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THE METABOLISM AND IDENTIFICATION
OF AN UNUSUAL NITROGEN METABOLITE
IN PEA SEEDLINGS.

by

© NORA KEIKO NISHIKAWA, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Masters of Science.

Carleton University

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August, 1982.

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Dedicated
to
my family,
with love always

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ABSTRACT

An unusual nitrogen metabolite was detected in pea leaf extracts and in this study is denoted "X3". The pattern of X3 distribution indicates that throughout development X3 accumulates in the whole shoot especially the growing leaves (includes shoot apex); a maximum level of 1.43 μmol per leaf being reached there at 20 days. In addition through analyses of xylem sap and phloem exudate (EDTA technique) X3 was found to be transported initially at high levels (ca. 5% of total amino acids translocated in each case) when the plant is 14-days old, while decreasing as the plant matures (ca. 2% of total amino acids translocated). Labelling studies were performed to determine precursors of X3. Of a number of amino and organic acid tracers only alanine and pyruvate showed significant X3 incorporation (2-3% of total radioactivity recovered). X3 was found to be synthesized in germinating cotyledons and growing leaves as indicated by alanine or pyruvate incorporation studies.

Structural identification of purified X3 indicates that it is similar to β -(Isoxazolin-5-one-2-yl)-Alanine, BIA. X3 is a strongly UV absorbing ($\lambda_{\text{max}}=265 \text{ nm}$, $\epsilon=11,700$) material. It also has sensitivity to chemical degradation by acid/base hydrolysis or photolysis and a detectable treatment product is a characteristic BIA breakdown product α,β -diaminopropionic acid.

Mass spectral data show that X3 and BIA have similar molecular weight (172) and fragmentation pattern, but NMR and mass spectral data also suggest that the compounds may exist in different forms. In particular, characteristic olefinic protons (δ 8.1; δ 5.1) of BIA were absent in the spectrum of X3.

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ABBREVIATIONS

AOA	-	)	Aminooxyacetate
BIA	-	/	β -(Isoxazolin-5-one-2-yl)-Alanine
DPA	-		α,β -Diaminopropionic Acid
DPM	-		Disintegrations per Minute
EDTA	-		Ethylene-Diamine-Tetraacetic-Acid
NCS	-		NCS Tissue Solubilizer
OAS	-		O-Acetylserine
SSA	-		5-Sulphosalicylic Acid
TQX	-	-	Triton-Omnifluor-Xylene

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INTRODUCTION

The uptake and utilization of nitrogen in plants involves a complex array of biochemical pathways and a great diversity of nitrogen-containing compounds. In the basic pattern of plant growth, the principal components synthesized include proteins and nucleic acids which are based on a variety of nitrogenous subunits. In addition to these universal subunits and their biosynthetic intermediates, some plants contain unusual nitrogen metabolites. These uncommon substances may have no apparent biochemical significance, however, they must fit into the overall pathways of nitrogen metabolism and by necessity bear some relationship to the better known nitrogenous compounds. This thesis describes studies of the metabolism and identification of an uncommon amino acid found in pea plants and initially known as "X3".

* * * * *

The discovery of new and unusual nitrogenous compounds increased with the advent of modern methods for amino acid separation. From early amino acid paper chromatography (Dent, 1948), many unknown substances were detected, but their identification was not always determined. The advent of ion exchange chromatography (Moore and Stein, 1954) allowed for the consistent

separation of these unidentified compounds, the common features of which were:

1. Positive reaction to ninhydrin indicating the presence of α -amino acid functionality.
2. Chromatographically distinct from known amino acids or their pathway intermediates.
3. Not present in proteins.
4. Unusual degree of accumulation, at certain times it may be a major part of free amino acid pool in certain organs or at specific stage of development.
5. Occurrence restricted to particular taxonomic groups.

These substances are called either non-protein or uncommon amino acids; as their numbers grew, attempts to characterize and identify them intensified. Many substances have now been structurally determined although the functional significance of most is yet unknown (Fowden et al., 1979; Bell, 1976, 1980b).

When an unidentified compound is detected, preliminary experimental data can indicate if the unknown appears to have been reported and identified elsewhere, circumstances which facilitate its study. A common practice is to compare chemical and physiological properties of the unidentified substance to different known materials to establish the structure of the unknown. An attendant problem is making the initial connection of the unusual metabolite to identified, authentic compounds of related form. The correlation of an unknown to previously documented substances can be difficult. There

is also the possibility that an unidentified compound is really an undiscovered substance with unique properties.

In 1967, Fowden et al. examined a short list of 100 uncommon metabolites that were amino acid analogues with toxic physiological effects. The identification of these compounds was simplified by their structural similarity to standard amino acids while their antagonistic biochemical behavior was produced by their substitution of normal amino compounds. Recently, Fowden et al. (1979) re-examined the reports of uncommon amino acids. Many more unusual substances had been discovered and identified during the interval between reviews and Fowden et al. noted that a number of these compounds were described which were unrelated to the protein amino acids. The review described important aspects of uncommon amino acid study including biosynthesis, metabolism and functional significance which are summarized here.

The simplest occurrence of uncommon amino acid production is the accumulation of a normally undetectable but recognized intermediate of a common biosynthetic pathway. Homoserine is an example of this case. Homoserine is a known intermediate in the synthesis of threonine, methionine and lysine, but it is only accumulated in detectable amounts in certain legumes (Lawrence, 1973). Bell (1976) proposed three other potential biosynthetic routes which would result in the

formation and accumulation of unusual amino compounds (Figure 1).

The first potential route is a post-biosynthesis modification of primary amino acids. Hydroxylation and methylation are common reactions that can alter pre-formed amino acids into unusual ones. The formation of 5-hydroxytryptophan occurs in this manner (Figure 1A; Bell, 1976).

The second possible type of uncommon amino acid synthesis arises from the modification of normal biosynthetic pathways (Figure 1B). To demonstrate this class of biosynthesis two specific cases are described. O-acetylserine is formed by the acetylation of serine where upon it can undergo condensation with certain appropriate heterocyclic compounds to yield β -substituted alanines. Part of the original serine moiety is transferred to the ring structure through the intermediate O-acetylserine. Mimosine and β -pyrazol-1-yl-alanine are two examples of this type of synthesis where serine is incorporated into an unusual product by a series of reactions which follow after its normal synthesis. A different case of pathway modification shows how a primary biosynthetic route may directly be utilized to form uncommon amino acids. Aromatic amino acids are synthesized via the shikimic acid pathway. The secondary metabolite, p-aminophenylalanine is produced through the

Figure 1. Types of Uncommon Amino Acid Biosynthesis
(from Bell, 1976; Fowden et al., 1979).

A. MODIFICATION OF PRIMARY AMINO ACIDS

B. MODIFICATION OF COMMON PATHWAYS

a) Additional modifying pathway for a primary amino acid.
e.g. β -Alanine Substitution of Mimosine and Pyrolysine

b) Substitution of an unusual precursor in a primary pathway.
e.g. Formation of p-Aminophenylalanine

C. NOVEL PATHWAYS

formation sequence of tyrosine (Fowden et al., 1979). p-aminophenylalanine biosynthesis requires the same precursors as tyrosine up to the intermediate step of chorismate formation at which point the normal 4-hydroxy group of chorismate is substituted with an amino group. The modified chorismate continues along the tyrosine pathway; its form recognized by the mediating enzymes and used to produce p-aminophenylalanine. The two given examples are representative of modifications that are made to normal pathways in order to produce unusual nitrogenous compounds.

The last alternative formation of uncommon amino acids is by a novel pathway. Evidence of this type of route is usually suggested by labelling studies. An example cited here is the biosynthesis of azetidine-2-carboxylate, a lower proline homologue (Figure 1C; Fowden et al., 1979). Azetidine-2-carboxylate does not appear to result from a modified pathway of proline. Unusual precursors of methionine, homoserine and aspartate were found to be incorporated into the unusual metabolite by ^{14}C -tracer experiments. A tentative scheme of azetidine-2-carboxylate formation has been proposed by Fowden et al. (1979). Novel pathways have thus been reported although there is some scepticism as to the validity of these proposals when experimental evidence is inferred by carbon labelling rather than biochemically

demonstrated (Bell, 1976).

The biosynthesis of uncommon amino acids may be categorized into one of the four types described above. Experimental evidence is usually obtained by tracer studies that investigate ^{14}C -incorporation into a compound from labelled precursors such as organic acids or amino acids. For example Fowden et al. (1979) described the elucidation of the potential biosynthetic route of cyclopropane containing compounds in the genus Aesculus where ^{14}C -labelling studies were exclusively used. To distinguish individual novel reactions or a series of unusual modifying steps, further biochemical investigations are required. These type of reactions may be characterized by identifying the mediating enzymes and their properties.

Metabolic studies on uncommon amino acids have generally been investigations of the biosynthetic pathway. The fate of these compounds is not easily discerned and experimental evidence is often difficult to obtain. A typical approach involves the ^{14}C -labelling of the uncommon amino acid and subsequent study of its incorporation into other plant constituents. For example Bauer et al. (1977b) utilized labelled amino acids including an uncommon amino acid, homoserine to study amide amino acid metabolism in pea. In performing this kind of experiment, the problem is preparing a labelled

metabolite of sufficient specific activity. Studies of this type also require a knowledge of the biosynthetic pathway in order to introduce a labelled precursor into the desired compound. These conditions make biosynthetic information important to the study of metabolic function. Other evidence may be gathered by studying transport of the metabolite or concentration changes upon alteration of environmental or nutritional conditions of the whole plant. Interpretation of these results may help to predict the assimilation of the compound, but the experiments are limited in that these observations are not linked to specific biochemical evidence of the compound's metabolic activity.

The ecological significance of secondary plant substances has also been examined (Bell, 1980a). Early reports of uncommon amino acids often noted their toxic effects. The different substances may act as deterrents to animal or insect predation. Alternatively, the plant may produce these compounds for release into its surrounding soil area to chemically prevent competitive growth by microbes and other plants. The importance of these uncommon amino acids is shown by their function as plant defenses, however since little is understood about their metabolism in the plant the relative significance of this ecotoxicological role is not known.

