A PRELIMINARY STUDY ON THE FATE OF FENITROTHION IN FOREST SEEDS

I. DETERMINATION OF RESIDUES IN EASTERN WHITE PINE (*Pinus strobus* L.) AND THEIR EFFECTS ON AMINO ACID METABOLISM

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INTRODUCTION

Fenitrothion (0,0-dimethyl 0-(4-nitro-m-tolyl) phosphorothioate has been used in place of DDT for operational control of lepidopterous defoliators in Canadian forests (Fettes 1968). Experimental tests and field observations have shown that fenitrothion will control the spruce budworm (Choristoneura fumiferana (Clemens) at a dosage of 2 to 4 oz/acre applied by aircraft at one application. It is recommended that no more than 6 ozs/acre be applied if two treatments are required. Treatment is recommended in two dosages firstly when larvae are hatched and secondly a week later just before the peak of the fifth instar stage (Nigam, 1972). The spruce budworm problem arises early in the spring and spraying begins usually in May. Card spotting tests indicate that a fine mist of fenitrothion (60 µm in diameter) generally penetrates to the floor of the forest at a level slightly lower than 4 oz/acre (Randall, 1970).

Germination is a critical stage of plant morphogenesis. Adverse environmental factors may have long lasting effects on seedling vigor and subsequent growth to maturity. The primary focus of this study was, therefore, directed towards determining whether fenitrothion or its break down products accumulated within the germinating seed to affect its subsequent germination and early seedling growth. Also such studies might have some ecological repercussions in the sense that many forest seeds and cones constitute food sources for wildlife (small rodents and game birds) and if the pesticide or its metabolites accumulated in the seeds or cones, it would be very pertinent to investigate their environmental impacts.

Further, the study was designed to determine whether there was any recognizable metabolic interaction between periods of stratification and exposure to fenitrothion. The fenitrothion concentrations used were comparable to field exposure levels.

MATERIALS AND METHODS

(i) Seed Germination: The seeds used in this study were obtained from the Petawawa forest in the fall of 1972 by picking fallen cones from the forest floor from unsprayed (control) areas and from areas sprayed previously since 1969. Forty seeds derived from 4 morphological groups were used at each time period. Population homogeneity was optimized by rigidly monitoring the air dried weights of seeds in each groups of 10 to within 0.005 grams. Then seeds were weighed at each sampling time to compare any changes brought about by the stratification process. Germination and stratification was carried out in sterile 9 cm. plastic petri dishes to which 15 ml of distilled water or one of the two fenitrothion emulsions had been added. For this purpose five layers of Whatman No. 1 filter paper saturated with distilled water were employed.

(ii) Seed Stratification: Seeds of many temperate forest species require a prechilling treatment for successful regeneration and stratification is the process of subjecting imbibed seeds to alternating (cold & warm) temperature for breaking of their dormancy. Accordingly, three methods of treatment were examined and compared with the control (non-treated) seeds. Representative seeds from each treatment were either stratified at 10^0C for 21 days and germinated at a diurnal variation temperature of 68^0 F and 86^0F (night:day) or germinated immediately without prior stratification. These treatments may be identified by a double notation system, namely: CS (Control stratified), CNS (Control non-stratified), SpS (field sprayed seeds stratified), SpNS (field sprayed seed non-stratified), CSF_5 (control seed stratified and germinated in added fenitrothion at a field strength of 10 ppm or 4 oz/acre fenitrothion), $CNSF_5$ (non-stratified seeds as above treated with 10 ppm

(1.5×10^{-4}M) fenitrothion, CSF_3 (control seed stratified and germinated in fenitrothion with 1000 ppm (1.5×10^{-2}M) fenitrothion and $CNSF_3$ (non-stratified seeds germinated in 1.5×10^{-2}M fenitrothion).

Fenitrothion was obtained in the form of an emulsifiable concentrate from Sumitomo Co. Japan. Before use the purity of the sample was checked by the G.C. method and was found to contain 100% fenitrothion with no other breakdown products. Appropriate dosages approximating a field concentration (10 ppm) and a higher concentration (1000 ppm) were prepared and shaken vigorously to achieve a good emulsion.

(iii) Seed Dissection: Whole seeds as well as seed parts were employed in the uptake studies and were dissected into three parts with the aid of a dissecting microscope:- (i) embryo (ii) endosperm plus gametophtye and (iii) seed coat. This permitted localization of the pesticide in the seed during the course of germination and early seedling growth.

(iv) Extraction: Ten seeds and seed parts were homogenized for 1 minute with a Polytron sonicator in 50 ml of acetonitrile. This homogenate was filtered through celite (Johns-Manville Co. Ltd.) to remove seed debris. Another 50 ml of acetonitrile was sonicated in the flask to remove any residual pesticide on the sonicator and on the walls of the flask. This was also filtered through the same celite to give a final volume of 100 ml.

(v) Cleanup: Seed oils and fats were removed by partitioning the acetonitrile fraction with 50 ml of hexane 3 times. The acetonitrile was then flash evaporated almost to dryness and taken up in 10 ml of hexane. This cleanup was sufficient for detecting fenitrothion by the gas chromatography. However, other metabolites such as fenitro-oxon are not easily cleaned up and are masked by the interfering molecules.

(vi) Column Separation: Fenitro-oxon was separated from fenitrothion by gas chromatography using a modification of the method of Bowman & Beroza (1969) and Yule & Duffy (1972). Apparently the separation of fenitrothion from forest seeds is somewhat different. Five percent deactivated Florisil was replaced by silica gel on the column. This column was then eluted with 100 ml benzene to remove fenitrothion and 100 ml acetone:benzene (1:3) to remove fenitro-oxon. Each eluant was flash evaporated to near dryness and taken up in 10 ml of hexane. They were then concentrated under forced air. Using this modified procedure a 100% recovery of fenitrothion and fenitro-oxon was obtained whereas only about 60% was recovered by the old methodology of the previous workers. This modified method is sensitive to 0.01 ppm fenitrothion and 0.1 ppm fenitro-oxon.

(vii) Gas-Liquid Chromatography: Following the cleanup, the concentration of fenitrothion and fenitro-oxon was determined by gas chromatographic analysis. A Pye Model No. 124 gas chromatograph fitted with an alkali flame detector (AFD) and a cesium bromide annulus was used. The operating conditions were as follows:

Column: glass, 6 ft. x $\frac{1}{4}$ inch packed with 4% SE 30 and 6% QF-1 on Chromosorb W.

Column Temperature: $220^{0}C$

Gas Flow: Nitrogen 40 ml/min.

Hydrogen 35 ml/min.

Air Flow 500 ml/min.

