an aqueous emulsion containing 1000 ppm of fenitrothion ($0.4 \times 10^{-2}$M). Stratification and germination procedures were the same as in Figure 6.

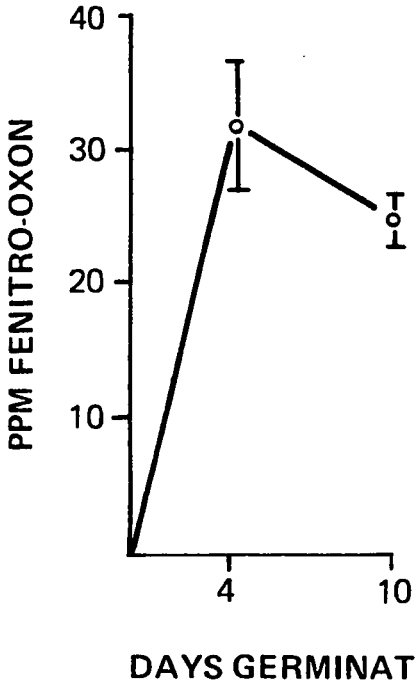
Fenitrothion in Birch
1000 ppm Fenitrothion




S-methyl Fenitrothion
in Birch 1000 ppm
Fenitrothion




Fenitro-oxon in Birch
1000 ppm Fenitrothion




Des Methyl Fenitrothion
in Birch 1000 ppm
Fenitrothion

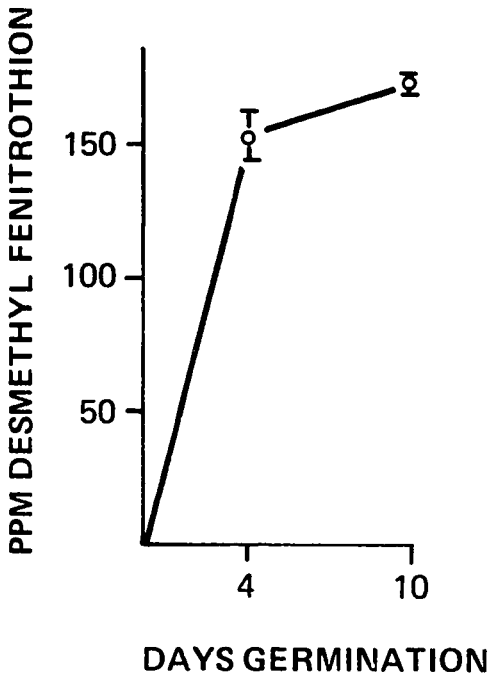


FIGURE 11

Mass spectrum of S-methyl fenitrothion found in white pine seedlings treated with fenitrothion. GC conditions: a 0.9m X 4.0mm ID glass column packed with 100/120 mesh Gas Chrom Q coated with 3 percent OV-17 was used isothermally at 202$^{o}$C.

# EI/MS OF S—METHYL FENITROTHION

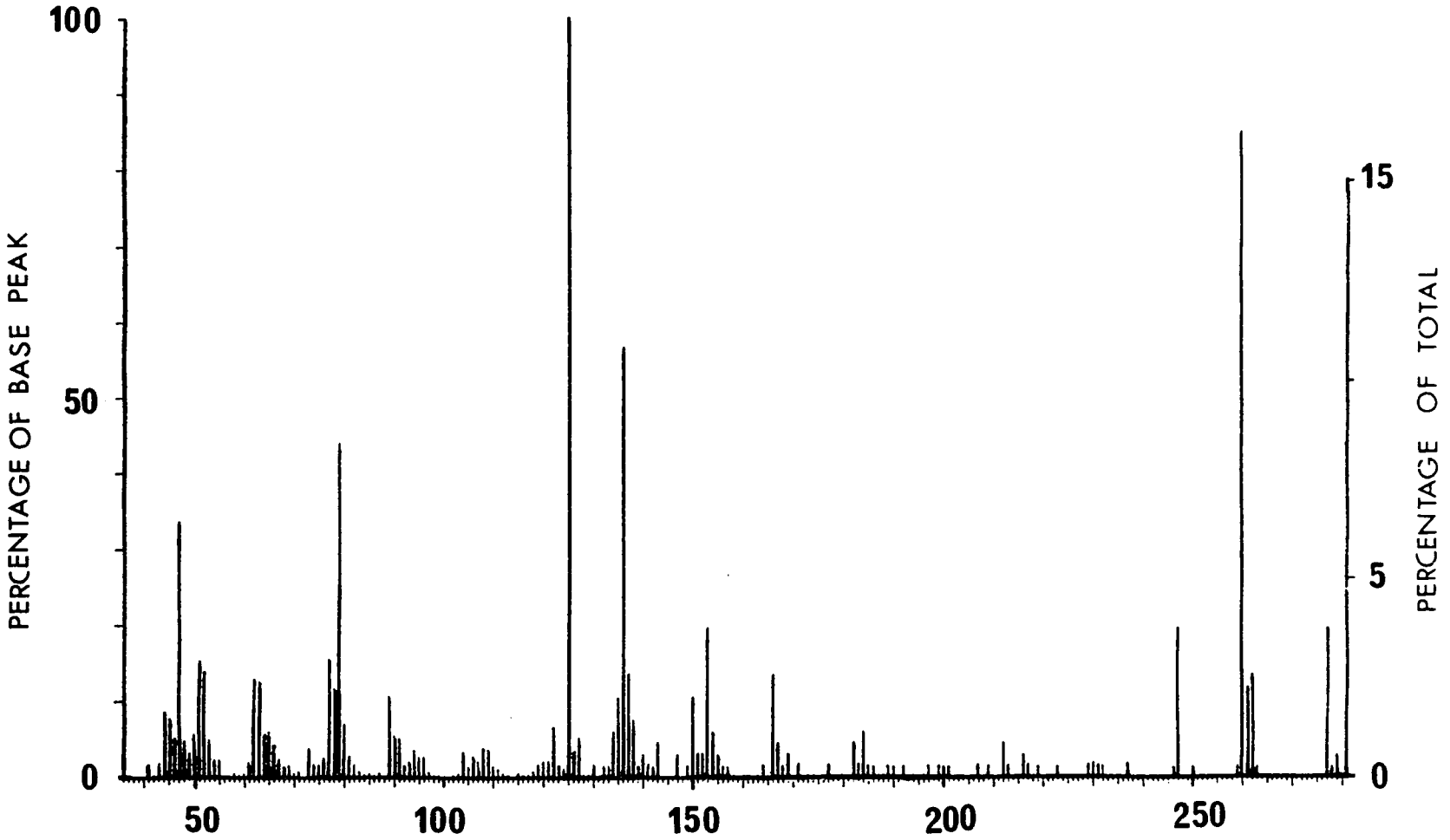



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FIGURE 12

GC/MS fragmentation pattern of S-methyl fenitrothion. The major ions at m3e 277, 260, 136, 125 and 79 are evident. The absence of a peak at 109 corresponding to the $CH_3O$-P-$OCH_3$ moiety found in fenitrothion and fenitro-oxon indicates a $CH_3SP$ bond rather than a P=S.