The areas of uncommon amino acid study have been

briefly introduced. Investigation of an unknown can be based upon these same areas of formation, metabolism and function. Biosynthetic information is the most useful. An unidentified substance may be recognized by elucidation of its biosynthetic pathway. Steps in its formation may correlate to those previously reported for a known compound or pathway. Metabolic studies may also be made feasible if the unknown has a recognized precursor which can be used for ^{14}C -incorporation experiments. These combined investigations may uncover the relationship of an unknown to existing normal pathways of nitrogen assimilation. The unidentified substance of this thesis appears to be an uncommon amino acid. Study of its biosynthesis, metabolism and its structural determination were therefore undertaken.

Early metabolic studies with pea seedlings indicated a remarkable number of unknown nitrogenous compounds. Homoserine, a non-protein amino acid was among the first to be identified (Virtanen et al., 1953). Absent in dry seeds, homoserine rapidly accumulated to predominate amino acid chromatograms of seed samples after five days imbibition. The high concentration of homoserine was unusual and many workers became interested in this feature. Larson and Beevers (1965) investigated the metabolism of amino compounds in germinating pea cotyledons. Using ^{14}C -labelling experiments, these

workers found that as seed reserves were mobilized, a considerable proportion of carbon incorporated into homoserine. These carbon skeletons originated from a number of compounds including glutamate and aspartate as traced by their ^{14}C -labelling patterns. The flow of carbon label indicated that seed biosynthesis of homoserine required enzymes and precursors formed during the mobilization of seed reserves. The newly-formed homoserine did not appear to be subsequently metabolized. Larson and Beevers found no evidence of catabolism when labelled homoserine was fed to germinating cotyledons. These results suggested that homoserine was accumulated in the seed because its biosynthesis increased without a change in the turnover of the compound.

Prior to the above report, Lawrence and Grant (1963) published the free amino acid patterns of developing pea seedlings of a different cultivar. Similar trends of amino acid accumulation were found in both studies including the high concentration of homoserine in the germinating seed. The work of Lawrence and Grant also documented several compounds that were unidentified ninhydrin positive substances. The seven unknown compounds were detected by amino acid analysis and tentatively identified however they were not authenticated at that time. The accumulation pattern of these compounds was observed to be similar to that of homoserine but with

lower concentrations. The authors considered the high levels of non-protein amino acids to be unusual. In early development there is a need for an array of free amino acids for de novo protein synthesis, however the young plant also seems to accumulate compounds such as homoserine and the unknowns whose function or further metabolism was not discernible. Their conclusion was that the developing pea balances its nitrogen assimilation between the production of essential biosynthetic requirements and secondary uncommon substances.

It was later reported that homoserine accumulation was only found in Pisum and Lathyrus species of Leguminosae (Lawrence, 1973). Further study on the fate of homoserine was carried out by Grant and Voelkert who investigated the effect of environmental conditions on pea metabolism (1970) and ^{14}C -homoserine incorporation in pea seedlings (1972).

The outcome of this early work on homoserine metabolism was a greater attention to the presence of unknown substances in peas. The detection of other unidentified nitrogenous compounds also raised questions as to their biosynthesis and function in the early growth stages of peas.

In 1975, Van Egeraat reported a study of pea germination that was designed to determine the effect of nutritional or environmental conditions on the levels of

amino compounds. During his experiments, Van Egeraat was able to detect and separate two unknown substances which were ninhydrin positive. The amino acid analysis of these compounds indicated an identical separation time as that of glutathione and aspartate however co-elution was not demonstrated with authentic glutathione or aspartate when separation conditions were adjusted by changing elution buffer pH. These unidentified compounds were designated as "X" and "Y". Their detection was thought to explain discrepancies in reported amino acids levels of other reports. In those studies, these compounds were not distinguished and their concentration could have been erroneously included in the determination of aspartate and glutathione concentrations.

Van Egeraat made further study of X and Y in pea root exudates where they were the main constituents. The identity of X was determined to be γ -glutamyl-alanine by co-chromatography experiments (Van Egeraat, 1976). The presence of γ -glutamyl-alanine in pea seedlings was first described by Virtanen and Berg (1954). In a later report, Van Egeraat substantiated his identification of X by the results of enzyme and chemical degradation studies (Van Egeraat, 1977). Upon acid hydrolysis X disappeared from treated pea extracts while the levels of glutamate and alanine increased in proportion to its loss. The addition of D-amino oxidase or L-glutamate decarboxylase to

hydrolysate preparations indicated the specific configuration of X components to be L-glutamate and D-alanine.

In the above study, the reactions of Y were also reported for various treatments. Y was degraded by L-amino oxidase with a detectable breakdown product of alanine. This reaction indicated the analogous structure of Y to compounds such as tyrosine or phenylalanine that the oxidase normally attacks. When an extract was acid hydrolyzed, a new ninhydrin positive compound was detected in conjunction with the disappearance of Y. This breakdown product of Y was found to be α, β -diaminopropionic acid. From these investigations Van Egeraat suggested that Y was either a pyrimidine based amino acid similar to that identified by Brown and Mangat (1969) or possibly an isoxazolinone derivative (Lambein et al., 1969). Further studies would determine the correct structure.

In 1977, Bauer et al. examined pea leaves as an ideal experimental system for nitrogen metabolism studies. During the course of their amino acid analysis they determined the presence of several unusual ninhydrin positive compounds, one of which had a significant concentration relative to standard amino acids. This unknown closely eluted after aspartate upon amino acid separation by an automated instrument. The substance was

denoted "X3" as it was the third unknown to be recorded and investigated by Joy and his co-workers. Adriaens et al. (1977) documented the elution pattern of over a hundred compounds using an analytical system similar to the one used by Bauer et al. They reported several compounds with elution times that were in the same period as X3. Joy co-chromatographed X3 with authentic of the following: cysteic acid, methionine sulfoxide, sulfone or sulfoximine, glutathione and γ -glutamyl-alanine, however it did not co-elute with any of these (Bauer et al., 1977a). Subsequent experiments with other known substances also did not identify X3 (Joy, unpublished).

Mills and Joy (1980) reported other characteristics of X3. It proved to be an ultraviolet light absorbing material with an absorption maximum at 265 nm (pH 2.0). X3 also showed sensitivity to basic pH (above pH 10.0) and short wave ultraviolet light (254 nm). Under these experimental conditions X3 underwent rapid degradation.

As previously described, Van Egeraat suggested a group of heterocyclic compounds to which his unknown Y might be a member. X3 and this compound Y correlate in several of their properties especially their separation near aspartate upon amino acid analysis. The possible association of Y and X3 lead to an examination of the published reports of these heterocyclic plant products.

The heterocyclic compounds of certain legumes may be

divided into two families, pyrimidine or isoxazolinone derivatives. The former are substituted uracils.

Willardine (β -uracil-1-yl-alanine) was found in peas by Lambein et al. (1968a). Independant studies concurrently reported the isolation and identification of isowillardine (β -uracil-3-yl-alanine) also from peas (Lambein and Van Parijs, 1968b; Brown and Mangat, 1969). Lambein and Van Parijs (1968b) reported the occurrence of β -(5-ribosyl-6-amino-uracil-3-yl)-alanine in germinating pea seeds. Later these workers re-examined their identification of this compound because of new information obtained from the NMR spectra of the substance and other known uracil derivatives. This evidence suggested that the compound did not have a uracil based structure. From its NMR spectrum and some new experiments, Lambein et al. (1969) proposed that the compound was β -(isoxazolin-5-one-2-yl)-alanine. This was the first report of the second family of heterocycles, isoxazolin-5-one derivatives.

Additional isoxazolin-5-ones have since been discovered by Lambein and his co-workers. They include sugar-substituted isoxazolin-5-ones such as 2-(β -D-glucopyranosyl-isoxazolin-5-one-4-yl)-alanine (Lambein and Van Parijs, 1970) and a variety of more unusual derivatives such as 2-cyanoethyl-isoxazolin-5-one (Van Rompuy et al., 1974). Representative compounds are

shown in Figure 2. The general class of isoxazolin-5-ones has been reviewed elsewhere (Lambein et al., 1976).

This new group of heterocyclic compounds has several distinct features. First, the isoxazolin-5-one derivatives are only detected in certain legumes of the genera Pisum, Lathyrus and Lens. The distribution of these compounds has been investigated in Pisum (Lambein et al., 1976) and Lathyrus species (Ikegami et al., 1981). Isoxazolin-5-ones are characteristically sensitive to alkali and short wave ultraviolet irradiation. These compounds undergo rapid degradation with exposure to either conditions. Although both treatments are irreversible, the photolysis reaction results in the accumulation of specific breakdown products that can be isolated and identified. These breakdown products are formed by certain isoxazolinone ring cleavages. When an unknown is suspected to be an isoxazolinone, examination of photolysis products is a useful method for reconstructing original structure. Specific fragments are predictable for a particular isoxazolinone derivative and their detection may help to identify the unknown. Another feature of isoxazolin-5-one compounds is their characteristic ultraviolet spectrum where the range of absorption maxima is between 260-265 nm depending upon the structural chromophore. Finally, NMR spectroscopy is used to distinguish isoxazolin-5-one

Figure 2. Structures of Representative Isoxazolin-5-ones
(from Lambein et al., 1976).

β -(Uracil-3-yl)-Alanine
Isowillardine

β -(Uracil-1-yl)-Alanine
Willardine

free isoxazolin-5-one ring

β -(Isoxazolin-5-one-2-yl)-
Alanine

β -(2- β -D-Glucopyranosyl-
Isoxazolin-5-one-4-yl)-
Alanine

β -(Isoxazolin-5-one-2-yl)-
Ethylamine

β -(Isoxazolin-5-one-2-yl)-
Propionitrile

derivatives from uracil compounds. The coupling constant of the ring olefinic protons (3-H, 4-H) is consistently 3.5 Hz for different isoxazolin-5-ones while uracil compounds have a coupling constant of 7 Hz for their ring olefinic protons (5-H, 6-H). The unique chemical environment of each ring structure affects the chemical shifts of the double bond protons when the compounds are in an applied magnetic field.