Instrument Settings: Attenuation 5×10^{2}

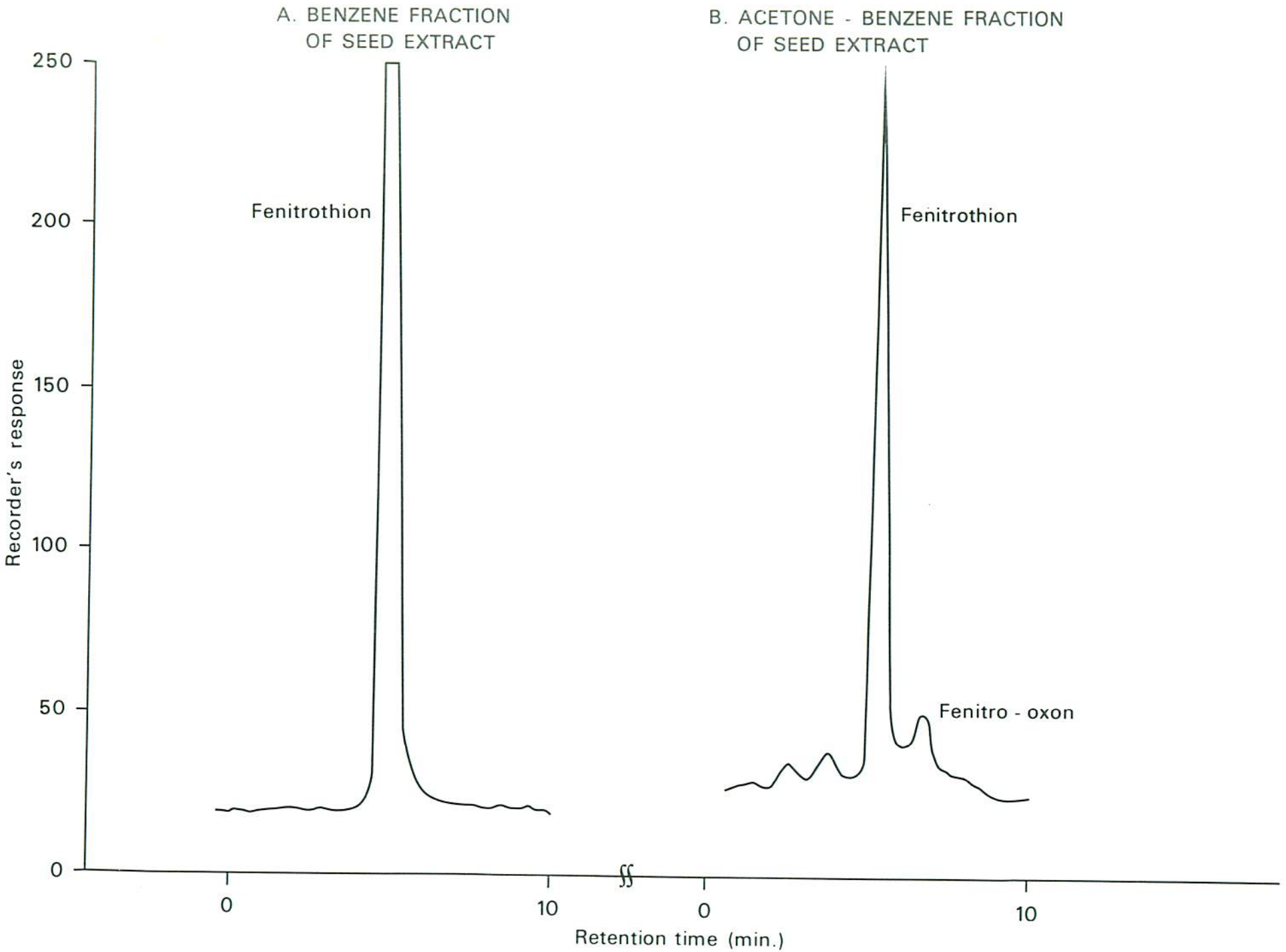

Fig. 1. A diagrammatic sketch of the old (A) and modified (B) gas liquid chromatographic procedure for separation of fenitrothion and fenitro-oxon from white pine seeds.

Duplicate determinations were carried out bracketed by injection of a sample standard. Under these conditions fenitrothion has a retention time of 5.0 minutes while fenitro-oxon of 5.5 minutes (see Fig. 1).

(viii) Amino Acid Analysis: Stocks of seeds were treated in the same manner as in the previous experiment and were then taken from the same groups as those assayed for fenitrothion uptake. Two replicates of five morphologically comparable seeds were used at each sampling period. Soluble free amino acids were extracted exhaustively from the whole seed by homogenizing in 2 ml of 60 percent ethanol for six minutes. The particulate material was then removed by centrifugation at 2000 rpm at the room temperature and the clear yellowish supernatant was flash-evaporated to dryness. Subsequently this material was taken up in 0.5 ml of 0.6 N HCl and divided into two equal portions. One portion was freeze-dried, redissolved in a N HCl and hydrolyzed for 12 hours at 110°C. The other portion was analysed on a Technicon Amino Acid Analyser according to methods of Ramiah et al (1971). Peaks were integrated with an Infotronics integrator and the amount of all amino acids were calculated and the results were expressed on the percentage of this total for each amino acid.

RESULTS

For the sake of convenience the findings are arranged under two categories:

(i) Fenitrothion Uptake and Accumulation by Stratified and Non-Stratified Seeds: The course of uptake and accumulation from low and high concentration of fenitrothion was followed up to 14 days and the data presented in Fig. 2 indicate that seeds and seed parts absorb the pesticide to a considerable extent. Unexpectedly high levels of absorption of fenitrothion were observed when whole seeds were assayed after 4 and 8 days of germination (Table I). There was also clear evidence of accumulation of fenitrothion against a concentration gradient.

TABLE I

Uptake and Accumulation of Fenitrothion by Non-Stratified and Stratified Whole Seeds During Germination Following Treatment with a High Concentration (1000 ppm).