## FRAGEMENTATION PATTERN OF S-METHYL FENITROTHION

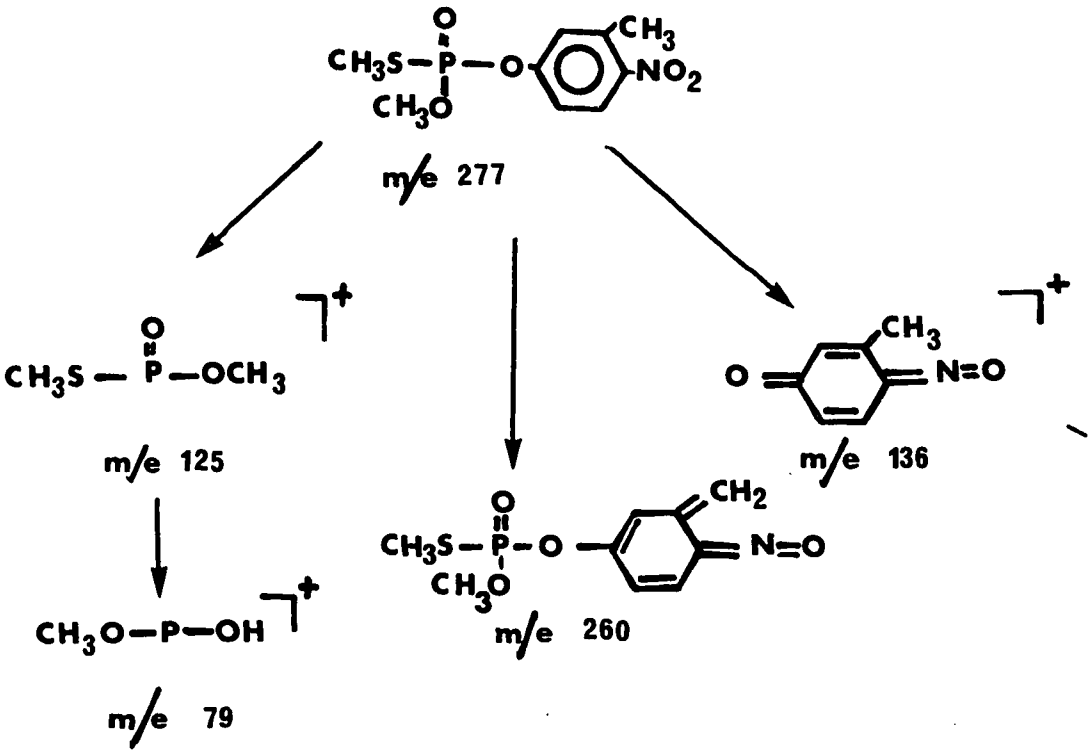
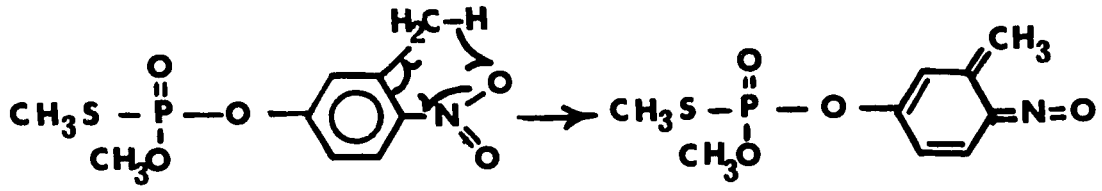


FIGURE 13

Ortho rearrangement, diagnostic for the methyl group ortho to the nitro group on the benzene ring. This is a characteristic of fenitrothion and metabolites with this configuration. The elimination of the hydroxyl ion (m-17) is initiated by the abstraction of a hydrogen from the methyl group and by an oxygen of the nitro group.

## ORTHO REARRANGEMENT — X



m/e 277 m/e 260

FIGURE 14

Direct probe electron impact mass spectrum of S-$CH_3$-glutathione extracted from germinated white pine seeds treated with fenitrothion.

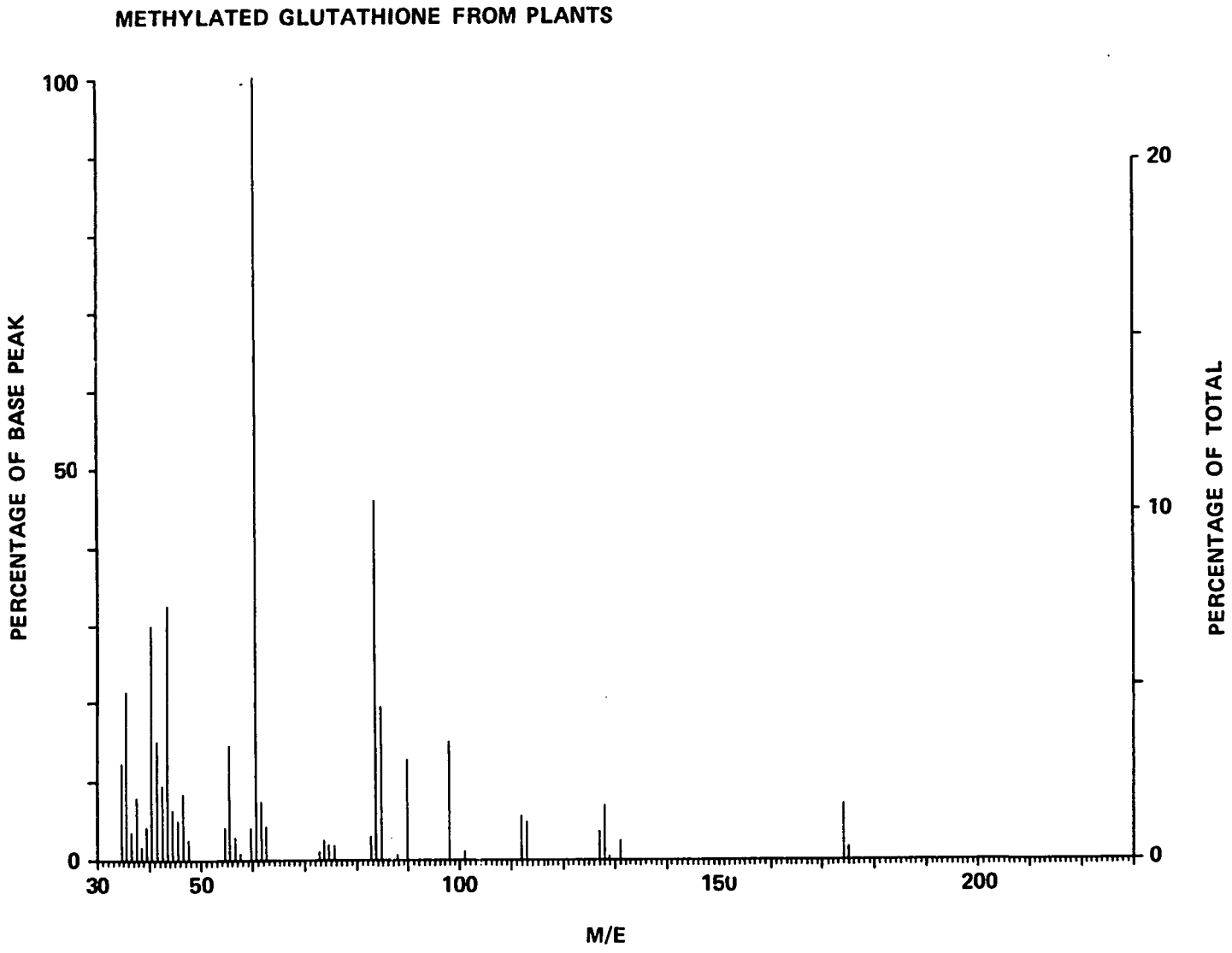

METHYLATED GLUTATHIONE FROM PLANTS
100
50
0
PERCENTAGE OF BASE PEAK
20
10
0
PERCENTAGE OF TOTAL
30
50
100
150
200
M/E

FIGURE 15

Six month old white pine seedlings Pinus strobus grown under identical conditions derived from control seeds (left) and seeds germinated in a 10ppm ($0.4 \times 10^{-4}$M) solution of fenitrothion in water.