The determination of natural isoxazolin-5-one compounds came from the observation of the chemical behavior of synthetic isoxazolin-5-ones. De Sarlo and co-workers (1966a) prepared methyl-isoxazolin-5-ones by two routes: 1) methylation of isoxazolin-5-ones and 2) reactions of N-methylhydroxamine and β -oxoesters. Products of these syntheses were either 3-aryl or 4-aryl substituted 2-methyl-isoxazolin-5-ones. Structures were assigned by results of catalytic hydrogenation, NMR spectroscopy and reaction of N-bromosuccinimide. Other properties were also reported including the investigation of compound alkaline hydrolysis (De Sarlo *et al.*, 1966b). A specific comparison of natural and synthetic isoxazolin-5-ones was made by Van Rompuy *et al.* (1973). They investigated alternate methods of 2-alkylation of 3,4-unsubstituted isoxazolin-5-ones. The workers confirmed the structural determination of a natural isoxazolin-5-one. A plant substance had been tentatively

identified as 2-cyanoethyl-isoxazolin-5-one (Van Rompuy et al., 1974). A comparison of chemical properties was made between the synthesized 2-cyanoethyl-isoxazolin-5-one and a sample of the natural plant product. They were found to be identical by correlation of their NMR, UV, IR and mass spectral data. In addition to the above experiment, Van Rompuy et al. prepared a number of other isoxazolin-5-ones. All of these products had UV spectra with absorption maxima of 265 nm. IR spectral analysis provided evidence of substituted functional groups. The NMR data showed consistent chemical shift values for the olefinic protons of the heterocyclic ring (3-H, δ -ca. 8.0; 4-H, δ -ca. 5.0). Molecular weights were determined by high resolution mass spectroscopy with agreement of experimental and predicted values. In addition, certain peaks at m/e 98 and 85 appeared characteristic of all isoxazolin-5-one mass spectral analyses although the fragment ions were not specified. From the report of Van Rompuy and co-workers it is evident that the properties of synthetic isoxazolin-5-ones and plant derivatives are the same.

Murakoshi and co-workers have investigated the biosynthesis of isoxazolin-5-ones. In 1972, Murakoshi et al. found that the formation of different β -substituted alanines involved a common modification step. They studied the biosynthesis of

β -N-(3-hydroxy-4-pyridone)-alanine and β -pyrazoyl-alanine in Leucaena, Mimosa and other plant species. In each case, the appropriate heterocycle received its alanine moiety from O-acetylserine in an enzyme-catalysed condensation. Murakoshi et al. went on to investigate a similar synthesis in isoxazolin-5-one derivatives. The first of these studies reported that β -(isoxazolin-5-one-2-yl)-alanine was formed from O-acetylserine and isoxazolin-5-one in a crude extract from pea seedlings (Murakoshi et al., 1973). This type of biosynthesis was also observed in the formation of α -(2- β -D-glucopyranosyl-3-isoxazolin-5-one-4-yl)-alanine (Murakoshi et al., 1975a; 1975b) and uracil alanines (Murakoshi et al., 1978). The Japanese workers have found considerable evidence of the reactions which are involved in isoxazolin-5-one modification however there is little information about the original ring formation. In the above cited papers, the free isoxazolin-5-one had to be exogenously supplied to the experimental preparations. It was synthesized by the method of De Sarlo et al. (1971). The free isoxazolin-5-one ring has not been detected in or isolated from plant sources however labelling studies have reported potential precursors. Callebaut et al. (1977) studied the incorporation of ¹⁴C-methionine derivatives into 2-(2-amino-3-carboxypropyl)-isoxazolin-5-one, a homologue of β -(isoxazolin-5-one-2-yl)-alanine. The

experiments showed that the compound did incorporate labelled S-adenosyl methionine and methionine into its structure. Other workers have tried to identify possible free ring precursors. A number of ^{14}C -labelled organic acids have been fed to plants in tracer experiments with pea (Kuo and Lambein, 1981a) or sweet pea (Kuo and Lambein, 1981b). Label was transferred to isoxazolinone derivatives from the following test materials: glycerol, glycerate, pyruvate and malonate. Unfortunately the original ^{14}C -tracers were not uniformly labelled at all carbon positions which made determination of the incorporated ^{14}C -position in the heterocycle difficult. Preliminary investigations of isoxazolin-5-one biosynthetic pathways are limited to the previously described studies. Further work is however in progress by these authors.

Although biosynthetic information is slowly being assembled, the metabolic fate or function of these isoxazolin-5-ones remains a question. The high concentration of these heterocycles is unusual and their accumulation suggests a possible imbalance between synthesis and turnover (Lambein *et al.*, 1976). This specific investigation of isoxazolin-5-one assimilation has yet to be done however toxic effects of these compounds has been demonstrated on animals. Neurolathyrism describes a condition of nervous

derangement which results from the ingestion of certain Lathyrus species. Lambein and De Vos (1981) found that neurolathyrism could be induced in young chicks by dosing with α -amino- β -(isoxazolin-5-one-2-yl)-butyrate.

Osteolathyrism is a condition where skeletal deformities occur as the result of abnormal collagen formation caused by interferrance of toxic metabolites. Rats exhibited symptoms of osteolathyrism when fed 2-cyanoethyl-isoxazolin-5-one. These reports illustrate the physiological toxic effects that isoxazolin-5-one derivatives may produce in predators. Bacteria have also been found to be inhibited by these compounds (Van Egeraat, 1977; Lambein et al., 1976). These investigations of metabolite toxicity indicate a considerable chemical defense as produced by the plants, however their overall significance remains unknown.

Isoxazolin-5-one derivatives have unusual chemical and biological properties. Aspects of their biosynthesis and assimilation are not fully understood. Upon chemical analysis the unknown X3 has shown similar properties to those of isoxazolin-5-ones. In this thesis, comparison of isoxazolin-5-ones and X3 is made with the purpose of possible identification of X3 as a member of this heterocyclic family.

* * * * *

X3 is an unusual nitrogen metabolite that has been found in *Pisum sativum*. This thesis will outline aspects of its metabolism and attempt to determine its structure. The distribution of X3 in plant organs has been examined while its transport was shown by analyses of xylem and phloem exudates. Incorporation of ^{14}C -labelled compounds into X3 was studied as a preliminary investigation of X3 biosynthesis. Finally, structural determination of X3 was made from the analysis of physical features.

* * * * *

METHODS

1. MATERIALS

A. Seeds

Pisum sativum (L. c.v. Little Marvel) from Stokes, St. Catharines Ontario or Mackenzie Steele Briggs Ltd., Brandon Manitoba.

B. Chemicals

Aminooxyacetate, Sigma Chemical Co.

Amino acids, Sigma Chemical Co.

Dowex 50 X8, 200-400 mesh, Sigma Chemical Co.

Dowex 1 X8, 200-400 mesh, Sigma Chemical Co.

α, β -Diaminopropionic acid, Sigma Chemical Co.

NCS Tissue Solubilizer, Amersham Searle

Ninhydrin, Sigma Chemical Co.

Omnifluor, New England Nuclear

Triton X-114, Rohm and Haas Canada Ltd.

Other chemicals Reagent Grade supplied by Fisher Ltd. or Canlab Ltd.

C. Radioisotopes (Supplied by the Radiochemical Centre, Amersham)

$H^{14}CO_3$ (sodium salt), 60.1 mCi/mmol

L-(U- ^{14}C)-Alanine, 75 mCi/mmol

L-(U- ^{14}C)-Aspartate, 10 mCi/mmol

L-(U- ^{14}C)-Asparagine, 105 mCi/mmol

L-(U- ^{14}C)-Glutamate, 10 mCi/mmol

L-(U-¹⁴C)-Glutamine, 57.3 mCi/mmol
L-(U-¹⁴C)-Glycine, 50 mCi/mmol
L-(U-¹⁴C)-Homoserine, 40 mCi/mmol
L-(U-¹⁴C)-Serine, 75 mCi/mmol
L-(U-¹⁴C)-Threonine, 100 mCi/mmol

L-(U-¹⁴C)- α -Ketoglutarate (sodium salt), 17.1 mCi/mmol

L-(U-¹⁴C)-Pyruvate (sodium salt), 10.7 mCi/mmol

D. Authentic Preparations

β -(isoxazolin-5-one-2-yl)-alanine was kindly provided by Dr. F. Lambein of the University of Ghent, Belgium. The compound was isolated from Pisum sativum or Lathyrus odoratus using a method previously published by Lambein et al. (1969). It was 99.8 % pure.

2. GROWTH OF PLANTS

Pisum sativum (L, c.v. Little Marvel) seeds were surface-sterilized in 5% (v/v) sodium hypochlorite for 10 minutes, thoroughly rinsed in distilled water and then soaked in aerated distilled water for 12 hours. Following imbibition, the seeds were placed in 10 inch pots of perlite:vermiculite (1:4) and germinated (covered) in the dark for 5 days. Uniform germinated seedlings (average shoots 2.0 cm tall; roots 8.0-10.0 cm long) were transferred to 9L containers of aerated modified Hoagland nutrient solution (Appendix 1) which provided nitrogen as

calcium and potassium nitrates (4 mM each). The plants were grown in cabinets with a 12 hour photoperiod (26-28 °C) and 12 hours of darkness (18 °C). The light source was a combination of Westinghouse 9GT 12 Daylight, High Output tubes and Westinghouse 100 watt incandescent bulbs. These lights supplied 50 watts per m² intensity at the level of the plants.

The plants were not nodulated. After 15 days of germination, cotyledons were removed. At this time, the plants have three fully-expanded leaves and the seed reserves are exhausted. Over the course of plant growth, the nutrient solution was replenished once a week.

For most experimental procedures, 20 day old seedlings were used. Plants of this age have four fully-expanded leaves and the fifth leaf is half-expanded. For most experiments 3-4 plants of a certain age (days after germination) and uniform sized were selected. When experimental procedure required a period of incubation, the work was performed in the growth cabinet under the same light and temperature conditions as previously described.

In investigations of pea fruit metabolism, soil-grown peas were used. Uniform pea pods were collected at 7, 14 and 21 day intervals after flower opening commenced. The seeds and pods were immediately separated and the tissues were extracted for amino acid analysis. In some cases,

the samples were quickly frozen in liquid nitrogen and stored at -21°C for later extraction.

3. XYLEM SAP

At various stages of plant development, xylem sap was obtained from bleeding root stumps of detopped plants. Plants were cut at the lower epicotyl. The root end was then rinsed and blotted dry. Fifteen to twenty plants were prepared and sap was collected in a capillary from those roots which were actively exuding. Collections were made over a 30 minute period. The pooled fractions were frozen and stored (-21°C) before amino acid analysis.