Treatment	Germination Period (days)	Content in Whole Seeds (ppm)
Non-stratified ($CNSF_3$)	4	438.5
	8	527.2
Stratified (CSF_3)	4	250.3
	8	172.5

Subsequently, the seeds were dissected and separated into seed coat and integument, endosperm gametophyte and embryo. Samples of 10 seeds were taken at the appropriate interval, dissected and weighed. The seed coat comprised approximately 32% of the endosperm gametophyte dissections. As shown in Table I, between 8 and 14 days, there was a considerable variation in the pattern of localization in as much as the contents of pesticide in the seed coat dropped from 35% to 16% (Fig. 2A) of the total amount absorbed. Concomitantly the amount in the endosperm rose slightly from 42% to 49%. Most significant was the concentration in the embryo which increased from 16.5% to 37%. At this time the radicles began to emerge from the seed. These results tended to indicate that the seed coat initially absorbed a certain amount of the pesticide from the emulsion onto its surface and subsequently donated some to the embryonic parts. The distribution and accumulation pattern at the high concentration (Fig. 2B)remained similar to that of low concentration (Fig. 2A). The concentration of the pesticide was then calculated in terms

of parts per millions in the following manner:

$$ppm = \frac{\text{concentration std } \mu g/ml. \text{ X number of } \lambda}{\text{number of } \lambda \text{ in sample}} \times \frac{\text{peak area sample}}{\text{peak area standard}}$$

$$\times \frac{1 \text{ gram}}{\text{sample weight}} \times \frac{\text{final volume sample}}{1}$$

In the low concentration experiment (Fig. 2A) there was only 10 ppm fenitrothion in the external emulsion. However after 8 days germination there was 71 ppm on the seed coat. With further water absorption and germination to 14 days, the pesticide evidently diffused from the seed coat to the endosperm and embryo and localized in the embryo to give a final concentration of 81.3 ppm. The pattern of accumulation at higher concentration (Table III and Figure 2B) appeared to be similar to that of the lower (field) concentration. The total amount absorbed remained fairly constant again over 14 days of germination. However, the actual amount absorbed was only twofold greater than the amount absorbed at the field concentration. This could indicate a saturation effect on absorption.

TABLE II

Uptake of Fenitrothion by Non-stratified and Stratified Pine Seeds During Germination Following Exposure to 10 ppm Fenitrothion

Treatment	Exposure Period	Contents - ppm Seed Parts		
	Days	seed coat	endosperm	embryo
Non-Stratified	14	34.5	102.7	81.1
(CNSF)	8	60.9	82.8	46.6
Stratified	8	22.0	25.6	155.6
(CSF)	4	44.5	22.9	4.8

TABLE III

Patterns of Distribution in Non-Stratified and Stratified Seeds and Seed Parts During Germination in a High Concentration of Fenitrothion (1000 ppm)

Treatment	Germination (Days)	Contents in ppm Seed Parts			
		seed coat	endosperm	embryo	Total
Non-Stratified (percentage distribution)	4	226.1	78.5	132.9	438.5
		(56)	(14)	(30)	(100)
Stratified (percentage distribution)	8	220.3	220.2	86.9	527.4
		(42)	(42)	(16)	(100)

The stratified seeds absorbed approximately one half the amount of fenitrothion from both the 10 ppm and the 1000 ppm solution (Fig. 3 and Table II and III). During the cold treatment for 20 days as shown in Figure 3, the bulk of the pesticide remained on the seed coat with very little reaching the embryo; but as soon as the stratified seeds were exposed to higher temperature (76^0 F) after 20 day chilling period for germination, the content of fenitrothion also increased, suggesting a positive effect of temperature on uptake. At 4 days germination, the stratified seeds had absorbed 31.7% of the total pesticide into the endosperm and 6.6% into the embryo. At 8 days, the seeds had protruded very short radicles. At this stage, the amount absorbed by the embryo seemed to double up. On the other hand, the level remained about the same in endosperm and decreased in the seed coat fraction by more than 50%. This situation parallelled in 8 and 14 days seeds germinated without stratification (Figure 2A). The concentrations found in the embryo however, had increased rapidly and accounted for over 90 per cent of the total amounts

absorbed between 4 and 8 days of germination.

Subsequently the water that these seeds were germinating in was assayed and was found to contain only 1.73 ppm fenitrothion. This seemed to indicate that the pesticide was being actively taken up by the embryo.

TABLE IV

Distribution of the Breakdown Product (Fenitro-oxon) in Stratified and Non-Stratified Seeds During Germination Following Treatment with Fenitrothion (10 ppm).

Treatment	Germination (Days)	Contents - ppm Seed Parts		
		seed coat	endosperm	embryo
Non-Stratified	14	-	0.3	12.3
($CNSF_5$)	8	-	-	-
Stratified	8	-	-	9.4
(CSF_5)	4	-	-	-

The oxon form of the fenitrothion occurred only in the embryo and in rather significant amounts (12.3 ppm) after 14 days of germination (Table IV). No oxon was found in any quantity in water samples (external emulsions) of these seeds after a total of 30 days (21 days for stratification, 9 days for germination) exposure to the pesticide at both high and low temperatures. This finding together with those from a similar experiment at 14 days germination suggest that the formation of fenitro-oxon was most likely due to biochemical breakdown by enzymes in the embryo.