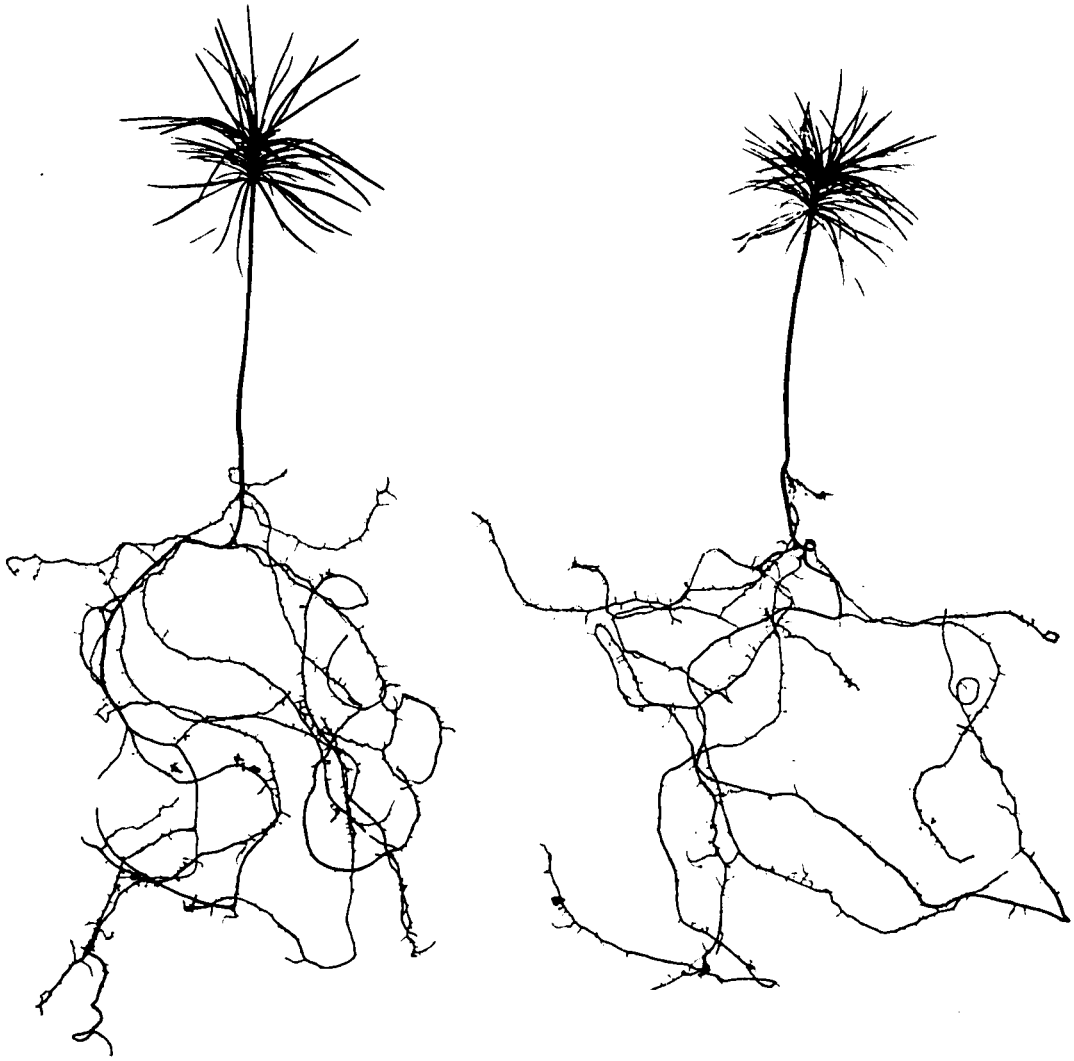


TABLE 1

Thin Layer Chromatogram of Extract from White Pine Seedlings Treated with $OC^{14}H_3$ Fenitrothion (Ninhydrin Positive Substances).

| Compound | Solvent System A | | | Solvent System B | |
|---|---|---|---|---|---|
| | Standard | Extract | Hydrolysis Product | Standard | Extract |
| S-Methyl Glutathione | .55 | .55 (1,500 dpm) | | .59 | .59 |
| Glutathione | .47 | | | .55 | |
| Cysteine | .46 | | .49 | .54 | |
| S-Methyl Cysteine | .59 | .59 (500 dpm) | .59 | .62 | |
| Glutamic Acid | .375 | | .38 | .53 | |
| Glycine | .325 | | .32 | .27 | |

TABLE 2

Thin Layer Chromatography of Extract from White Pine Seedlings Treated with $OC^{14}H_3$ Fenitrothion

| Compound | Rf | | Radioactivity dpm |
|---|---|---|---|
| | Standard | Extract | |
| Fenitrothion | .58 | .58 | 300,000 |
| S-methyl Fenitrothion | .15 | .15 | 1,500 |
| Fenitro-oxon | .09 | .09 | 1,000 |

TABLE 3

Concentration of Glutathione in 12 Day Old Tree Seedlings treated with Fenitrothion.

| | Glutathione ug/gram seed | | |
|---|---|---|---|
| Species | Control | 10 ppm | 1000 ppm |
| White Spruce | 901.1±9.2 | 883.2± 6.1 | 662.5± 4.7 |
| White Pine | 828.3±8.5 | 796.5±32.1 | 459.0±17.5 |
| Yellow Birch | 656.9±6.3 | 625.6±14.7 | 168.1±25.0 |

TABLE 4

NMR Spectra of Fenitrothion, Desmethyl Fenitrothion, and S-methyl Fenitrothion

| | |
|---|---|
| Fenitrothion | $\tau$ 6.1 ($CH_3OP$,d,6H,$J_{CH_3OP}$=14Hz) |
| | $\tau$ 7.41 ($CH_3Ar$,s,3H) |
| | $\tau$ 2.22 (Ar,m,3H) |
| Desmethyl Fenitrothion | $\tau$ 6.15 ($CH_3OP$,d,3H,$J_{CH_3OP}$=13Hz) |
| | $\tau$ 7.48 ($CH_3Ar$,s,3H) |
| | $\tau$ 2.24 (Ar,m,3H) |
| S-methyl Fenitrothion | $\tau$ 6.05 ($CH_3OP$,d,3H,$J_{CH_3OP}$=10Hz) |
| | $\tau$ 7.46 ($CH_3Ar$,s,3H) |
| | $\tau$ 7.40 ($CH_3SP$,d,3H,$J_{CH_3SP}$=16Hz) |
| | $\tau$ 2.29 (Ar,m,3H) |

$\tau$ (tau) = chemical shift downfield using tetramethyl silane as reference at 10.0 ppm

J = coupling constant

H = equivalent hydrogens

d = doublet

s = singlet

m = meta

## DISCUSSION

During the period of stratification, white pine seeds adsorbed fenitrothion from aqueous solution onto the seed coat, where it was accumulated during the first 4 days of germination. No metabolites of the pesticide were detected during this period, either in the water, filter paper, or on the seed coat surrounding the seeds. The pesticide did not penetrate the seed coat into the inner seed parts nor were any metabolites detected in the inner seed parts. During this initial phase of the experiment there was no detectable photodegradation of the fenitrothion (Ohkawa et al, 1974), as expected, since the experiment was carried out totally in the dark. There was no isomerization at the temperatures involved (Jaglan and Gunther, 1970), or hydrolysis of fenitrothion in the aqueous solution at neutral pH (Zitko and Cunningham, 1974). There was also no evidence of biodegradation due to microbial degradation of the pesticide neither on the seeds nor in the aqueous solution or filter paper (Yasuno et al, 1965, Miyamoto et al, 1966, and Zitko and Cunningham, 1974). The seeds were surfaced sterilized and the seeds were maintained under aerobic conditions. With all of these experimental variables eliminated one could then follow the true absorption and metabolism of fenitrothion by the seeds themselves during early seedling growth with reproducible results.

After the seeds had germinated and started to grow rapidly fenitrothion was absorbed first through the seed coat into the endosperm and embryo and later directly as the endosperm and embryo were exposed to the aqueous solution containing the pesticide. Fenitrothion rapidly penetrated and accumulated in the growing pine seedling tissues. This

accumulation was similar to that found for rice plants (Miyamoto and Sato, 1965), and for cocoa tree leaves (Sundaram and Sundaram, 1969). The rapidly growing young seedlings were capable of metabolizing the fenitrothion particularly in the embryo and endosperm. The rate of normal metabolism was associated with the rapid growth of the seed embryo.

The endosperm and embryos contained oxidative desulfurase enzymes capable of converting fenitrothion to fenitro-oxon, and dealkylase enzymes capable of converting fenitrothion to desmethyl fenitrothion. S-methyl fenitrothion was also evident but it is doubtful that the seedlings contained a fenitrothion isomerase enzyme. Oxygen analogues of phosphorothioates are commonly formed in seeds such as wheat germ (Rowlands, 1968). Fenitro-oxon was also found to be formed in the leaves and sheaths of rice plants (Miyamoto and Sato, 1965) and cocoa trees (Sundaram and Sundaram, 1969). Desmethyl fenitrothion was found as a water soluble metabolite in the leaves and sheaths of rice plants treated at the preheading stage of growth (Miyamoto and Sato, 1965) and was shown to be transferred to the rice grains. There were no previous reports of the biological formation of S-methyl fenitrothion in plants, animals, or bacteria.

It was evident that all of the metabolites were accumulating in the pine seedling tissues as they were formed. The concentrations increased with time and metabolites were not detectable in the water or filter paper supporting the seedlings. The fenitro-oxon and S-methyl fenitrothion produced in the endosperm and embryo were shown to be active microsomal enzyme inhibitors as shown by TLC (Figure 2) and are toxic cholinesterase inhibitors (Hollingworth 1967, a, and Kovacicova et al, 1973).

With these metabolic patterns established in white pine which is a large easily dissectable seed it was useful to expand the study to white spruce which is environmentally and economically more relevant in Canadian forests sprayed with fenitrothion, and yellow birch as a representative angiosperm of these forests. These seeds were too small to dissect easily. The smaller size of spruce and birch seeds would present a greater surface area per equivalent weight of pine seed, thus allowing for greater absorption of fenitrothion on seed surfaces. However, tissue distribution and metabolic patterns in the different seed parts were assumed to be similar to white pine.

When comparative uptake and metabolism of all three species was studied it was shown that yellow birch absorbed 1.5 to 2 times more fenitrothion than the other species. This was possibly due to the larger surface areas presented by an equivalent weight of the smaller birch seeds.