4. PHLOEM EXUDATE

Uniform pea material was selected in which the youngest leaf was half-expanded; the mature leaflet immediately below this was used for phloem exudation. These mature leaves were detached by severing the petioles under a solution of 20 mM Na EDTA (adjusted to pH 7.0 with NaOH). The cut leaves were then placed in small vials (0.25 ml capacity) containing 0.2 ml of 20 mM EDTA, pH 7.0 (King and Zeevart, 1974). The leaf vials were supported on polystyrene trays in a humid chamber either in light (27°C) or in the dark (20°C). After two hours, the EDTA solutions were collected. This solution contained the exudate of the leaf phloem. The solution was adjusted to pH 2.0 and stored at -21°C . Immediately before analysis,

the precipitated EDTA was removed from the sample by centrifugation (25,600 Xg, Eppendorf Micro Centrifuge Model 5412). In some cases, the leaf tissue was frozen with liquid nitrogen and treated by SSA extraction for amino acid analysis.

5. RADIOACTIVE LABELLING EXPERIMENTS

A. Feeding Experiments of Detached Shoots or Shoot Apices

The growing leaves (fifth expanding leaflet and shoot apex) or whole shoots of 20 day old plants were supplied with different labelled ^{14}C -compounds that entered the material through the xylem during transpiration.

Experimental feeding solutions contained the following: 4 mM nitrate (normal Hoagland solution); 0.5-1.0 μCi of ^{14}C -labelled compound plus 1 mM concentration unlabelled amino or keto acid carrier. This solution was adjusted to pH 6.5 and aliquots ranging from 0.2 ml (growing leaves) to 1.0 ml (detached shoots) were supplied to the cut end of the plant material. Each experimental replicate consisted of 3-4 plants. After specified feeding intervals, the tissues were removed from the radioactive solution and the ends rinsed in distilled water. The plant samples were then weighed, frozen in liquid nitrogen and stored for later extraction.

In some experiments, a transamination inhibitor was supplied with the labelled material and as a pretreatment. Sample material was first supplied with a solution of 4 mM

sodium aminooxyacetate and 4 mM nitrate for two hours. Afterwards, their cut ends were transferred to the labelled feeding solution which also contained 4 mM aminooxyacetate. Experimental procedure was then followed as previously described.

B. ^{14}C -Labelling of Cotyledons

Two methods of feeding cotyledons were tried. The experiment utilized 6 day germinated plants from which the seeds were removed and separated into individual cotyledons. One cotyledon was used in the experimental control while the other was prepared for the labelling study. In one experimental system, intact cotyledon halves were incubated in a humid (moist filter paper-lined cover), parafilm-lined petri dish. Individual inner cotyledonary surfaces were placed over a 20 μl drop of a radioactive solution of ^{14}C -alanine (0.5 μCi in 200 μl of 4 mM nitrate and 1 mM carrier as described before). In the other case, 2 mm cotyledon slices were floated in 0.5 ml of ^{14}C -alanine solution (as in the first system). After 2 or 4 hour incubation periods, cotyledon samples were washed with distilled water to remove excess labelling solutions then weighed and frozen for later extraction.

C. $^{14}\text{CO}_2$ Fixation by Whole Plants - Isolation of X3

Trays of peas were germinated as previously

described, however the plants were left growing in vermiculite for two weeks. The peas were at the 2-1/2 leaf stage by this time.

At the beginning of a light period, the 14 day old plants were transferred to a fume hood and left under a light source (floodlights shielded by a water barrier to prevent overheating). After an hour of equilibration, the trays were sealed in clear plastic bags. The ^{14}C - CO_2 was injected as a mixture of 100 μCi ^{14}C - HCO_3 (1 $\mu\text{Ci}/\mu\text{l}$) and concentrated H_2SO_4 in order to produce the radioactive gas. Incubation time was 30 minutes. After the initial ^{14}C -feeding pulse, the plastic bag was removed and post-labelling metabolism was allowed to occur over the rest of the light period for a total of 12 hours. The trays of shoots were then quickly harvested, weighed and immediately frozen in liquid nitrogen before extraction.

6. EXTRACTION OF ETHANOL-SOLUBLE COMPOUNDS

To determine the amino acid content of the plant material, experimental samples were usually extracted with ethanol. Fresh or frozen plant tissue was ground in cold 80% (v/v) ethanol (40-60 ml per g fresh weight) in a Ten Broeck homogenizer. The homogenate was centrifuged at 10,000 Xg for 10 minutes and the pellet was re-extracted with one or two 5-10 ml washes of ethanol. The ethanolic fractions were pooled and concentrated by vacuum roto-evaporation at 30 °C. A water:chloroform partition

was then performed on the concentrated extract. The ratio of water to chloroform was 2:5 (by volume). The solution was vigorously mixed and then poured into glass centrifuge tubes. Subsequent settling of the partition layers was allowed to occur at 0 °C. After one hour, the extracts were centrifuged at 5,000 Xg for 2 minutes. The aqueous layer was removed; the chloroform layer was re-extracted with water. The aqueous fractions were combined to make up a final known volume containing the equivalent of 0.2-0.5 g fresh weight per ml.

Alternatively, aqueous plant extracts were obtained by homogenizing the tissue in a solution of 5-sulphosalicylic acid, SSA (50 mg per ml solution) and centrifuging the sample at 17,000 Xg for 10 minutes. The resultant supernatant showed similar amino acid content to those obtained by ethanolic extraction (Urquhart, 1980).

7. SEPARATION OF WATER-SOLUBLE COMPOUNDS

For labelling experiments, the estimation of radioactivity distribution in water-soluble compounds was determined. Separation of these compounds was achieved by passing through Dowex 50(H) and Dowex 1(formate) resins (X8, 200-400 mesh) in 7 mm X 80 mm columns (Canvin and Beevers, 1961). Samples were initially passed through Dowex 50. The neutral and anionic components remain in the solution washed through the column. This water eluant was then run through Dowex 1. The amino acid fraction of

the sample absorbs onto Dowex 50 and was eluted with 2N HCl. Neutral compounds such as sugars are not retained on either Dowex 50 or Dowex 1 thus were found in the final water wash. Use of Dowex 1 isolates organic acids from neutral components of the Dowex 50 aqueous eluant. This fraction was collected by elution of Dowex 1 with 2N to 4N formic acid.

Dowex 1 also proved useful for separation of aspartate from X3. Aspartate and X3 elute closely together during amino acid analysis (3-4 minutes apart, peak to peak) and often it was difficult to determine X3 concentration alone. Between pH 6.5-8.0, acidic amino acids such as aspartate are sufficiently anionic to be separated out by ion exchange (Pruisner and Milner, 1970). Therefore, some sample extracts were adjusted to pH 7.0 and passed through Dowex 1 to reduce or eliminate aspartate. X3 did not absorb to Dowex 1 at pH 7.0 and was a component of the initial water wash from the column. This aqueous extract was subsequently used for X3 analysis.

8. ESTIMATION OF RADIOACTIVITY IN ETHANOL-INSOLUBLE MATERIAL

In some labelling experiments, ^{14}C -incorporation was determined in the ethanol-insoluble fraction. NCS Tissue Solubilizer was used to prepare the material for radioactivity estimation. After ethanol extraction, the

pellet of insoluble material was removed and air-dried. Samples of the material were suspended in a small volume of water and mixed with NCS in a 1:9 proportion of $H_2O:NCS$ (by volume). Digestion was allowed to proceed at 50 °C overnight. Samples were then cooled after digestion and stored in the dark (reduction of chemiluminescence) until prepared for counting. One ml aliquots of the NCS-tissue preparation were mixed with 10 ml of toluene-based scintillation cocktail (Omnifluor, 4 g per L toluene) for counting.

9. ISOLATION OF X3

For X3 purification, either 6-day-old shoots or the shoot apices of 14 to 20-day-old pea seedlings were used. The average amount of processed material was 30-40 g fresh weight per extraction. The sample was extracted in distilled water with SSA (50 mg per ml solution). Homogenization was done with a Polytron homogenizer (Brinkmann, Rexdale Ontario) or a Waring blender (Canlab, Ottawa Ontario). The crude extract was then filtered through four layers of cheesecloth. The remaining protein precipitate was centrifuged out at 10,000 Xg for 10 minutes. The solution was adjusted to pH 4.0 and the sample layered onto a Dowex 50 column (2.50 cm by 50 cm; 245 ml bed volume). After sample loading, the column was washed with 300 ml of 0.5N HCl. It was further eluted with a linear gradient of HCl (0.5-2.0N, 2L total volume).

The fractions of X3 were detected by UV-absorption at 265 nm. The compound was eluted out with other amino acids after 800 ml (ca. 1N HCl). The pooled fractions of maximum UV-absorbance were then roto-evaporated at 40 °C to concentrate the sample and remove the HCl. The dry crude X3 was resuspended in ca. 2.0 ml of distilled water. Aliquots of this solution were subsequently separated by paper chromatography. Chromatograms (Whatman 1 paper) were developed by the ascending method using 2-propanol:formic acid:water (20:1:5, v/v) as the solvent system. They were run for 36 hours then removed and air-dried. Test strips were sprayed with ninhydrin-cadmium reagent to detect separation. X3 was a dark orange-brown band upon reaction with ninhydrin. Its R_f value was 0.165. The band of X3 was removed from individual chromatograms and the compound eluted out of the paper with distilled water. Solutions of crude X3 bands were pooled and roto-evaporated to yield crude X3 crystals. The final purification step was recrystallization of X3 from ethanol:water (60-80%, v/v).

10. AMINO ACID ANALYSIS

A. Quantitative Estimation of Amino Acid Content

A Beckman Amino Acid Analyzer model 119BL was used to determine amino acid composition of experimental samples. The instrument was normally used in the physiological mode where amino acid separation is by lithium citrate buffers.

Aliquots of plant extracts (ca. 14 mg fresh weight of tissue) were routinely diluted with lithium citrate buffer and adjusted to pH 2.1 prior to loading on the analyzer. A modified running program was utilized for greater resolution of certain amino acids including asparagine, glutamine and homoserine (Joy et al., 1980). The program modification involves an early temperature increase (44 to 66 °C) 44 minutes after sample injection.

In some experiments, the hydrolysate mode of the analyzer was utilized to resolve basic amino acids. Using a sodium citrate buffer system, basic components of a plant extract were eluted from the hydrolysate column in a shorter period of time than required for separation in the physiological mode. The hydrolysate system was not normally used for quantitative determinations because acidic amino acids tended to co-elute from its column.