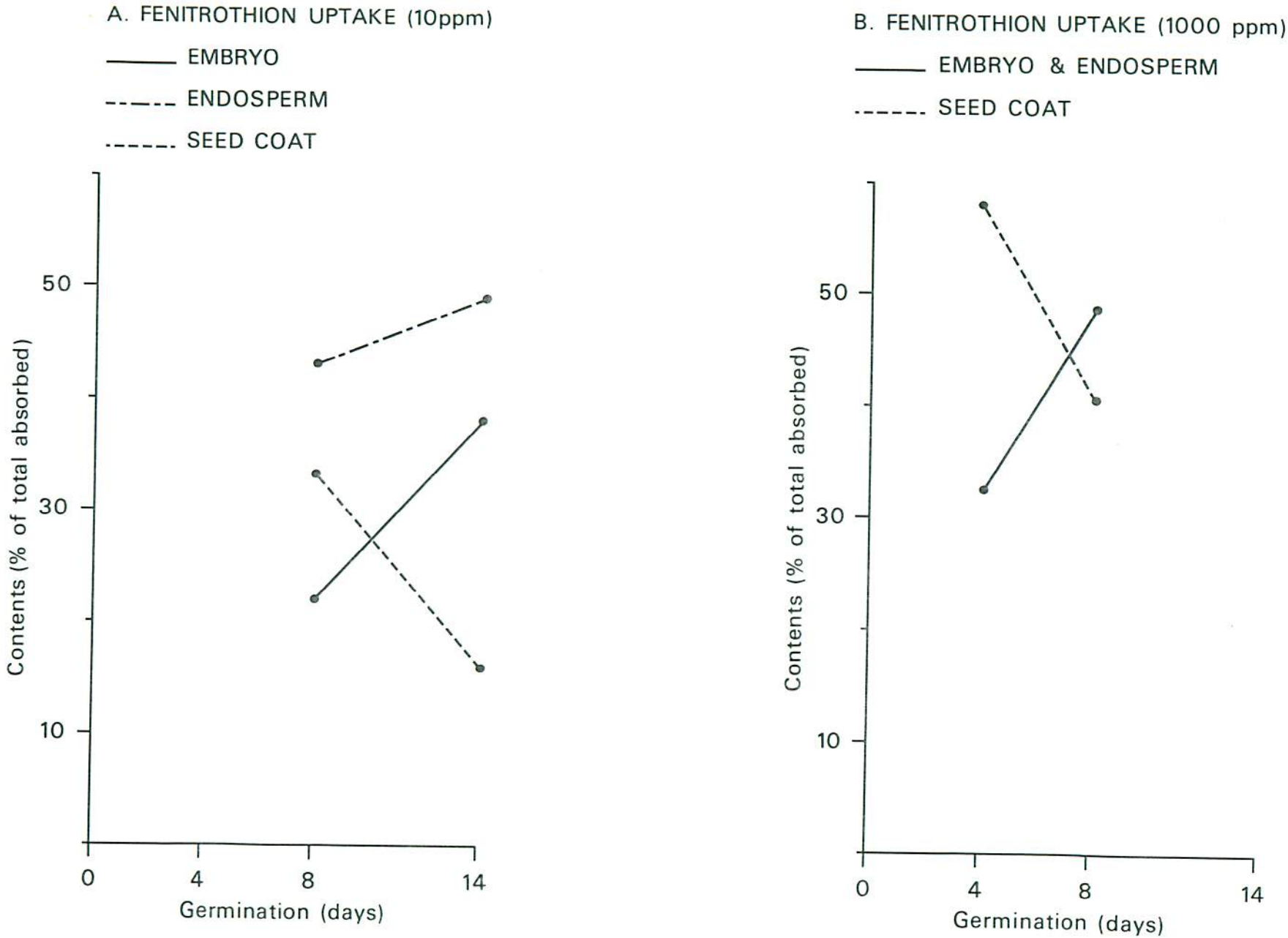

Fig. 2 Distribution of fenitrothion in non-stratified seed & parts following uptake from a low (A) and high (B) concentration of fenitrothion.

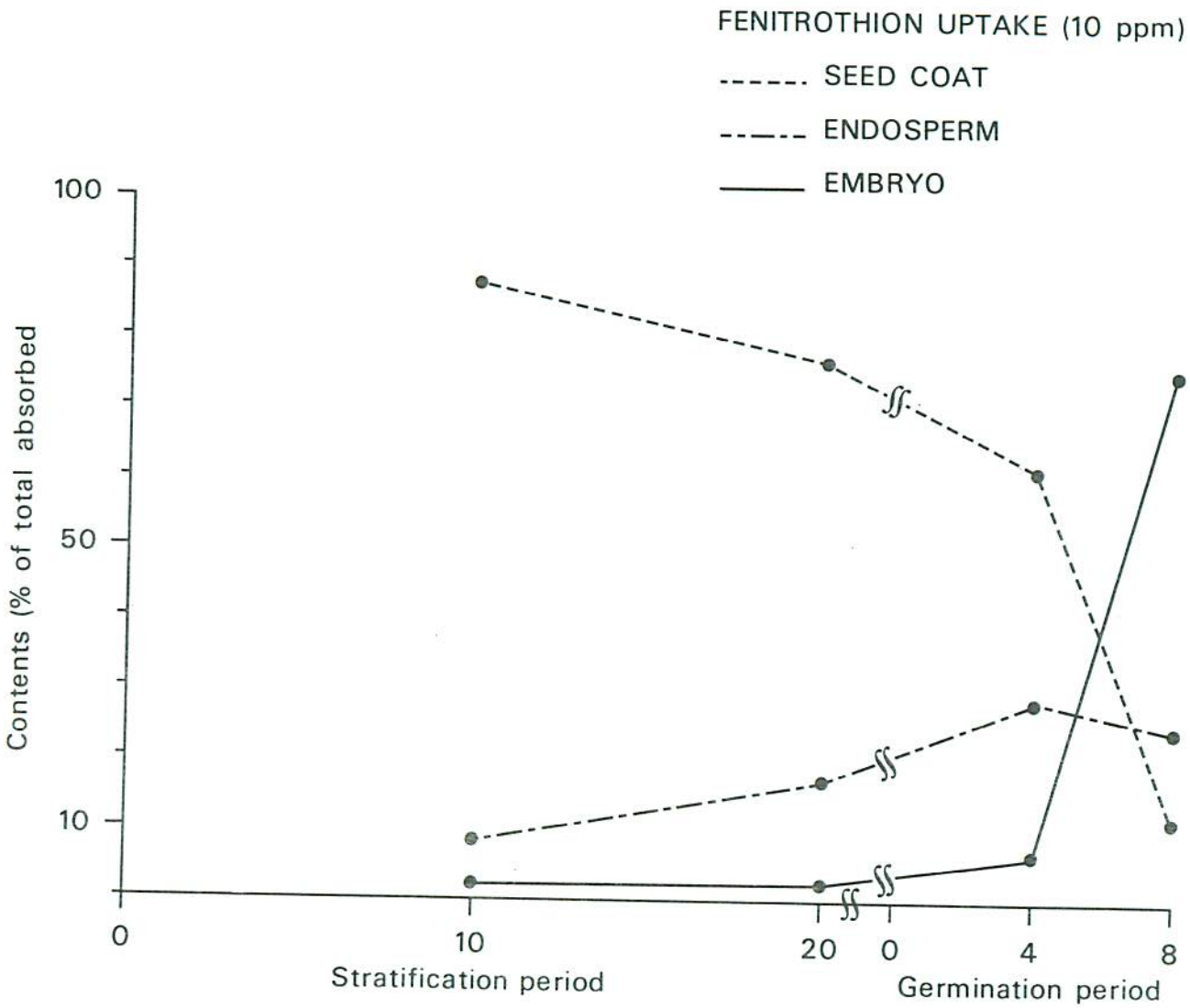

Fig. 3 Distribution of fenitrothion in pine seeds & parts following uptake from a low concentration after stratification for 20 days. Note the greater amounts of accumulation.

(ii) Fenitrothion effects on amino acid metabolism: The changes brought about by fenitrothion in levels of amino acids and their standard deviations are shown in Figures 4, 5, 6, 7 and 8. On the whole the data points out that glutamate, glutamic acid, alanine, arginine, glycine and serine are the most sensitive indicators of amino acid changes under the present experimental conditions. There is a wide fluctuation in the amino acids of control seeds and these were thought to be due to variation in the germination characteristics. However, the effects of fenitrothion are clearly marked and it seems there is a concentration effect: the changes in amino acids contents are greatest when the concentration of fenitrothion is highest (1000 ppm).