Yellow birch metabolized the pesticide into fenitro-oxon, S-methyl fenitrothion, and desmethyl fenitrothion more rapidly than the two coniferous species. The white pine and white spruce seeds paralleled each other in the formation of lower concentrations of these metabolites.

Germination of yellow birch seeds in the presence of very high levels of fenitrothion (1,000 ppm) resulted in wilt and senescence after only 4 days of germination. This level had no apparent effect on white pine or white spruce.

Birch seeds absorbed more fenitrothion, more rapidly than the two coniferous species but of greater importance is their ability to metabolize the fenitrothion into more toxic acetylcholinesterase inhibitors; fenitro-oxon and S-methyl fenitrothion, and to de-alkylate the insecticide into desmethyl fenitrothion.

Studies on the role of acetylcholinesterase in plants are limited. Jaffe (1970) has proven the existence of acetylcholine in all organs of both light-and-dark-grown mung bean seedlings. The highest concentrations were found in tissues containing active growing points, buds and secondary roots. Red light caused an efflux of acetylcholine from secondary root tips within 4 minutes, increasing tissue concentrations. Subsequently, a further four minutes of far red light reduced the level of acetylcholine to levels comparable to those in the dark. Acetylcholine, given for 4 minutes in the dark, was able to substitute for red light in reducing the formation of secondary roots, inducing increased $H^+$ efflux, and causing the root tips to adhere to a negatively charged glass surface. Acetylcholinesterase inhibited this phenomenon. It was concluded that acetylcholine functions in mung bean roots as it does in animal systems: by mediating changes in ion flux across cell membranes. In plants these phenomena are phytochrome mediated and acetylcholine probably acts as a local regulatory hormone. Jaffe (1973) later showed that cholinergic drugs such as atropine were capable of modifying the acetylcholine mediated ion flux in bean roots. It is therefore possible that the high levels of acetylcholinesterase inhibitors, fenitro-oxon and S-methyl fenitrothion, which were formed in the yellow birch were responsible for the senescence and wilt in the seeds treated with 1000 ppm fenitrothion. Acetylcholine mediated ion fluxes were likely thrown into imbalance by acetylcholinesterase inhibition. Studies on acetylcholinesterase in yellow birch seedlings were initiated by L. Pomber and P. Weinberger in regard to this problem. The

growth effects of these two metabolites on forest seedlings was also being studied since they are likely to occur naturally in the environment not only as plant metabolites, but fenitro-oxon has also been shown to occur as the result of UV irradiation by sunlight (Ohkawa et al, 1974). It is very unlikely however that forest overspray would create concentrations in forest plants as high as that found with the 1,000 ppm treatment. This experiment was carried out to demonstrate the extreme case of metabolism on the pesticide for better understanding of the metabolism at lower concentrations.

The highest concentrations of all metabolites evident in the treated seedlings was that of desmethyl fenitrothion. This metabolite is not considered toxic in mammals. It is a polar acid which is water soluble and is not as lipophilic as fenitrothion. As such, it occurs as a major urinary excretion product in mammals (Hollingworth, 1969). Plants, particularly seeds during germination and early seedling growth, do not possess this efficient system for selectively excreting water soluble metabolites such as desmethyl fenitrothion. They most likely store it in vacuoles. Fenitrothion was shown to be rapidly metabolized into water soluble metabolites by rice plants (Miyamoto and Sato, 1965). Most of these products resulted from hydrolysis of fenitrothion and fenitro-oxon but about 10 percent resulted from dealkylation of fenitrothion and fenitro-oxon to form the desmethyl derivatives. These polar metabolites were translocated from the rice leaf blades and shoots into the rice grains. Fenitro-oxon, which is lipophilic was not translocated. Fenitrothion was not shown to translocate to any significant extent in cocoa trees (Sundaram and Sundaram 1969). In this study transport

of polar metabolites was not examined. Transport of desmethyl fenitrothion was not considered relevant to the study of early seedling growth but should be considered when examining more mature trees. It should definitely be included in all environmental analyses of biota since desmethyl fenitrothion persists as a distinct, diagnostic residue of fenitrothion much longer than any other known unhydrolysed environmentally formed derivative of the parent compound (Zitko and Cunningham, 1974). The effects of its persistence in plant tissues is unknown.

It was demonstrated with $OC^{14}H_3$ labelled fenitrothion that desmethyl fenitrothion was formed in white pine seedlings with glutathione as the acceptor of the $C^{14}H_3$ methyl group forming S-$C^{14}H_3$ glutathione. In plants, the presence of a glutathione mediated enzyme system was shown to exist by the isolation of glutathione-atrazine conjugates in corn (Shimabukuro et al, 1970) and sorghum leaf sections (Lamoureux et al, 1970). The role of glutathione in detoxifying peroxacetyl nitrate, a phytotoxic oxidizing component of photochemical air pollution forming S-acetyl glutathione is well documented (Mudd, 1966, Mudd and McMannus, 1969). Extensive studies carried out with mammalian liver and kidney showed that fenitrothion was dealkylated via a glutathione alkyl transferase enzyme to form desmethyl fenitrothion and S-methyl glutathione (Hollingworth, 1969). The presence of high levels of desmethyl fenitrothion in white spruce and yellow birch suggested that the glutathione S-alkyl transferase pathway was a major detoxification pathway of fenitrothion in the seedlings.

Glutathione has been implicated in the induction of enzyme synthesis in key metabolic pathways in both plant and animal cells (Webb,

1966, Boyland and Chasseaud, 1969). Glutathione and ascorbic acid take an active part in enzyme regulation during the early stages of pea seedling germination (Spragg and Yemm, 1958). Mapson and Moustaffa (1956) indicated that about one-quarter of the total respiration of the young seedling may be mediated by glutathione and ascorbic acid.

Depletion of glutathione levels in the growing seedlings was correlated both with the amount of fenitrothion used in the treatment and with phytotoxicity to the species. Seed germination and seedling growth were not affected by treatment with fenitrothion at the 10 ppm level in any of the three species examined. Glutathione levels were also not affected with this low level treatment. Seed germination and growth were also not affected by treatment with fenitrothion at the 1000 ppm level in the two gymnosperm species white spruce and white pine. Glutathione levels were moderately reduced by 25 percent and 43 percent respectively in these species. By comparison, yellow birch suffered a severe depletion of 75 percent of glutathione at this extreme treatment and seedlings did not progress past the 10 day stage of growth. The phytotoxicity of high levels fenitrothion to yellow birch seeds and seedlings may be partially accounted for by depletion of tissue glutathione.

No known enzymatic mechanisms exist for the direct isomerization of fenitrothion to form S-methyl fenitrothion. However, the presence of a glutathione alkyl transferase detoxification mechanism for fenitrothion was shown in the growing seedlings to form desmethyl fenitrothion. As well, desmethyl fenitrothion in the presence of an excess of fenitrothion

(similar to that found in the seedlings) was shown to form S-methyl fenitrothion in relatively short periods of time (21h) when compared to 21 days of germination and early seedling growth. Fenitrothion does not chemically isomerize under the conditions of darkness and low temperature imposed by stratification and germination (Ohkawa et al 1974, Jaglan and Gunther, 1970) and no S-methyl fenitrothion was detected in the aqueous solutions in which the seeds were germinated. It can therefore be concluded that S-methyl fenitrothion was formed in the germinating seedlings via the reaction of absorbed fenitrothion with enzymatically created desmethyl fenitrothion. S-methyl fenitrothion is much more active acetylcholinesterase inhibitor than fenitrothion (Kovacicova et al, 1973).

McLeod (1975) reported a possible residual effect of fenitrothion on Swaine jack pine sawfly 42 days following aerial application of the pesticide. This delayed toxicity of fenitrothion may be partially explained by enzymatic formation of S-methyl fenitrothion in the tree. Slow isomerization of fenitrothion absorbed by the pine foliage via the desmethyl form, would produce small concentrations of the more active toxicant S-methyl fenitrothion which then would kill the sawflies. This could be simply investigated by analysing the supposedly toxic vegetation 42 days after application for residual fenitrothion as well as desmethyl and S-methyl fenitrothion.

Yule (1974) reported that up to 1 ppm of fenitrothion persisted in coniferous foliage throughout the year when the foliage was subjected to repeated annual field applications of the pesticide for up to 5 years. Assuming the analysis to be accurate, this would imply that the routes

of hydrolysis and dealkylation of the absorbed pesticide are bound up after several applications. Desmethyl fenitrothion could persist in the foliage for long periods if it is not translocated, since it is unreactive and will not hydrolyze in the ionic state. Fortunately, it is this property which makes it non toxic. Its enzymatic binding properties particularly for aryl transferases have not been investigated to date. The persistence of desmethyl fenitrothion in foliage treated similarly to that investigated by Yule should also be studied.