B. Separation of Amino Acids for Radioactivity Estimation

For determination of ^{14}C -labelling, amino acids could be separated using the analyzer in a preparative mode. The eluant was diverted before the colorimetric determination and was collected as one minute fractions. From each fraction, 0.5 ml was used for liquid scintillation counting. Positions of radioactive peaks were then compared to the elution pattern of an standard amino acid calibration or an analytical sample of the

labelled extract. In some cases, where there was sufficient leftover volume, samples of the fractions could be returned to the analyzer for correlation of radioactivity to a particular amino acid component.

11. ANALYTICAL TECHNIQUES

A. Ninhydrin Reagent for Amino Acids

A ninhydrin-cadmium reagent was used to visualize amino acids after paper chromatography separation (Section 9.). The ninhydrin-cadmium solution consisted of the following: 0.5 g ninhydrin; 6.0 ml cadmium acetate; and 50 ml acetone. The reagent was used as a spray and allowed to develop over 30 minutes at 40 °C.

12. LIQUID SCINTILLATION SPECTROSCOPY

Radioactivity was estimated by liquid scintillation counting. Routinely, 0.5 ml aliquots of aqueous samples were acidified with 0.1 ml 2N HCl. The preparations were then combined with 5.5 ml (when 7 ml vial used) or 10 ml (20 ml capacity glass vial) of TOX (Triton X-114, 25% by volume; Omnifluor, 4 g per L; Xylene, 75% by volume). The optimal water content of the cocktail is 0.6 ml sample to 10 ml TOX (Pande, 1976), but the capacity of aqueous incorporation was not found to be diminished in the smaller volume of 6.0 ml used. For the non-aqueous tissue (NCS solubilized) samples, the scintillation cocktail was Omnifluor:toluene (4 g Omnifluor per 100 ml toluene). A

1.0 ml aliquot of tissue sample was mixed with 10 ml of Omnifluor:toluene plus 0.1 ml of acetic acid to acidify the preparation. Liquid scintillation counting was performed using one of the instruments described below:

A. Beckman LS150 Liquid Scintillation Counter

In early experiments, samples were counted on a Beckman LS150 Liquid Scintillation Counter. A modified ^{14}C -window, 150-500 channel settings were used. The range of 0-150 was eliminated to reduce phosphorescence interference and the effect on counting efficiency was minimal (Wigfield, 1974). All samples were counted in glass vials. Radioactivity was measured and corrections made for background fluorescence. Counting efficiency was determined by use of a cesium external standard. The relationship of quenching and counting efficiency was calibrated by the change in external standard ratios when a series of sealed quenched ^{14}C -standards were measured for radioactivity. The range of maximum counting efficiencies was 70-75%.

B. Beckman LSC5000 Liquid Scintillation Counter

In later experiments, use of a programmable Beckman LSC5000 was made. The full ^{14}C -window (0-655 channel settings) was routinely used for counting, but the other windows were split into 0-397 and 397-655 channel settings to check for low level fluorescence (chemiluminescence).

To determine counting efficiency a method utilizing "H-number" was employed. H-number calculation requires calibration by estimation of an external cesium standard and a series of quenched ^{14}C -standards as previously described. Normally channel ratios are used as measurement of quenching effects, however the H-number method determines the difference or shift in the pulse height distribution for Compton (recoil) electrons that are generated by the external standard. The location of the edge of the pulse height spectrum relative to the redistribution shift after quenching is monitored. A standard curve of shift difference, the H-number and counting efficiency is then calculated by the instrument for use in dpm (disintegrations per minute) estimations of experimental samples. This H-number method was routinely used; the counting efficiency for samples was generally in the range of 80-85%.

13. ULTRAVIOLET SPECTROSCOPY

For experiments of X3 isolation, a Carl Zeiss model PMQ II was used to detect ultraviolet absorption by X3 in ion exchange column eluant. Maximum absorbance at 265 nm was taken as an indication of the presence of X3 in column eluant fractions. Concentration of X3 was also estimated by ultraviolet absorption (Beer's Law). For structural analysis, ultraviolet spectra were obtained on a Varian

model Cary 219.

14. INFRARED SPECTROSCOPY

Infrared spectra were run on a Beckman IR model 237. Samples (1.5-1.7 mg) of X3 were prepared as KBr disks. Polystyrene was used as an external reference; peaks at 2851, 1601 and 1028 cm^{-1} are indicated on experimental spectra.

15. ^1H -NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

NMR spectroscopy was performed by Dr. G. Buchanan, Department of Chemistry, Carleton University. Samples (ca. 15 mg) of X3 and BIA were prepared in deuterated water with tetramethylsilane (TMS) as internal standard. D_2SO_4 (ca. 5 μl) was required to dissolve BIA and was also included in the X3 sample to maintain a similar pH. The ^1H -NMR spectra were recorded in a Varian model XL200 at 200 MHz.

16. MASS SPECTROSCOPY

Mass spectra of X3 and BIA were obtained from three sources. First, spectra were obtained with an AEI.MS9205 at recording parameters of 60 eV/150-180 °C by Dr. J. Krauss, Ottawa-Carleton Institute of Chemistry Graduate Studies. Comparative analyses were done by Judy Lockwood, Environment Canada. These spectra were recorded on a Finnegan 4000 or 4500(modified ion source) with recording parameters of 70 eV/150-200 °C. The final set of mass

spectral data was obtained through Dr. L. Weiler, Department of Chemistry, University of British Columbia. Both low and high resolution mass spectral analyses were done with samples sent to Dr. Weiler. The instruments used were a Atlas CH-4B Mass Spectrometer, AEI.MS-9 or MS-50 which was operated at 70ev/150-180 °C.

RESULTS

DETECTION AND ESTIMATION OF X3

The unknown, X3 was first detected in pea leaf. The comparison of leaf extracts with standard amino acid mixtures showed that two unidentified compounds were present (Figure 3). The first unknown eluted immediately following aspartate and was termed "X3". The second unknown was termed "Y" and overlapped threonine and serine in amino acid separation; it was not further investigated.

In early experiments, the concentration of X3 was estimated by assuming that it had the same extent of ninhydrin colour reaction as aspartate (absorbance at 570 nm) i.e. using the calibration factor determined for an aspartate standard. This calibration factor of aspartate is re-calculated in every calibration run of amino acid analysis. Later in this study, an authentic sample of β -(isoxazolin-5-one-2-yl)-alanine (BIA) was obtained (Lambein, Belgium). Early X3 data was then converted to concentrations relative to BIA by using known quantities of BIA and determination of its calibration factor, compared to that of aspartate. The ratio of factors was 1.2:1 (BIA:aspartate) which indicates a more intense reaction of BIA with ninhydrin. Thus all reported X3 concentrations are based on calibration with BIA.

Figure 3. Detection of Unknowns X3 and Y
in Pea Leaf Extracts.

An extract from the growing leaves of 20-day old pea plants (ca. 14 mg fresh weight) was amino acid analyzed. Two unknowns were detected when this analysis was compared to a standard amino acid calibration (50 nmol of each amino except for asparagine which is 75 nmol) which is shown in the overlay. The unknowns were denoted "X3" and "Y".

DISTRIBUTION OF X3

The distribution of X3 was examined to determine potential sites where X3 may be synthesized, accumulated or metabolically active. Germinating pea seeds were the first plant parts to be studied. In cotyledons, X3 was not detected until after 48 hours of imbibition (Figure 4). From this trace amount, the concentration of X3 rapidly increased to a maximum at 8 days after germination then the pool of X3 (1.2 μmol per seed) decreased. The decline of X3 coincides with the changes in seed fresh weight, an indication of the mobilization of seed reserves.

In the germinating seed, the major components of the free amino acid pool are homoserine, asparagine, glutamate and glutamine. The pattern of X3 accumulation was compared to some of these amino acids (Figure 5). Homoserine and X3 are absent in the dry seed and are undetectable until the seeds are germinated for two days. Although homoserine and X3 make delayed appearances in the cotyledons, they differ in proportion with a much higher level of homoserine (6.8 μmol per seed) present. Homoserine accumulation also peaks earlier. Aspartate, alanine and asparagine are present in the dry and the germinating seed. They range in maximum concentrations from 1.0-5.0 μmol per seed. X3 and these amino acids have similar trends during the course of germination; X3 is comparable in quantity to aspartate while X3 and alanine

Figure 4. Formation of X3 during Seed Germination.

The levels of X3 (—▲—) were determined during the course of seed germination as indicated by changes in seed fresh weight (g per seed; —●—). X3 data is expressed as μmol per seed.

Figure 5. Levels of Various Amino Acids during Seed Germination.

At certain intervals during germination, seed samples were taken for analysis of the amounts of various amino acids present. Data is expressed as μmol per seed.

X3 ---●---
Asp ---▲---
Asn ---▼---
Ala ---■---
Hse ---◆---

peak simultaneously. Glutamate and glutamine (not shown) are similar in amounts and trends to homoserine. At their maximum glutamate is 6.1 μmol per seed (at 5 days) and glutamine has 7.8 μmol per seed (at 4 days)

Other plant organs were examined for evidence or trends in metabolism of X3 during growth. Three representative stages of pea plant development were chosen for sampling of individual plant parts. These plant stages were: 1) germinating seedlings, 6-day old pea plants, 2) a young plant at 14-days old and 3) a mature pea plant 20-days old. The 6-day old seedling has only its main root and a shoot where the first leaf is beginning to develop. At this stage of plant development, the seedlings are normally transferred after germination in perlite:vermiculite to hydroponic culture for further growth. The 14-day old plants are at the stage where they are forming extensive root systems and their shoots have two fully expanded leaves while the third is opening. The seedlings' cotyledonary reserves are nearly exhausted. At this time, remains of the seed are removed and the young plant is independent of its original source of nutrients (seed protein nitrogen). The last representative stage is a mature, established plant at 20-days after germination (Figure 6). These seedlings have four fully developed leaves while the growing leaf (leaf 5) is only half expanded; its stipules enclose the shoot apex (leaf 6). Leaves 3 and 4 are considered mature while the remaining

Figure 6. Representative 20-Day Old Pea Plant.

leaves 1 and 2 are typically close to senescence. Bauer et al. (1977a) used the 20-day old plant for extensive nitrogen metabolism studies. The active growth and metabolism of the developing leaf 5 is well characterized.