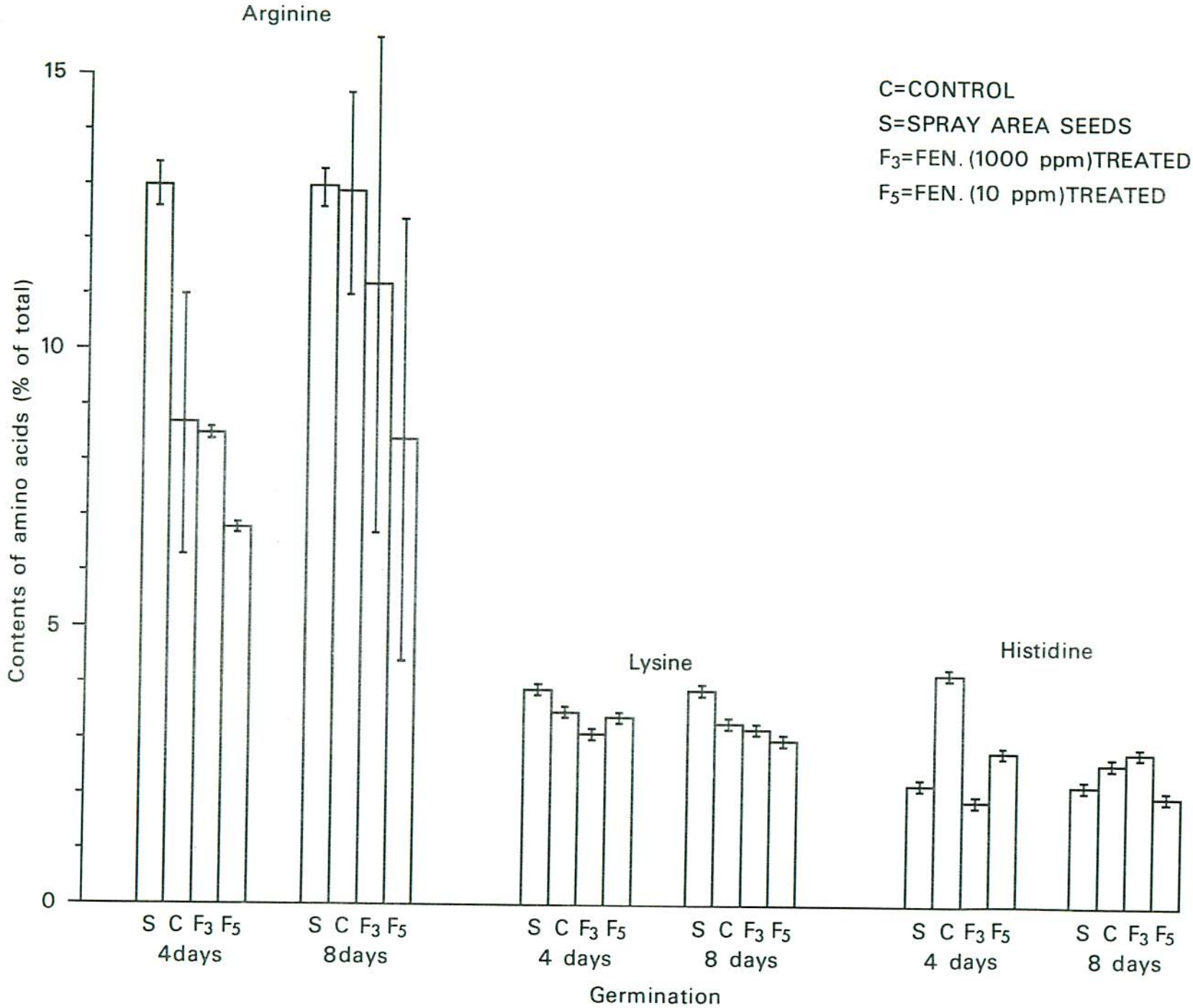

Fig. 4 Changes in amino acids (arginine, lysine & histidine) induced by low and high concentration of fenitrothion in pine seeds.