Although fenitrothion was shown to be capable of alkylating, by reacting with desmethyl fenitrothion to form S-methyl fenitrothion, none of the fenitrothion derivatives tested showed any mutagenic potential to the Ames bacterial strain sensitive to alkylation activity. It has been possible to show alkylation of DNA with organophosphate pesticides such as dichlorvos yielding in particular 7-methylguanine. The molar ratios of pesticide to nucleotide units were stressed unrealistically in favour of alkylation (Bedford and Robinson, 1972). It was considered that mutagenic alkylating agents must be strong 'spontaneous' alkylators while organophosphorus pesticides are relatively weak alkylators. The Ames test is not perfect in predicting mutagenicity. Control alkylating agents used, which are strong mutagens, such as N'-methyl-N-nitro-N-nitrosoguanidine, did show strong positive reactions validating the tests. It seems unlikely that fenitrothion or its derivatives would either persist long enough or bioaccumulate in a high enough ratio to alkylate nucleic acids <u>in vivo</u>.

Fenitrothion has also been shown to be non-mutagenic when administered in the diet of rats at dosage rates of 10, 40, and 80 ppm. No chromosomal abberations were found in the bone marrow of second generation progeny (Benes et al, 1973). It, therefore, seems doubtful that the genetic health of coming generations of forest mammals would be threatened by long term eating of foliage and seeds sprayed with fenitrothion alone.

The metabolic pathways investigated in this thesis are limited to pure fenitrothion and do not account for possible synergistic toxicities caused by their combination with carrier mixtures used in aerial spraying. I am confident that they represent solely plant metabolism and are not confused by bacterial or fungal artifacts which, however, could also occur in addition during field spraying. The reactions involved in the formation of toxic and non-toxic metabolites of fenitrothion by white pine, Pinus strobus, white spruce, Picea glauca, and yellow birch, Betula alleghaniensis are summarized in Figure 16. The insecticide was metabolized to form the toxic fenitro-oxon, and also deactivated by a glutathion alkyl transferase to give the polar non-toxic desmethyl fenitrothion. A second toxic metabolite, S-methyl fenitrothion, was also detected in the seeds. It was thought to result from the alkylation of desmethyl fenitrothion with fenitrothion. Hydrolysis products were not studied since they are not cholinesterase inhibitors.

It has been suggested that the pesticide could act as a

nitrogen supplement for the trees. No desnitro derivatives of fenitrothion or 3-methyl-phenol have ever been discovered in any environmental or chemical study. It is, therefore, doubtful that low dosage spraying of fenitrothion on forests or experimental treatment of plants with fenitrothion would result in increased growth due to an additional nitrogen source provided by the pesticide.

FIGURE 16

Metabolic pathways of fenitrothion in germinating seeds and young seedlings of white pine; Pinus strobus, white spruce; Picea glauca, and yellow birch; Betula alleghaniensis.

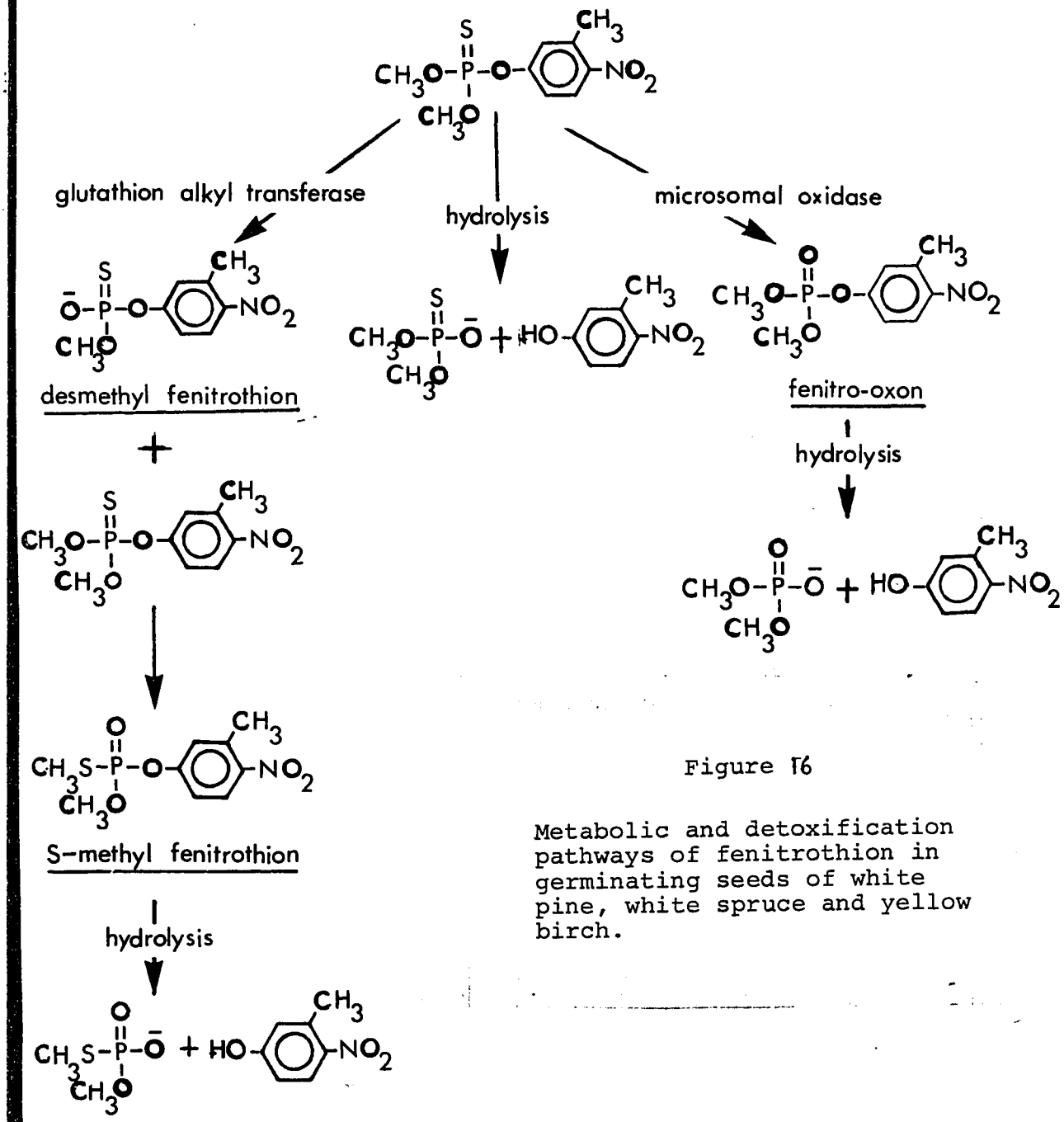



Figure 16

Metabolic and detoxification pathways of fenitrothion in germinating seeds of white pine, white spruce and yellow birch.

## CONCLUSIONS AND CONSIDERATIONS FOR FUTURE RESEARCH

1. These metabolic studies on tree seedlings indicate that environmental monitoring of forest tree foliage for fenitrothion residues should include not only the parent compound but also the more active cholinesterase inhibitors fenitro-oxon, and S-methyl fenitrothion, and the more persistent residue desmethyl fenitrothion. Monitoring of the former three compounds should be mandatory since they represent the actual potential for organophosphorus pesticide poisoning from excessive contact with the sprayed area. Perhaps a more efficient routine method, where feasible, may entail an enzymatic approach by measuring total cholinesterase inhibition in samples. This may however, not be practical.

Further it should be noted that,

a) unless adequate precautions are taken during sampling, residues of the more labile fenitro-oxon, and S-methyl fenitrothion will be lost before either chemical or enzymatic analyses have been performed.

b) although these residues, particularly the more toxic metabolites, will not likely persist in the environment for long periods, the potenital damage they may cause through direct contact to all biota in sprayed areas during the short period in which they are active cholinesterase inhibitors is considerable.

2. It seems unlikely that long term environmental effects will be due to bioaccumulation of fenitrothion or for other short lived modern pesticides, including other organophosphates or the carbamates class of pesticides. However, long term effects of these pesticides are possible.

Marked loss of species diversity in the environment due to the peculiar sensitivity of certain species or classes of plants, animals, and invertebrates may occur. Sensitive species should perhaps be monitored as selective indicators of long term ecological damage.

3. To this end, environmental models peculiar to the various biomes of Canada should be developed so that the long term effects of pesticides can be studied with more accuracy before millions of acres of the Canadian environment are subjected to their use.