With these three representative stages of pea growth, the changes in the X3 pool for different organs were examined. Table 1 outlines the results. The X3 levels range from 0.2 to 1.73 μmol per organ. The percentage distribution of total X3 was calculated to provide a balance sheet of X3 accumulation in the whole plant.

In the 6-day old plant, the root and shoot maintain similar amounts of X3: 30.7% and 46.6% of the total respectively. X3 level in the root (1.14 μmol per root) does not increase from 6 to 14 days and its proportion of total plant X3 declines. This is also the trend of X3 metabolism in the germinating cotyledons. The seed pool of X3 is past its peak by 14 days after germination (Figure 2). The greatest change of X3 levels is reflected by the data of the pea shoot. By 20 days of plant growth, 82.7% of X3 is localized in the whole shoot where the shoot apex and growing leaf contained the highest amount, 38.8% of total X3. Comparison of data for leaves 1 to 3 from 14 to 20 days growth shows that in these lower leaves the concentration of X3 declines as they mature. The senescent leaf 1 of the 20-day plant has only 0.02 μmol per leaf of X3 while in the corresponding leaf of the 14-day old plant there is 0.88 μmol of X3 per leaf. This

Table 1. Distribution of X3 in the Pea Plant.

Three representative stages of pea development (days after germination) were sampled by SSA method of extraction. The average X3 amount is given for each part and also individual percentages of estimated total X3 content were calculated.

Plant Material	Amount of X3 (umol per organ)		
	<u>6 Day Stage</u>	<u>14 Day Stage</u>	<u>20 Day Stage</u>
Roots	1.14	1.15	0.62
Cotyledons	0.84	0.75	ND ^a
Whole Shoot	1.73	3.80	ND
Growing Leaf plus Shoot Apex	ND	0.85	1.43
Mature Leaf	ND	1.32	1.03 (4) ^b 0.50 (3)
Old Leaf	ND	0.88	0.09 (2) 0.02 (1)
	<u>% of Total X3</u>		
Roots	30.7	20.2	16.8
Cotyledons	22.6	13.2	—
Whole Shoot	46.7	66.7	—
Growing Leaf plus Shoot Apex	—	14.9	38.8
Mature Leaf	—	23.3	27.9 (4) 13.6 (3)
Old Leaf	—	15.5	1.8 (2) 0.5 (1)

ND^a - Not Determined

(n)^b - n refers to leaf number as shown in Figure 5.

is almost a 50-fold difference. The growing leaf thus appears to be the major sink for X3 in older plants as fully-expanded leaves do not maintain a stable level of X3 after they mature.

Developing fruit of flowering pea plants were also examined for the presence of X3 (Table 2.) The plants were 25-30 days old before flowering occurred. Samples of seed and pod were taken at representative stages of pea seed formation. The stage of fruit development was based on the number of days that had elapsed after flowering. The 6-day old fruit is an example of early seed formation when the seed reserve materials are being transported into the fruit tissue. The 14-day old pea seed is at its maximum fresh weight and size. The process of seed formation is complete and the seed and pod begin to dry out. The last sampling is at 20 days after flowering.

From these three periods of fruit development, it is evident that fruit levels of X3 are low, at a maximum 0.25 μmol per seed and 0.63 μmol per pod for 14-day old samples. The amount of X3 in the pod is higher than in the seed. During fruit maturation, the seed/pod pools of X3 decline in size, eventually disappearing (data not shown) as the fruit dries completely. Although X3 is not a major fruit tissue component, the data does suggest that X3 is either transported from the shoot to the developing fruit or is synthesized there during development.

Table 2. Distribution of X3 in Pea Fruit.

Three representative stages of pea fruit development were sampled for X3 content. The stage of development was based on the period after the flowers opened and the fruit emerged (days after flower opening). The concentration of X3 is given as amount per organ and the percentage of total X3 is also calculated.

Plant Material	Amount of X3 (μ mol per organ)		
	Days after Flowering		
	<u>6 days</u>	<u>14 days</u>	<u>20 days</u>
Seed	0.02	0.25	0.16
Pod	0.34	0.63	0.23
<u>% Distribution of X3 in the Fruit</u>			
Seed	7.1	28.5	41.1
Pod	92.9	71.5	58.9

TRANSPORT OF X3

A. Xylem Sap Analysis

When seed reserves are no longer supplied to a developing shoot, the plant becomes dependant upon its roots as a means of obtaining nutrients such as sources of nitrogen. Compounds are transported from the root to the shoot in the transpiration stream via the xylem. Analysis of xylem sap provides information about the assimilatory activity of the roots and the nature of transported nitrogenous compounds.

X3 was found to be a component of pea xylem sap. Figure 7. is a representative analysis of the bleeding sap of 20-day old detopped roots. Major constituents of this exudate are asparagine, aspartate, glutamine and homoserine. X3 is a minor element of the xylem sap with a concentration (3.25 nmol/root after 30 minutes exudation) comparable to the level of transported glutamate.

The concentration of X3 varies in the xylem exudates of plants at different developmental stages. Three representative stages of pea and two time periods of sampling were utilized to examine changes in X3 transport (Table 3) The recovery of X3 is expressed with respect to the total concentration of amino acids shown in Figure 7. Analyses were routinely performed up to homoserine because 85% of the xylem amino acid content was recovered in these compounds (Urquhart, 1980) In a complete xylem sap

Figure 7. Representative Xylem Sap Analysis
from 20-Day Old Pea Roots.

Xylem sap was collected from detopped
20-day old pea roots for a 30 minute period.
From this exudate the levels of various amino
acids were determined and expressed as
nmol per root.

Table 3. Distribution of X3 in Xylem Sap.

The exudates of bleeding detopped roots of plants at various stages of development were collected over a 30 minute period and analysed for their amino acid content.

Stage of Development (Days after Germination)	Sampling Times			
	9 a.m.		3 p.m.	
	nmol per root	% Recovery ^a	nmol per root	% Recovery ^a
14 Days	1) 2.93	5.57	1) 3.12	5.03
	2) 1.80	4.00	2) 0.82	4.78
	3) 2.06	2.11	3) 1.33	3.52
17 Days	1) 2.45	2.15	1) 1.15	2.54
	2) NS ^b	—	2) 1.70	0.50
20 Days	1) 2.58	3.77	1) 2.12	4.31
	2) 3.84	2.54	2) 1.43	2.60
	3) 3.27	3.47	3) 3.20	1.39

a-% Recovery= % of the total content of amino acids shown in Figure 7.

^bNS=Not Separated

analysis, X3 represented 2.7% of the free amino acid pool; this value is comparable to the percentage calculations of the shortened analytical runs. The range of X3 concentrations decreased with plant development from an average 4.18% recovery in experiments from 14-day old plants to 2.75% in older 20-day old (Table 3). Results were relatively consistent between replicates and sampling times as well. Different sampling times were used to determine if X3 movement varied during the photoperiod. It had been found that serine and aspartate levels do differ in their proportion of the xylem sap, but X3 did not change in its relative amount for either time sampling.

B. Phloem Exudate

Phloem exudate analysis provides information about the nature of compounds which are transported from mature photosynthetic leaves to the developing tissues of growing leaves or fruit. As for xylem sap experiments, differing stages of pea plants were used to determine whether X3 was among the amino acids which can be exported in the phloem of mature detached leaves. A representative analysis of 20-day old plant mature leaf phloem exudate is shown in Figure 8. The major amino acids found in the exudate were asparagine, glutamate, aspartate and homoserine. Threonine and X3 were about the same level ca. 0.48 nmol per leaf (2 hour exudation period). Table 4 shows the

Figure 8. Representative Phloem Exudate Analysis
from Detached 20-Day Old Pea Leaves.

Mature leaves were detached from 20-day old pea shoots and the petioles submerged in a 20 mM EDTA solution (pH 7.0). The leaves were allowed to exude from the phloem for a 2 hour period. The EDTA solution was then taken for analysis of the amounts of various amino acids present. Data is expressed in nmol per detached leaf.

Table 4. Distribution of X3 in Phloem Exudate.

The phloem exudate was collected from detached mature leaves from pea plants at various stages of development. The detached leaves were transferred to a 20 mM EDTA solution and allowed to exude over a two hour period. Exudates were analyzed for their amino acid content.

Stage of Development (Days after Germination)	Sampling Times			
	9 a.m.		3 p.m.	
	nmol per leaf	% Recovery ^a	nmol per leaf	% Recovery ^a
14 Days	1) 0.624	5.40	1) 0.608	3.65
	2) 0.351	1.76	2) ND ^b	—
	3) 0.594	5.47		
17 Days	1) ND	—	1) 0.193	2.22
20 Days	1) 0.138	1.95	1) 0.097	0.93
	2) 0.480	2.70	2) 0.178	1.79

a- % Recovery= % of the total content of amino acids shown in Figure 7.

b-ND=Not Detected

distribution of X3 in phloem exudates of detached mature leaves taken at various ages. The level of X3 varied between replicates although the trend was towards a greater percentage of X3 in exudates from the mature leaves of younger plants. There is also a difference between sampling times with experiments later in the day having lower concentrations of X3 exported in the phloem exudate.

^{14}C -LABELLING OF X3 WITH ^{14}C -LABELLED AMINO OR ORGANIC ACIDS

Carbon-labelled tracers were used to study the potential for incorporation of common metabolic compounds into X3; a variety of organic and amino acids were tested as precursors (Table 5). Whole shoots (or in some cases shoot apices) were fed labelled amino acids over a prolonged period (4 hours). The subsequent analysis of the tissue extracts generally showed a large proportion 50-88% of the total radioactivity remained in the free amino acid pool.

Most compounds showed no incorporation into X3 where background or low (less than 0.5% total dpm) levels of radioactivity were detected in X3. For example, the distribution of radioactive label from ^{14}C -serine is shown in Figure 9. ^{14}C -serine was not metabolized into X3. Representative of a number of tracers listed in Table 5, labelled carbon from ^{14}C -serine was recovered largely in

Table 5. ^{14}C -Labelling Experiments of X3.

Detached whole shoots or shoot apices of 20-day old plants were fed a number of labelled amino acids and the incorporation of carbon label assessed for total recovery in different fractions of metabolites and individual amino acids.