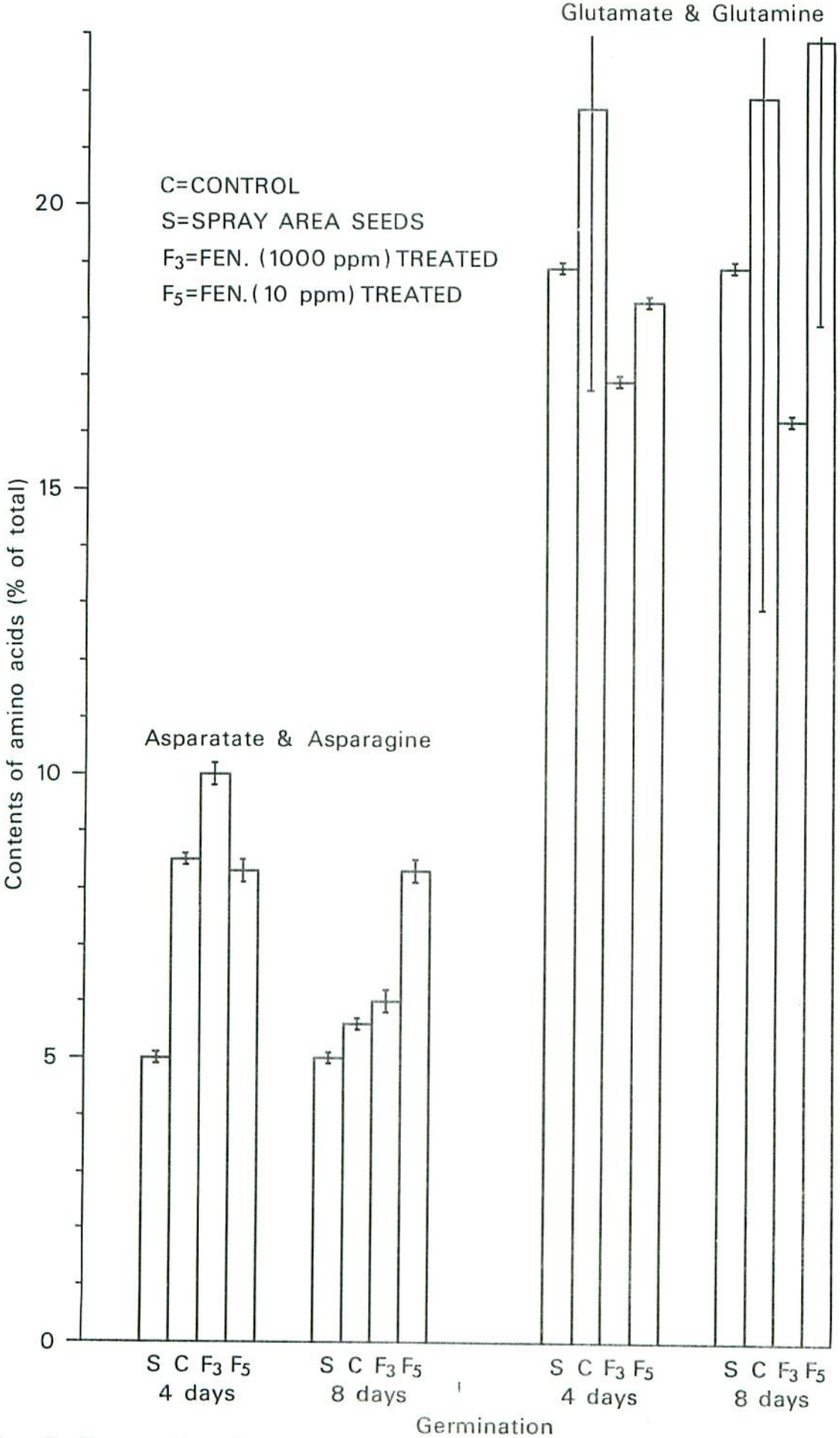

Fig. 5 Changes in amino acids (asparagine, aspartate, glutamine and glutamate) induced by low and high concn. of fenitrothion in pine seeds

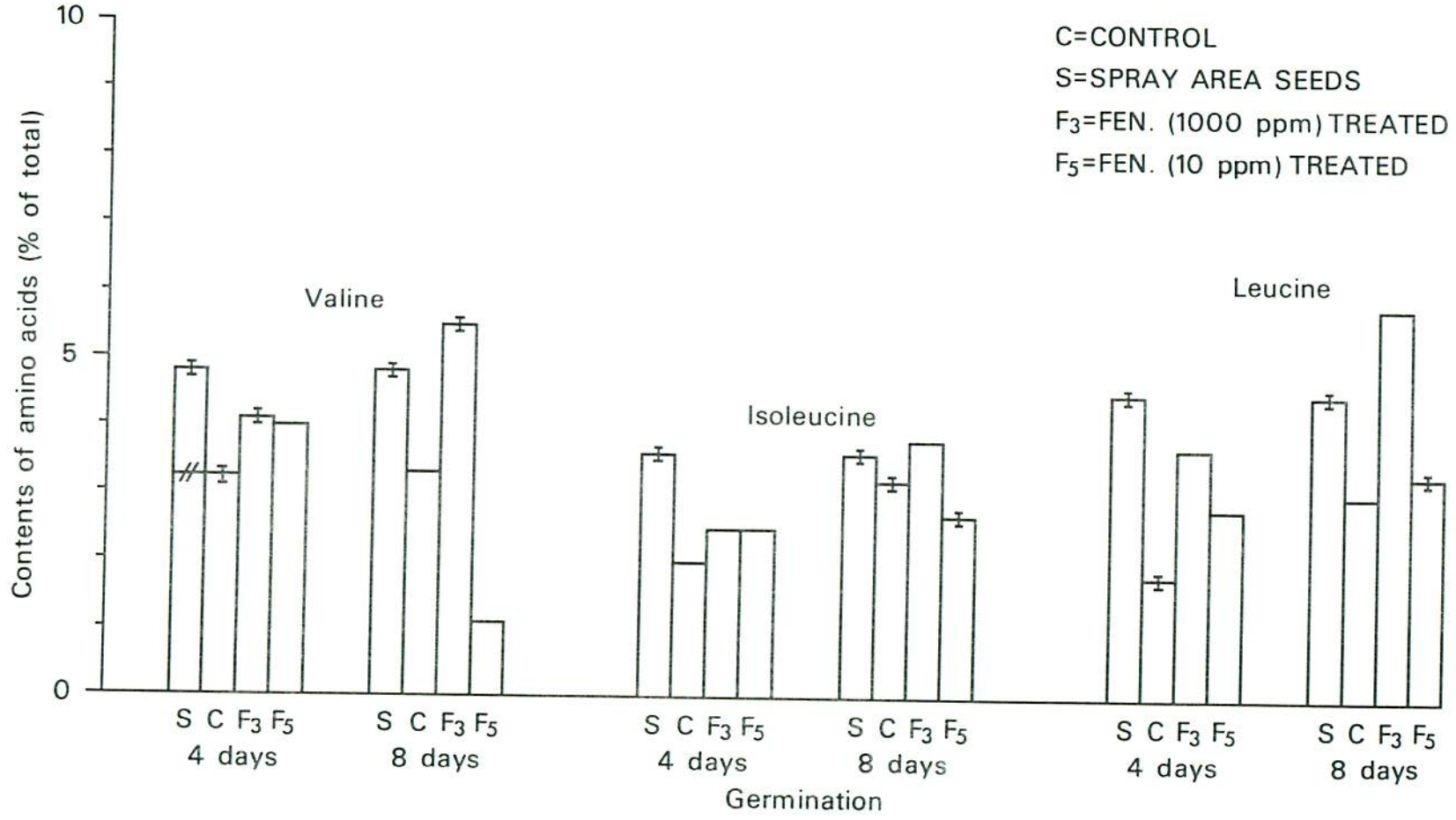

Fig. 6 Changes in amino acids (valine, isoleucine, (leucine) brought about by low and high concn. of fenitrothion in pine seeds.