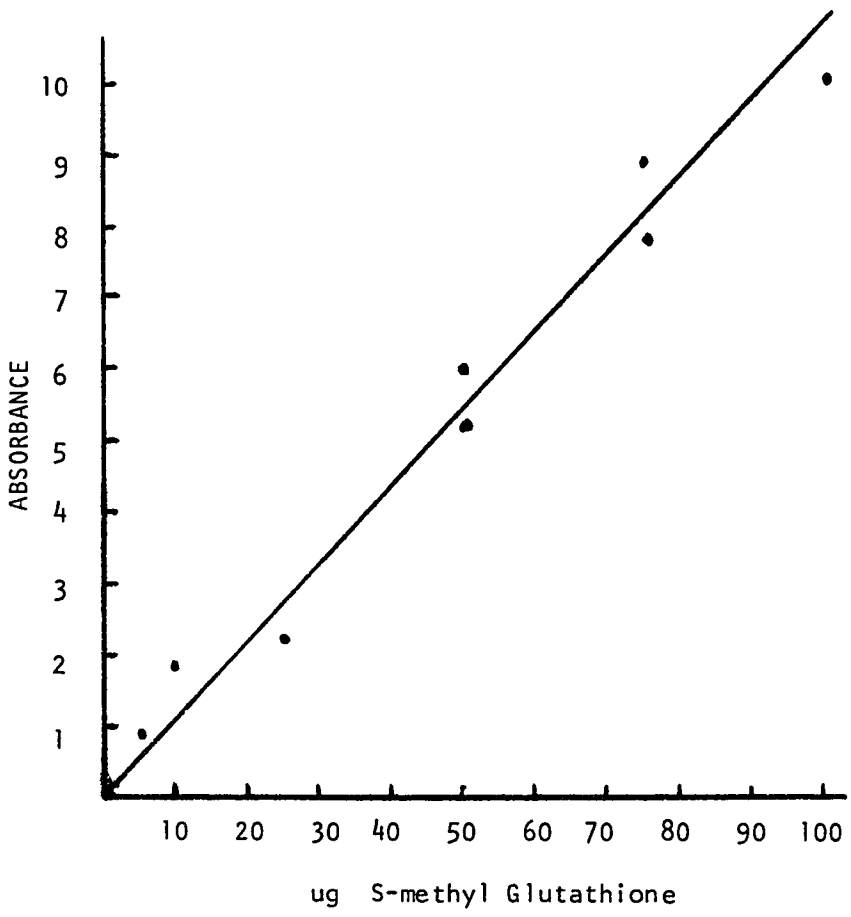
4. Reye's syndrome has recently emerged in the general press and in the scientific literature. A strong association has been made between the application of fenitrothion in Eastern Canada and the rise in the incidence of this syndrome in children from specific locations.

I bring up this syndrome not for scientific discussion, as many scientists believe that statistically valid corroborative evidence is lacking, I wish to point out that environmental studies particularly on man made chemical pollutants must become more thorough, scientifically accurate and therefore predictive, so that they may become a recognized warning to the public of the eventual detrimental effects on human health.

Appendix 1

Calibration curve for determination of S-methyl glutathione after Grunert and Phillips, (1951).

CALIBRATION CURVE
S-METHYL GLUTATHIONE



Appendix 2

## Fenitrothion Research 1976-1978

In April 1977 a Symposium on the long-term effects of the use of fenitrothion in forest ecosystems was held and written proceedings published and made generally available in February 1978. At least two of these studies have been republished in other journals, however, the data and conclusions have not changed.

The major contribution of this thesis was the discovery that fenitrothion can be metabolized by plant tissues found in Canadian forests where the pesticide is sprayed. Fenitro-oxon and S-methyl fenitrothion are more toxic than the parent compound and therefore were considered in detail. Although it is relatively non-toxic, desmethyl fenitrothion was also considered as an intermediary metabolite in the formation of S-methyl fenitrothion. These three metabolites are the major compounds formed in mammals, plants, and bacteria, and are also the major derivatives formed by photolysis or in aquatic ecosystems where the pesticide is exposed to both the photolytic action of sunlight, and bacterial metabolism. The only exceptions are the recently characterized derivatives; carboxy fenitrothion and formyl fenitro-oxon (Miyamoto, 1977).

Armstrong (1977) presented a complex report of spray deposition of fenitrothion in formulation on the forest surface. The spray is concentrated as it meets surfaces on the downwind side of the spray swath. This concentration pattern occurs on a small scale for individual trees and on a large scale for openings in the forest. Variations from 2.05 l/ha to 22.19 l/ha were found to occur across a single spray swath. The

data also indicated that under most operational conditions 50 percent of the emmitted spray was deposited on the forest. It is therefore possible to have many localized pockets of concentrated fenitrothion within a forest relative to an even spray as registered.

Moody et al, (1977) presented his final data which showed that the majority of fenitrothion deposited on foliage is lost by volatization. However, fenitrothion was absorbed by balsam fir, white spruce, and jack pine foliage, is systemically transported by xylem vessels to younger foliage. No large scale degradation of absorbed fenitrothion took place under the experimental conditions, although there was evidence of one metabolite which remained unidentified.

McNeil and McLeod (1977) continued their previous work with the apparent residual toxicity to Swaine Jack Pine Sawfly. It was determined that foliage treated in 1975 with fenitrothion was toxic to the sawfly. Although the detrimental effects of feeding the foliage to the sawfly including a decrease in cocoon size and weight was well documented, a thorough chemical approach to ascertain fenitrothion residues and metabolites present was not accomplished. Therefore a cause and effect relationship could not be established as in the aforementioned study by Moody et al, (1977).

Several other studies pertained to the Canadian environment Lockhart et al, (1977) found that detectable fenitrothion and its metabolites did not persist in running streams or stagnant ponds in sprayed forests. However, it was ascertained that caged Japanese quail were not protected by the forest canopy from the spray, and suffered a large drop

in serum cholinesterase activity. No detrimental effect on bird populations in New Brunswick was found directly attributed to forest spraying (Pearce and Peakall, 1977). However, conclusive cause and effect data was not collected and studies are continuing. Varty (1977) concluded that there has been no long term effect of fenitrothion forest spraying since 1968 on non-target terrestrial arthropods. Population recovery was generally rapid.

Buckner et al (1977) presented data which indicated that fenitrothion is non-toxic to the masked shrew, red-backed vole, and white footed mouse by exposing caged animals to oil-based fenitrothion formulated sprays. Levels 2X and 3X the registered maximum rate of application (4 oz/acre, 280 g/ha) showed a dose related lethal response to all three species. Reproduction was also affected at the same levels which one would expect from a dosage which was lethal to some of the population. The size of each experimental group was too small to allow statistical interpretation. Field studies on spray plots which were operationally treated for up to 5 consecutive years indicated little effect on small mammal populations although the author does not account for population stress caused by sampling in a relatively small area with snap-back traps which kill the animal, or for the probable influx of animals from non-sprayed areas.

Environmental modelling of the impact of pesticides is the most recent approach in predicting rather than assessing the possible impact of fenitrothion forest spraying in the Canadian environment. A tentative simulation model for the distribution of pesticides including fenitrothion was proposed by Marshall and Roberts (1977). This appears to be the most

useful direction for environmental research associated with pesticides at this time. Since fenitrothion, its metabolites, and degradation products are well characterized chemically, it remains that the various physical, biological, and chemical parameters be characterized for each particular environment within spray zones in order to estimate persistence and distribution patterns within each unique ecosystem. Modelling will aid in synthesizing and selecting new pesticides and may also better demonstrate the effects of biodegradable pesticides in ecosystems.

The toxic effects of fenitrothion and particularly technical formulations of fenitrothion have become better understood since 1976. Toxicity studies on S-methyl fenitrothion, a contaminant of technical fenitrothion and a metabolite shown in this thesis, showed it to be as potent a cholinesterase inhibitor as fenitro-oxon. However, due to its chemical instability relative to fenitrothion it took a longer time at subacute dosages to cause the same lethal response as fenitrothion (Hladka et al, 1977).

Miyamoto (1977) presented data to show that there was no retention of fenitrothion and its metabolites in rats, rabbits and dogs at subacute or chronic dosages. The mutagenicity of fenitrothion was tested with Ames type testing using revertant strains of Salmonella typhimurium and confirmed the lack of activity as shown in this thesis. In addition 100 mg/kg and 200 mg/kg dietary dosage rates of fenitrothion were shown to be non tumorigenic to rats when given for 24 and 18 months respectively.