¹⁴ C-Labelled Tracer used in each trial	Radioactivity supplied X 10 ⁻⁵ DPM	Radioactivity recovered X 10 ⁻⁴ DPM	Percentage Distribution ^a AA Neut. OA		
<hr/>					
Detached Whole Shoots 4 Hour Labelling					
<hr/>					
1)Asparagine	3.70	9.39	68.0	32.0 ^b	
2)Glutamate	3.72	21.9	71.0	29.0	
3)Glutamine	4.20	0.85	68.0	32.0	
4)Glycine	5.42	2.36	28.6	20.8	50.6
5)Serine	5.18	2.45	55.6	15.4	29.2
6)Alanine	4.16	2.44	38.8	19.0	42.2
<hr/>					
Detached Shoot Apices 4 Hour Labelling					
<hr/>					
1)Aspartate	11.6	53.1	50.6	0.9	48.5
2)Homoserine	56.9	2.04	88.0	2.5	9.5

a- Distribution between 3 major categories:

AA - Amino Acids
Neut - Neutrals (e.g. sugars)
OA - Organic Acids

b- Neutral and organic acid fractions were not separated in these experiments.

Figure 9. Distribution of Radioactivity in Compounds of the Aqueous Extracts of Detached 20-Day Old Whole Pea Shoots Fed ^{14}C -Amino Acids.

In each experiment detached whole pea shoots were fed a labelled amino acid via transpiration for a 4-hour period. The sample was then treated to ethanol extraction and the final aqueous solution used to estimate the radioactivity incorporated into different compounds. The data is expressed as a percentage of the total radioactivity recovered in the extract.

major amino acids (alanine or aspartate) or in its original form. Only two of the amino acids tested showed significant labelling into X3. The recovery profiles of ^{14}C -glycine and ^{14}C -alanine are also shown in Figure 9. The ^{14}C -glycine experiment showed that glycine was rapidly metabolized into numerous compounds especially serine (19%) and threonine (10%). X3 incorporation was a small fraction (0.65%) of the originally supplied radioactivity. The ^{14}C -alanine tracer also metabolized into X3, to a slightly greater degree than ^{14}C -glycine, 1.1% of the total radioactivity. The other major fractions labelled from ^{14}C -alanine were asparagine-glutamate and glutamine-homoserine. Another ^{14}C -alanine experiment with growing leaf tissue (data not shown) showed similar incorporation of carbon label into X3 (2.0% of total radioactivity).

The biosynthesis of X3 was shown to occur during seed germination (Figure 2). Cotyledons of 6-day germinated peas were tested for their ability to incorporate labelled carbon from alanine into X3. In detached shoots, ^{14}C -tracers are normally introduced into the tissue by the uptake of the radioactive compound in the transpiration stream of the plant. With cotyledons, this method cannot be used, so the cotyledons were allowed to float in radioactive tracer solution where uptake is expected to occur at the seed surfaces. The uptake of the labelled precursor was poor in comparison to the uptake of

solutions by transpiring detached shoots (Table 6). The minimum recovery of label was in the intact cotyledon experiment where only 9.0% of supplied radioactivity was found in the seed extract. When the cotyledons were sliced into small segments, the uptake was improved two-fold by the increase in surface area exposure to the labelled compound. In the three experiments, ^{14}C -alanine remained largely unmetabolized (58-84%); the other major proportion of the tracer was in the organic acid fraction (Figure 10). Of the minor labelled components, X3 comprised 1.1% of the total radioactivity of both four hour experiments and 0.98% in the two hour one.

In addition to amino acid tracers, two labelled keto acids were fed to growing leaf tissue of mature 20-day old plants (Table 7). There is a greater degree of metabolism of pyruvate than of α -ketoglutarate. Most of the radioactivity from ^{14}C -pyruvate is recovered initially in amino acids after 30 minutes of feeding the tracer (54%) while further incubation redistributes radioactivity between amino and organic acid fractions. X3 is shown to have incorporated carbon label from pyruvate at a similar level to that found when alanine was the precursor. Two experiments using ^{14}C -pyruvate labelling are shown in Figure 11. In the α -ketoglutarate experiments little tracer becomes incorporated into neutrals or amino acids (6.9 % to 14%), the majority (85%) of the labelled carbon remains in the organic acid pool. Figure 11. gives the

Table 6. Distribution of Radioactivity in Germinating Seed Tissue Following Incubation of 14 C-Alanine on Cotyledonary Surfaces.

$\times 10^{-4}$ DPM	Cotyledon Slices 2-hour Labelling	Cotyledon Slices 4-hour Labelling	Cotyledon Halves 4-hour Labelling
Total Radioactivity of Fed Solution	11.0	11.0	9.28
Amount Recovered in Aqueous Extract	1.53	2.31	0.90
% Total Recovery in Aqueous Extract			
Amino Acids	71.0	74.1	90.0
Neutrals	9.6	5.6	2.2
Organic Acids	19.4	20.3	7.8

a-SSA method of aqueous extraction was used.

Figure 10. Distribution of Radioactivity in Compounds of the Aqueous Extracts of 6-Day Old Germinating Pea Cotyledons Fed ^{14}C -Alanine.

Either intact individual cotyledon halves or cotyledon slices were incubated in solutions of ^{14}C -alanine for different periods of time. Afterwards the samples were SSA extracted and the final aqueous extract used to estimate the radioactivity incorporated into various compounds. The data is expressed as a percentage of the total radioactivity recovered in the extract.

Table 7. Distribution of Radioactivity from ^{14}C -Organic Acids fed to 20-Day Pea Shoot Apices.

Detached growing leaves (including shoot apices) of 20-day old plants were fed either labelled pyruvate or α -ketoglutarate via their transpiration stream. Incubation periods were for 30 and 60 minutes. After ethanolic extraction, the aqueous extract was sampled for ^{14}C -incorporation into various metabolites.

X 10^{-4} DPM	^{14}C -Pyruvate		^{14}C - α -Ketoglutarate	
	30 min	60 min	30 min	60 min
Total Amount of Radioactivity Supplied	3.12	6.24	37.5	56.2
Amount of Radioactivity Taken Up	1.35	2.79	15.9	36.4
Amount Recovered in EtOH-soluble Fraction	0.71	1.17	9.6	11.1
Amount Recovered in Aqueous Extract	0.50	1.44	8.37	10.6
% Total Recovery in Aqueous Extract				
Amino Acids	54.3	33.6	6.9	14.2
Neutrals	20.1	29.1	9.5	5.0
Organic Acids	25.6	37.3	84.6	80.6

Figure 11. Distribution of Radioactivity in Compounds of the Aqueous Extracts of Detached 20-Day Old Shoot Apices Fed ^{14}C -Organic Acids.

In each experiment, detached growing leaves including shoot apices were fed a labelled organic acid via transpiration for shortened feeding periods. After ethanol-extraction, the final aqueous extraction was used to estimate the radioactivity incorporated into various compounds. The data is expressed as a percentage of the total radioactivity recovered in the extract.

percent recoveries of total radioactivity found in various ethanol-soluble compounds. The major labelled fractions of α -ketoglutarate experiments are alanine, glutamate and aspartate. Originally it was thought that traces of labelled X3 were detected but later separation on ion exchange (Method 7) indicated that the label was actually in contaminant aspartate.

Table 8 provides the experimental results of ^{14}C -pyruvate feeding experiments where incubation time of the tracer was shortened. The sampling times were 7.5, 15 and 30 minutes after supplying detached growing leaf tissue (shoot apex) with ^{14}C -pyruvate solutions. The 30 minute experiment contained the greatest radioactivity due to the longer period allowed for uptake of the tracer. Distribution of the labelled pyruvate carbon was rapid as the 7.5 minute sample had a recovery profile similar to that of Figure 10 although minor incorporation fractions (X3, threonine and glycine) were lower. There was carbon label in X3 at all sampling times (1.5-1.7%).

During the period of feeding the ^{14}C tracer rapid equilibration of the carbon label may occur between the alanine and pyruvate pools of the plant tissue via transamination. This leads to the possibility that either pyruvate and alanine may be precursors to X3 or that label must flow from one compound into the other (via transamination) before X3 incorporation will occur.

To investigate the route of ^{14}C incorporation from

Table 8. Distribution of Radioactivity in Detached Shoot Apices of 20-Day Old Pea Plants Fed ^{14}C -Pyruvate for Short Time Intervals.

Detached growing leaves (including shoot apices) of 20-day old plants were fed labelled pyruvate via their transpiration stream. Incubation periods were shortened to 7.5, 15 and 30 minutes. After ethanol extraction, the aqueous extract was sampled for ^{14}C -incorporation into various metabolites.

X 10^{-4} DPM	^{14}C -Pyruvate Labelling Period		
	7.5 minutes	15 minutes	30 minutes
Total Radioactivity of Fed Solution	5.52	5.52	11.0
Amount of Radioactivity Taken Up	2.68	1.47	7.54
Amount Recovered in EtOH soluble Fraction	0.64	1.47	1.85
Amount Recovered in Aqueous Extract	0.693	1.30	2.14
% Total Recovery in Aqueous Extract			
Amino Acids	51.8	43.2	38.1
Neutrals	8.7	14.4	14.1
Organic Acids	39.5	44.4	47.8

pyruvate/alanine into X3 the flow of label was monitored when a transamination inhibitor, aminooxyacetate was used to reduce the equilibration of ^{14}C label between pyruvate and alanine. The experiments were carried out with 20-day old shoot apices that were fed labelled solutions of alanine or pyruvate in the presence or absence of the inhibitor, aminooxyacetate (AOA). In the initial trial, AOA was introduced with the tracer during a short period (15 minutes) of uptake (Table 9, 10, Experiment 1.) A pre-incubation period (2 hours) in an AOA solution was found to be required since transamination inhibition in this shoot tissue was insufficiently induced during the short period of label uptake. During control experiments with ^{14}C -alanine or ^{14}C -pyruvate alone, the ^{14}C -incorporation into X3 ranged from 0.8-3.3% (Tables 9 and 10). Upon inhibition of transamination, ^{14}C -pyruvate incorporation was eliminated. The effect of inhibition was complete in the pyruvate experiments as the radioactivity of the extract was almost exclusively in the organic acid pool. Less definite results were obtained in the ^{14}C -alanine system where radioactive carbon was still distributed in the neutral and organic acid fractions. Traces of label were still found in different amino acids including the unknown X3 (0.8%) after inhibitor uptake in these ^{14}C -alanine experiments. These results suggest that alanine may be the more direct precursor to X3 while metabolism of ^{14}C -pyruvate occurs after transamination.