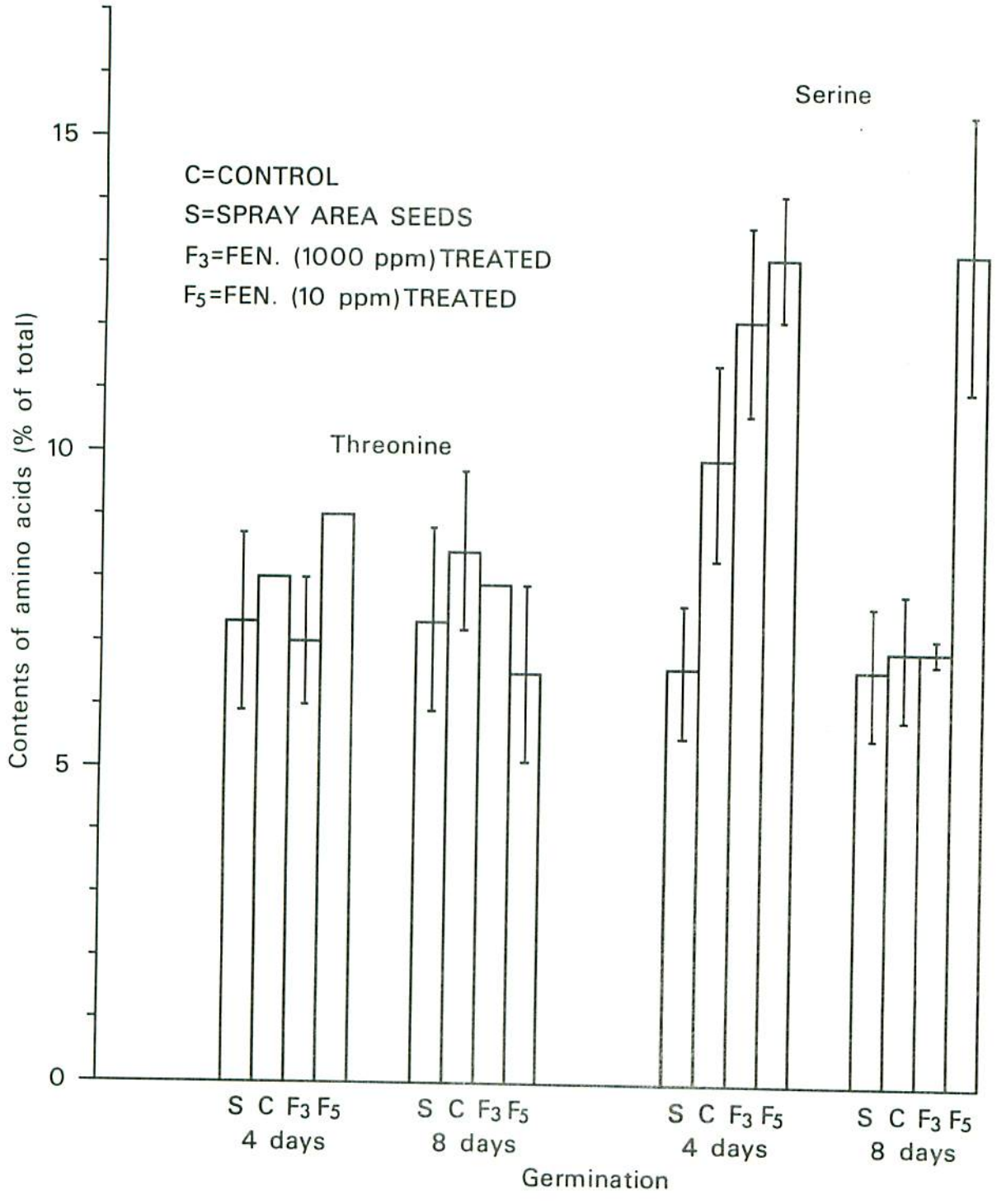

Fig. 7 Changes in amino acids (threonine and serine) induced by low and high concn. of fenitrothion in pine seeds

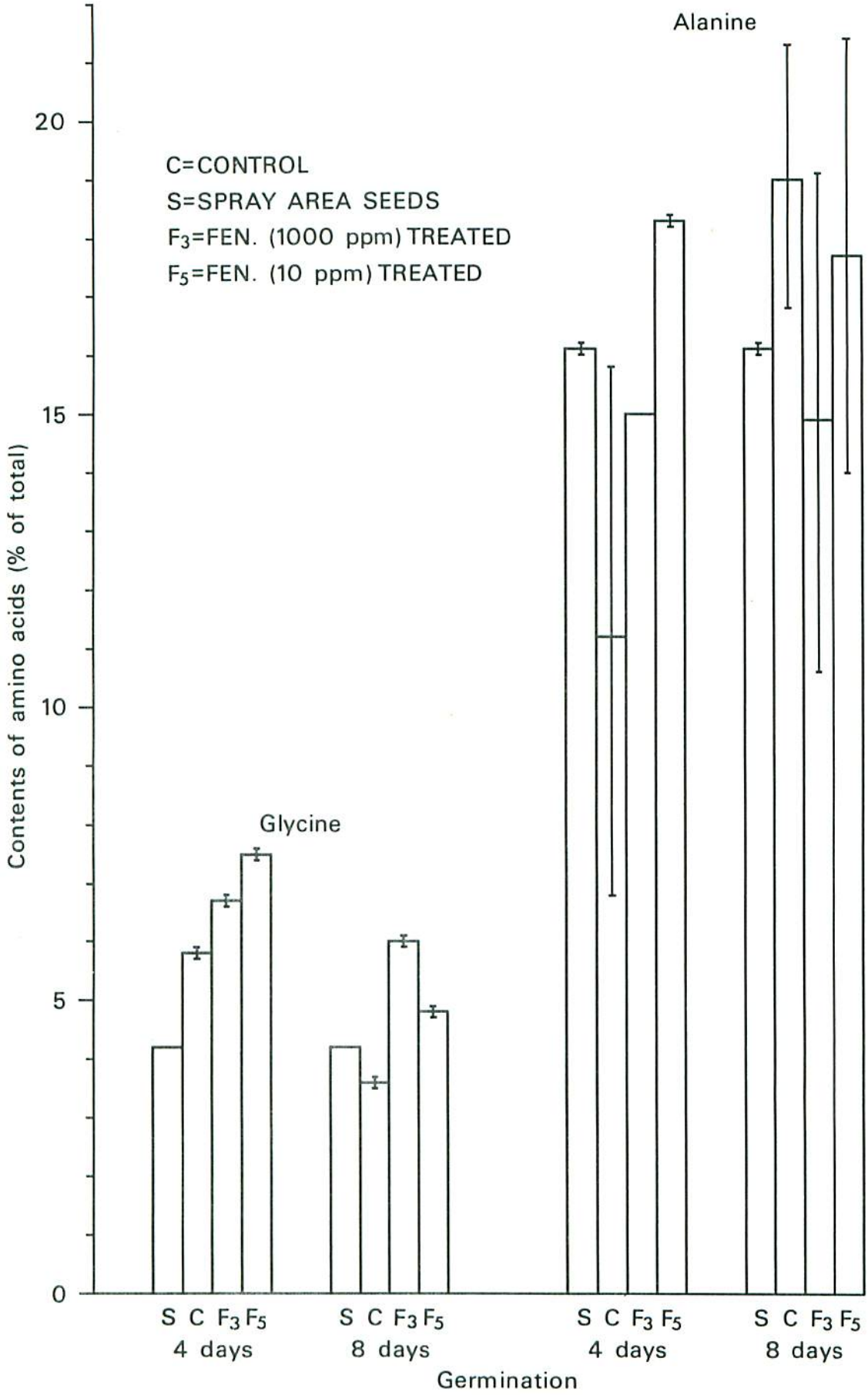

Fig. 8 Changes in amino acids (glycine and alanine) induced by low and high concn. of fenitrothion in pine seeds.