Iverson and Marshall (1977) showed the inhibition of acetyl and butyryl cholinesterase by m-hydroxymethyl, formyl, and carboxy fenitrothion analogues. M-hydroxymethyl, and formyl fenitrothion were shown to

increase overall inhibition of the cholinesterase with formyl fenitrothion being the most potent derivative described so far. Carboxy fenitrothion was shown to have a lower cholinesterase inhibition rate than fenitrothion by a factor of 100.

Crocker and Ozere (1977) restated their indication that viral infection by encephalomyo carditis virus in young mice is potentiated by emulsifiers particularly Toximal MP8. A parallel was drawn between the viral infection of these mice and Reye's syndrome which he suggests is a toxin-virus related syndrome in children.

Rozee et al (1977) showed that the enhanced response of the virus with emulsifiers was relatively specific for single stranded RNA genome-type virus such as polio, encephalomyocarditis virus, and vesicular stomatitis virus, but not for double stranded RNA or DNA genome-type virus. Enhancement was noted in established cell lines from monkey, mouse, and human sources.

Appendix 3

## Glutathione

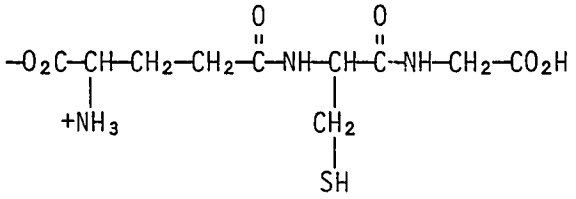
Sulfur containing isolates from yeast were first described in 1888 by de Rey-Pailhade and the impure substance named "philothion". Thiols were later demonstrated in 1908 by Heffter to be present in living cells by using the colorimetric reaction of sodium-nitro-prusside with mercaptans.

Glutathione was first crystallized from yeast in 1921 by Hopkins. The formula of glutathione was first suggested by Pirie and Pinhey (1929) who determined by electrometric titration four different pK values which they correlated with one sulfhydryl, one amino, and two carboxyl groups. Hopkins (1929) was able to improve the purity of his crystalline glutathione yeast isolate and better describe the molecule. Acid hydrolysis yielded glycine, glutamic acid, and cysteine. Amino and carboxyl groups were determined by alcohol-acid titration using methyl red to indicate amino groups and phenophthalein for carboxyl groups. Titrations were found to be within 2.7 percent of those values theoretically determined for a tripeptide containing cysteine, glycine, and glutamic acid which was within experimental accuracy. Carboxyl group determinations were confirmed by water titration and amino group determinations were confirmed by high acid end-point titration.

The relative positions of cysteine and glycine in the molecule were determined by several methods. Scission of the molecule with water at $42^{o}$ C liberated a dipeptide, not an anhydride, containing cysteine and

glycine which was then hydrolysed to free glycine indicating that the dipeptide was cysteylglycine (Kendall et al 1929). Glutathione was partly hydrolysed by carboxypolypeptidase (Grassman et al 1930) with liberation of glycine indicating that glycine was in the carboxy-terminal position. Harrington and Mead (1935) confirmed the structure to be a glutamylcysteylglycine by chemically synthesizing this molecule and comparing this compound with that characterized from Hopkins' crystallized yeast isolate. Elemental composition was consistent with a structure containing $C_{10}\ H_{12}\ O_6\ N_3\ S$, M.P. 195° C., optical rotation $[\alpha]_{5461}$ -21.0° in water for reduced glutathione (GSH), and $[\alpha]_{5461}$ -107°, M.P. 262°C, for oxidized glutathione (GSSG).

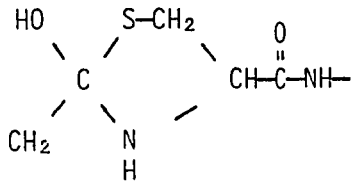
Thus they established the general structure:



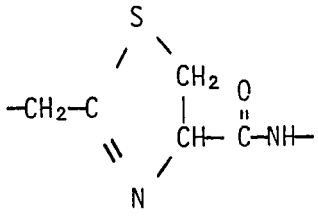
γ–glutamyl — cysteinyl — glycine

The molecule, however, was shown to be remarkably stable relative to cysteine and could not be this simple structure with a free cysteine mercaptan. The enhanced stability was shown to be due to an interaction between the carboxyl oxygen of the γ-glutamyl residue and the SH of the cysteine residue. Evidence for hydrogen bonding is taken from the infra-red spectrum of benzyl mercaptan in the presence of N-dimethyl acetamide where the S-H bond is shifted to 2525 $cm^{-1}$ in the presence of the amide. In addition to hydrogen bonding the reaction may proceed further to form

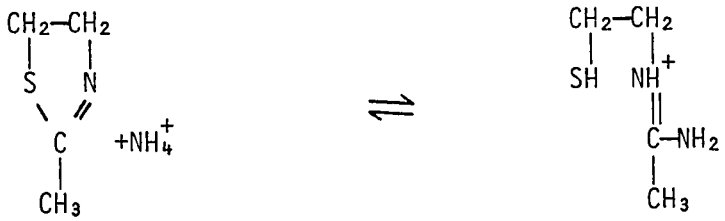
a hydroxy thiazolidine derivative:



which can further form a thiazoline by elimination of the amino H combining with the OH to form $H_2O$ thus:



All three thiazoline structures are consistent with the uv spectra of glutathione. One aspect of thiazolines was that they exist in a rapidly reversible equilibrium with ammonium ions allowing ring opening and closure thus:



In glutathione the ammonium ion is always present, in the $\alpha$ amino group of the glutamic residue which can be moved close enough to allow ring

opening and closure. All of the above mentioned structural forms are therefore possible for glutathione and account for the stability of the molecule.

Glutathione was considered important in cellular hydrogen and electron transport due to the ease of conversion of the sulfhydryl and disulfide forms $2\ GSH \rightleftharpoons GSSG + 2H$ through oxidation of GSH by molecular $O_2$ and reduction of GSSG in many tissues. However, it was found that conversions were not sufficiently rapid (40 µl of $O_2$/gram liver tissue/hr) (Hopkins and Elliot, 1931). It was concluded that GSH could not function as a hydrogen carrier for the bulk of tissue oxidations. As a respiratory catalyst the cytochrome system appeared to act independently of glutathione.

Glutathione reductase and TPN (NADP) - reducing systems of various tissues appear to be the major systems involved in keeping tissue glutathione in reduced form. Some higher plants, particularly green vegetables such as cauliflower, cabbage, and broccoli maintain ascorbic acid in reduced form by means of dehydroascorbic acid reductase plus GSH.

The presence of relatively large quantities of reduced glutathione in most tissues suggests that this compound might play an important role in biosynthesis of proteins. GSH is involved with both transpeptidase as well as transamidase reactions. In fact glutathione appears to have a multiple metabolic role since many enzymatic reactions are activated by SH compounds of which glutathione is the predominant one. Examples include γ-glutamyl transfer reactions, acyl transferase reactions, and cis-trans isomerization, as well as contributing SH groups and cysteine to peptide sequences.

Rapkine (1931) showed that SH compounds inhibited cell division but that the condition could be reversed with the addition of cysteine. It was later suggested by Harrington (1967) that glutathione or other soluble SH donors engage in a reversible reaction which first reduces the spindle protein precursor molecules to open up intramolecular SH groups and then oxidizes these sites to intermolecular S-S linkages, thus forming the spindle. GSH has been shown to be involved with pre-mitotic stages in pea-seedling cells. (Hughes and Spragg, 1958). Glutathione is also indirectly associated with plant meristeim growth in that auxin gradients are paralleled by SH gradients. The SH compounds such as glutathione have been shown to inhibit peroxidase-catalyzed oxidations of auxins such as IAA and therefore act as auxin protectors.

Carnegie (1963) showed the presence of a disulphide which he isolated from seedlings of Phaseolus aureus. The structure was bis-L-γ-glutamyl-L-cystienyl-bis-B-alanine which in the thiol form is a higher homologue of glutathione. It differs from glutathione by one methylene group in the C terminal amino acid. The compound substitutes for glutathione in certain enzymatic reactions such as the oxidation of NADPH but chromatographic and electrophoretic properties were different.