DISCUSSION

The actively metabolizing seeds of eastern white pine accumulate high quantities of fenitrothion against a concentration gradient. Uptake data using two levels of fenitrothion (field as well as a hundred fold greater concentration) suggest that at the higher concentration carrier-mechanisms are saturated by fenitrothion. The seed coat is the primary site of accumulation and subsequently the fenitrothion moves into the developing embryo. Degradation products, such as fenitro-oxon, accumulate in relatively high levels in seeds 14 days after germination and it seems there is an apparent metabolic mechanism (enzyme systems) for this breakdown process. The initial concentration of fenitrothion into the seed coat of white pine is similar for the rice seeds (Miyamoto and Sato 1965) where most residues were found on the bran rather than in the polished grain. Similarly fenitrothion when used as grain protectant in storage, leaves behind considerable residues on seeds of wheat and barley (Krehm 1972). It must be remembered here that pine seeds are different from cereal grains in as much as they contain more lipids than carbohydrates and so the accumulation and distribution pattern of fenitrothion and its products are likely to be divergent. Perhaps the pattern of penetration and accumulation in pine seeds is more akin to that on the waxy cabbage leaves where high degree of absorption and phytotoxicity by Tomizawa and Kobayashi (1964) was observed.

With further germination, oxon production is expected to increase. This form of the pesticide is about 10 fold more toxic to the insect than the parent form of the pesticide. Yule and Duffy (1972) did not find any significant amount of oxon in the foliage of a forest sprayed with fenitrothion under operational conditions, but they did find the parent compound to be fairly persistent in foliage after a rapid initial loss. This loss was likely to

be the result of evaporation and volatilization caused by air, sunlight, and rain. It appears from the data presented in this report that a rapid metabolism in the pine embryo is necessary for oxon production.

There is a high fat and liquid content in pine because of occurrence of the glyoxylate cycle (Firenzuoli et al. 1968) and glyoxysomes in the endosperm-gametophyte (Ching 1968). In fat rich seeds isocitrase activity provides a source of glyoxylate which yields glycine by transamination. Glycine and serine are interconvertable amino acids catalyzed by serine aldolase requiring tetrahydrofolic acid. It is noted that in most cases, glycine and serine levels increase from 4 to 8 days growth. This implicates gluconeogenesis as an important metabolic nutritional pathway in pine seed germination which is enhanced by the presence of fenitrothion. This probably reflects inhibition of other energy pathways. Serine is an important amino acid for being converted to hydroxypyruvate by transamination and to ethanolamine which is, in turn converted to choline, an important component of phosphatides. Alanine levels are shown to drop considerably in control seeds between 4 and 8 days but not in treated seeds. Fenitrothion treated seeds, again, had higher percentages of alanine at 4 days but not at 8 days. Aspartic acid is readily converted to alanine by decarboxylation. Alanine, glutamic and aspartic acid result in the formation of keto-acids which are intermediates in the tricarboxylic acid cycle. The glutamic-glutamate pool was found to be the most prevalent amino acid pool and is supported by a similar observation of Ramaiah et al (1971). Fenitrothion treatment did upset the balance of arginine at 4 days and thus increasing its level above that of the control seeds. Schulze (1896, 1898, 1907) observed that arginine is a primary product of protein hydrolysis and serves as a main nitrogenous reserve. Changes in arginine levels then may

reflect metabolic changes caused by fenitrothion uptake which was prevalent in the endosperm and embryo at 4 days and at higher levels in the embryo at 8 days.

Glutamine and glutamic acid were the most prevalent amino acids found even though asparagine and aspartic acid were also found in relatively large quantities. This supports the work of Ramaiah et al (1971) with jack pine and early work of Mothes (1929) whose studies indicated that pines were amide plants. Ramaiah et al. (1971) showed that during seed imbibition and in the seedlings of Jack pine, glutamine-N was the main amide when seed reserves were abundant, whereas when the radicle emerged and the gametophyte reserves declined, asparagine accumulated. Glutamine and glutamic acid levels have been shown in white pine to be stable at 4 and 8 days in all treatments. Further resolution of these amino acids by hydrolysis to the amide form may show changes. There was considerable variability of these amino acids which may reflect fluctuations of metabolic rate in the seed samples. A significant decrease in asparagine and aspartic acid were shown between 4 and 8 days in control and a greater decrease with high conc. fenitrothion treated seeds but not in low fenitrothion treated seeds. This would indicate a fluctuation in asparagine and asparatate levels caused by the fenitrothion treatment. Glutamine has been shown to contribute to the synthesis of purines, and via carbamyl phosphate for pyrimidines. It also contributes the amide group to asparagine (Chibnall 1939; Meister 1965; Streeter 1970). Fluctuations in levels of glutamine, serine, alanine and asparagine should therefore, be looked at in later development of these germinating seeds in various parts of the embryo, and the depleting endosperm under treated and control conditions.

The pathway of serine metabolism to choline is important with high levels of fenitrothion in the endosperm as well as in the embryo at 8

days after germination. It is known in animals that fenitrothion inhibits cholinesterase which degrades acetylcholine in the synapses of nerve endings. It may be of some interest to note here that it is the serine residue on the cholinesterase active site which is phosphorylated by methylated toxic forms of organophosphate pesticides although this has no relation to serine metabolism.

The emphasis of recent work on organophosphate pesticides has turned from phosphorylating to alkylating effects. Cysteine is a very important amino acid for protein structure and its sulfhydryl group is also important in mitosis. A R-SH group is termed a very soft nucleophile and is extremely susceptible to alkylation. It has been shown by Morello et al (1968) that detoxification of organophosphate pesticides in the liver of mammals is glutathione dependent. Therefore it seems as if it is the SH group of the cysteine residue in glutathion which is responsible for initiating detoxification by taking a methyl group from the pesticide and thus forming methylglutathione. As this work is carried further into the small animals of the food chain, this mechanism might deserve further study with fenitrothion. At least such a mechanism in plants has not previously been found.

SUMMARY

A new and modified GLC procedure for the cleanup and separation of fenitrothion and a metabolic break down product (fenitro-oxon) found in the eastern White pine (Pinus strobus L.) seeds is described.

Pine seeds, exposed to fenitrothion at a concentration comparable to field usage (10 ppm) and higher, preferentially accumulate the pesticide and its metabolic breakdown product into the embryo. This seems to affect the amino acid metabolism of the pine seeds as evidenced by significant changes in serine metabolism.

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