The biosynthesis of glutathione has been shown to occur in two independent enzyme dependent steps:

$$\text{L-glutamate + L-cysteine + ATP} \xrightarrow[\text{synthetase; } M^{2+}]{\gamma\text{ - glutamylcysteine}} \text{L-}\gamma\text{ glutamyl - L cysteine + ADP + Pi}$$

$$\text{L-}\gamma\text{ glutamyl - L-cysteine + ATP + glycine} \xrightarrow[\text{synthetase; } M^{2+}]{\text{glutathione}} \text{glutathione + ADP + Pi}$$

(Meister, 1973). When glutathione is incubated with crude kidney preparations, 5-oxoproline and cysteinylglycine are formed:

$$\text{glutathione + amino acid} \xrightarrow[\text{transpeptidase}]{\gamma\text{ glutamyl}} \gamma\text{-glutamyl amino acid + cysteinyl glycine}$$

$$\gamma\text{-glutamyl amino acid} \xrightarrow[\text{cyclotransferase}]{\gamma\text{ glutamyl}} \text{5-oxoproline + amino acid}$$

Of the 21 amino acids all but proline are active. Cysteinyl glycine is cleared by a peptidase to yield free glycine and cysteine.

$\gamma$-glutamyl transpeptidase is distributed widely in mammalion tissue and is a membrane bound enzyme. It is particularly abundant in the kidney and is localized in the brush border of the proximal convoluted tubule which is believed to be the major site of amino acid reabsorption. Isolates have been prepared from kidney tissue which catalyze the ATP dependent conversion of 5-oxo proline to glutamate.

$$\text{5-oxo-L-proline + ATP + } 2H_2O \xrightarrow{\text{5-oxoprolinase}} \text{L-Glutamate + ADP + Pi}$$

The $\gamma$-glutamyl transpeptidase cycle therefore appears to be responsible for amino acid transport across membranes. This function is particularly important in the kidney but $\gamma$ glutamyl derivatives are also found in the choroid plexus of the brain. Activity may also be present in the

intestinal mucosa. This glutathione dependent function is therefore very important physiologically.

Another of the more important biological functions of glutathione is the protection of the organism against foreign compounds (Chasseaud, 1973). This protection is achieved through glutathione S-transferases. These comprise five main classes:
glutathione S-alkyl transferases, which were demonstrated in dealkylation of fenitrothion to desmethylfenitrothion, glutathione S-ara-alkyltransferases, glutathione S-aryltransferases, glutathione S-epoxidetransferases, and glutathione S-alkenetransferases.

These glutathione dependent enzymes have two main roles. One is to conjugate potentially harmful electrophilic foreign compounds with the nucleophilic glutathione-SH. Glutathione conjugates are less toxic and more water soluble than the original foreign compound. They also provide a means of elimination since glutathione-SH conjugates are anionic and are large molecules having molecular weights exceeding 300. Biliary excretion is favoured as a consequence. Glutathione conjugates have also been demonstrated to exist for endogenous substrates such as oestradiol.

Erythrocytes or red cells contain a glutathione reductase enzyme which catalyzes two reactions.

$$NADPH + H^+ + GSSG \longrightarrow 2G\text{-}SH + NADP^+$$

and

$$NADH + H^+ + GSSG \longrightarrow 2G\text{-}SH + NAD^+$$

(Beutter, 1973). In vivo, however, it has been shown that the latter system is ineffectual in the red cell itself. The enzyme does not appear to have a limiting role in red cell metabolism. Deficiencies in red cell

glutathione reductase appear to be related to riboflavin deficiency and can be corrected by addition of flavine adenine dinucleotide or riboflavin.

Glutathione is considered to be the most abundant low molecular weight thiol of most living cells. However, the presence of homologues with similar physical and chemical properties cannot be ruled out, such as homoglutathione referred to previously. Mixed or unsymmetrical disulfides may be regarded as compounds formed by the oxidative coupling of the sulfhydryl groups of dissimilar thiols (Mannervick and Eriksson, 1973). Glutathione is also the most important low molecular weight moiety of naturally occurring mixed disulfides. Examples are, coenzyme A and glutathione, cysteine and glutathione, pantetheine and glutathione, hemoglobin and glutathione, serum albumen and glutathione, and crystallins of lens material and glutathione.

Glutathione peroxidase was first isolated in bovine erythrocytes and was assumed to protect hemoglobin against oxidative denaturation by $H_2O_2$ (Flohé and Günzler, 1973). Four gram atoms of selenium are present per mole of glutathione-SH peroxidase. Glutathione peroxidase consists of four sub units, which are apparently identical and likely each contain one selenium atom. The manifestations of selenium deficiency are caused by peroxidation of unsaturated lipids within biological membranes, the counteraction of which is a well established function of glutathione-SH peroxidase since it was later shown that the enzyme taken from rat liver mitochondria not only reduced $H_2O_2$ but also a variety of hydroperoxides.

$$R\text{-}O\text{-}OH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

The enzyme plays a strategic role in the protection of erythrocytes from oxidative stress caused by drugs or basal oxidative agents such as generated by the interaction of ascorbic acid and oxyhemoglobin The enzyme forms the final link between the hexosemonophosphate pathway and the detoxification of hydrogen peroxide. By competing for hydrogen perioxide via glutathione peroxidase, glutathione increases the half life of hemoglobin which can transport oxygen only if its iron is kept in a bivalent state. Glutathione peroxidase is also found in leucocytes and platelets, however, the function and importance is not well understood. Hemoglobin is an SH containing protein and is protected as well by glutathione which acts as a scavenger competing with other SH carriers (Aebi and Suter, 1973).

The ocular lens, like the erythrocyte contains a high level of reduced glutathione, a low level of oxidized glutathione, the capacity to synthesize glutathione from amino acids, and can excrete glutathione against a concentration gradient. Mixed disulfides of glutathione-SH and crystallin can be produced and are reduced by glutathione reductase and NADPH. Lens glutathione probably plays a central role in protection against denaturation of lens protein and thus formation of cataracts (Beutter and Srivastava, 1973).

Thiols have the ability to scavenge radicals, forming disulfides, which may be reduced again by metabolic reactions (Rink, 1973). Ionizing radiation produces very reactive radicals which react with thiol groups in cells and therefore interfere with SH-dependent cell functions and lead to cell death. Glutathione may then be regarded as a pool of radioprotective substances which can be released under certain conditions and enhance resistance of cells to ionizing radiation.

Living cells normally contain substantial concentrations of glutathione, with smaller amounts of glutathione disulfide, not including that present in the form of protein glutathione. The status of GSH-GSSG can be perturbed reversibly by enzymes such as glutathione peroxidase, other disulfides, or with other molecular structures such as diazenes (Kasower and Kasower, 1973). Perturbations in the GSH-GSSG status have been shown to be manifested by changes in protein synthesis (in supplying thiol groups etc.), neurotransmitter release, and muscle contraction.

Neural tissue has a relatively high concentration of glutathione. The involvement of glutathione in neurotransmitter release has led to proposals that glutathione may play a central role in the thinking of higher animals.

γ-glutamyl transpeptidase enzymes have been demonstrated in Phaseolus vulgaris fruits, and are probably responsible for the synthesis of the wide range of γ-glutamyl dipeptides encountered in higher plants. Glutathione is probably the natural form of the γ-glutamyl donor, but γ-glutamylanilide is frequently used in the enzyme assay.

$$\left.\begin{array}{c}\text{glutathione}\\ \text{or}\\ \gamma\text{-glutamylanilide}\end{array}\right\} + R{\cdot}NH_2 \rightleftharpoons \gamma\text{-glu. } NH{\cdot}R + \left\{\begin{array}{c}\text{cysteinylglycine}\\ \text{or}\\ \text{aniline}\end{array}\right.$$

R in this reaction normally contains a carboxyl group which means the acceptor is an amino acid. α-Amino acids are better acceptors

than the corresponding α or β-amino derivatives. Glutamine cannot participate itself as a γ-glutamyl donor in the reverse reaction (Fowden, 1967).

Non protein thiols are thought to be involved in other physiological processes in plants such as protoplasmic streaming, (Kamiya, 1959), and electron transport and phosphorylation during photosynthesis, Jocelyn, 1972). However, cysteine plays the major role connected with these reactions. Thiol compounds are also thought to be involved in frost hardiness in plants (Kohn and Levitt, 1966).

Stoiner and Yang, (1973), have linked glutathione as an effective inhibitor of peroxidase-catalysed oxidations of indole acetic acid, since glutathione is also oxidized by peroxidase. However, SH gradients have been shown to parallel indole acetic acid gradients in young plant tissues, (Pilet and Zyrd, 1966). Gradients of peroxidase inhibitors such as O-dihydroxyphenols, have been reported in many plant leaves and stems such as tobacco (Lavee and Galston, 1968). It therefore seems most probable that the auxin protectors also prevent glutathione oxidation, and allow the involvement of glutathione in other processes of cell division such as spindle protein formation (Rapkine, 1931, Harrington, 1967).

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