Table 9. The Distribution of Radioactivity in Detached Shoot Apices of 20-Day-Old Pea Plants. Fed ^{14}C -Alanine and Treated with the Transamination Inhibitor, Aminoxyacetate.

The growing leaves of 20-day old pea plants were detached and pre-treated to a two hour incubation in water (control) or 4 mM aminoxyacetate, AOA (except in Experiment 1 where this treatment was not done). The leaves were then transferred to a ^{14}C -alanine solution and incubated for 15 minutes. After ethanolic extraction, the incorporation of label into various metabolites was determined.

OF / DE

X 10 ⁻⁴ DPM	Experiment 1		Experiment 2		Experiment 3	
	<u>14C-Ala</u>	<u>14C-Ala+AOA</u>	<u>14C-Ala</u>	<u>14C-Ala+AOA</u>	<u>14C-Ala</u>	<u>14C-Ala+AOA</u>
Total Amount Radioactivity Supplied	4.71	4.90	35.0	12.3	9.32	7.04
Radioactivity Taken Up	2.48	2.83	18.7	10.4	3.28	2.12
Recovery in EtOH Extract	1.76	1.68	1.33	1.67	ND ^a	ND ^a
Recovery in Aqueous Extract	1.49	1.54	1.31	1.65	1.15	1.30
% Total Recovery in Aqueous Extract						
Amino Acids	88.0	97.7	73.7	97.2	79.4	99.0
Neutrals	3.2	2.1	6.7	0.9	3.7	≥0.1 ^b
Organic Acids	18.8	0.2	19.6	1.9	17.9	≥0.1
% Total Recovery in Major Amino Acids						
Alanine	35.8	86.9	61.8	95.0	66.8	97.8
Gln/Hse	14.6	0.8	5.2	0.4	4.2	0.1
x3	1.3	2.8	2.4	0.1	2.1	0.6
Asp	1.1	4.4	0.8	0.4	0.7	1.0

ND^a - Not Determined

b-Bkgd - Levels of radioactivity ≥0.1% or background (25 DPM) level.

Table 10. The Distribution of Radioactivity in Detached Shoot Apices of 20-Day-Old Pea Plants. Fed ^{14}C -Pyruvate and Treated with the Transamination Inhibitor, Aminoxyacetate.

The growing leaves of 20-day old pea plants were detached and pre-treated to a two hour incubation in water (control) or 4 mM aminoxyacetate, AOA (except in Experiment 1 where this treatment was not done). The leaves were then transferred to a ^{14}C -pyruvate solution and incubated for 15 minutes. After ethanolic extraction, the incorporation of label into various metabolites was determined.

X 10 ⁻⁴ DPM	Experiment 1		Experiment 2		Experiment 3	
	<u>14C-Pyr</u>	<u>14C-Pyr+AOA</u>	<u>14C-Pyr</u>	<u>14C-Pyr+AOA</u>	<u>14C-Pyr</u>	<u>14C-Pyr+AOA</u>
Total Amount Radioactivity * 6.30 Supplied		14.0	11.0	19.6	14.6	28.2
Radioactivity Taken Up	2.75	4.70	4.03	6.2	5.00	1.00
Recovery in EtOH Extract	1.76	6.10	2.60	3.08	ND ^a	ND
Recovery in Aqueous Extract	0.95	4.85	1.45	4.02	1.33	7.36
% Total Recovery in Aqueous Extract						
Amino Acids	67.0	5.0	2.1	0.7	25.1	1.0
Neutrals	24.8	28.1	29.2	16.5	7.1	≥0.1 ^b
Organic Acids	8.2	95.0	68.6	82.4	67.8	96.2
% Total Recovery in Major Amino Acids						
Alanine	23.7	1.46	14.0	Bkgd ^b	5.0	Bkgd
Thr/Ser	5.5	1.7	1.0	Bkgd	6.9	Bkgd
X3	3.3	1.0	3.0	Bkgd	3.9	Bkgd

ND^a - Not Determined
b-Bkgd - Levels of radioactivity ≥0.1% or background (25 DPM) level.

^{14}C -LABELLING OF X3 BY $^{14}\text{CO}_2$ FIXATION EXPERIMENTS

An attempt was made to produce ^{14}C -labelled X3 with a high specific activity. This labelled X3 was thought to be potentially useful in metabolic studies where its incorporation into other compounds could be traced. The results of the X3 labelling experiments are reported in Table 11. A initial trial of $^{14}\text{CO}_2$ fixation (data not shown) indicated that ^{14}C -incorporation into X3 did not occur when a short radioactive pulse (10 minutes) and a short post-fixation period (60 minutes) was used. It was thought that X3 incorporation of label may be enhanced by a longer chase after the $^{14}\text{CO}_2$ pulse.

The $^{14}\text{CO}_2$ pulse consisted of 100 μCi of radioactivity, however only 26% of that amount was recovered in the aqueous plant extracts. Ethanol-insoluble material represented 61.9% and 69.5% of these samples' total radioactivity. Of the ethanol-soluble material, a small percentage remained in the chloroform layer (ca. 1.1% for each trial) after chloroform:water partition of the ethanolic fraction. Most of the recovered radioactivity in the water-soluble extract was in the neutral fraction (ca. 70%) while the remainder of the radioactivity was divided between the amino and the organic acid fractions. The amino acid fraction contained 16.4% and 19% of the total radioactivity. There was a small amount of radioactivity

Table 11. ^{14}C -CO₂-Fixation Experiments -Labelling of X3.

An attempt was made to produce ^{14}C -X3 by labelling the compound indirectly through $^{14}\text{CO}_2$ -fixation labelling of whole plants. 14-Day old peas were exposed to 100 μCi of $^{14}\text{CO}_2$ for a one hour period and allowed a post-fixation chase of 12 hours. The plants were subsequently extracted and processed for the isolation of X3. The distribution of radioactivity was estimated for various metabolites.

X 10^{-6} DPM	<u>Experiment 1</u>	<u>Experiment 2</u>
Total Amount Radioactivity Supplied	220	220
Recovery in EtOH-insoluble Extract	35.4 (61.9%) ^a	41.6 (69.6%) ^a
Recovery in EtOH-soluble Extract	21.8 (38.1%)	18.2 (31.4%)
Recovery in Aqueous Extract	19.5	17.7
% Total Recovery in Aqueous Extract		
Amino Acids	16.4	19.0
Neutrals	69.8	70.0
Organic Acids	13.8	11.0

a-% of Total Radioactivity

incorporated into X3 (4.4%). Although the specific activity of X3 was low, isolation was attempted to recover labelled X3 for metabolic studies. The purified X3 did not contain sufficient radioactivity to be useful in subsequent labelling studies.

ISOLATION OF X3

The isolation of X3 utilised a three step purification process. First, ion exchange chromatography with a linear HCl gradient (0.5-2.0N) was used to prepare a crude X3 fraction (Figure 12). This X3 fraction consisted of the column eluant (ca. 100-150 ml) when the gradient reached a concentration of 1.0N HCl. The solution contained primarily X3 but other amino acids including glutamate, asparagine and alanine were major contaminants. To separate X3 from these compounds, paper chromatography was used. Table 12 gives the R_f values for standard amino acids which were separated in this second step. The final purification process was recrystallization of X3 (obtained from paper chromatography separation) from ethanol:water (80:20, v/v). Pure X3 was a creamy white crystal. The yield was low: 20-30 mg from 30-50 g fresh weight of starting material.

CO-CHROMATOGRAPHY OF β -(ISOXAZOLIN-5-ONE-2-YL)-ALANINE, BIA AND X3

On the amino acid analyzer, co-elution of purified X3

2

Figure 12. Elution Profile of X3.

Fractions ( ) of a maximum absorbance at 265 nm were collected as a crude X3 following ion exchange chromatography of a plant extract. The Dowex 50 column was eluted with a linear HCl gradient (0.5-2.0N). Concentration was measured by conductivity (mmho) using a Radiometer Conductivity meter.

Table 12. Rf Values of Amino Acids.

Rf values for standard amino acids after one-dimensional paper chromatography with solvent system: isopropanol: water: formic acid (10:5:1).

Amino Acid	Rf
α,β -Diaminopropionic Acid	0.078
Asparagine	0.119
Glutamine	0.160
X3	0.165
β -(Isoxazolin-5-one-2-yl)-Alanine	0.165
Aspartate	0.192
Glycine	0.200
Serine	0.215
Homoserine	0.256
Glutamate	0.267
Threonine	0.280
Proline	0.311
Tyrosine	0.320
Phenylalanine	0.474

and authentic β -(Isoxazolin-5-one-2-yl)-Alanine, BIA (provided by Dr. F. Lambein, Belgium) was demonstrated (Figure 13). The position of aspartate was determined by use of a standard calibration (50 nmol) sample which was followed by analyses of BIA, X3 and a mixture of these compounds. X3 and BIA also co-chromatograph on paper chromatography and their spots give the same orange-brown colour reaction when sprayed with ninhydrin reagent.

Since BIA and X3 were shown to co-elute, comparative structural analysis of these compounds was done. Structural information about BIA was either experimentally obtained with authentic BIA or cited from the reports of Lambein et al. (1969; 1976). The data was then compared to the experimental results of purified X3 analysis.

ULTRAVIOLET (UV) SPECTROSCOPY OF X3

A common characteristic of isoxazolin-5-ones is their strong UV absorption and their photosensitivity to short UV light. In an aqueous solution (pH 2.0) BIA has a high extinction coefficient, $\epsilon=12,200$ at $\lambda_{\max}=265$ nm (Lambein et al., 1969). Increasing the alkalinity of the solution to pH 10.0 caused a bathochromic shift of this spectrum to $\lambda_{\max}=267$ nm. A solution of BIA also underwent photolytic degradation when it was treated to successive periods of short UV light. This degradation was indicated by the disappearance of the original BIA UV spectrum upon irradiation.

Figure 13. Co-Chromatography of
 β -(Isoxazolin-5-one-2-yl)-Alanine
and X3.

Co-elution of β -(Isoxazolin-5-one-2-yl)-Alanine,
BIA (scan A; 35 nmol) and X3 (scan B; 30 nmol) was
shown by amino acid analysis of a mixture of the
substances (scan C; amount as in scans A and B).
Position of the scan peaks was relative to the
elution time of aspartate (-----; calibration run
before other analyses).

The UV spectrum of purified X3 (aqueous solution at pH 2.0) is shown in Figure 14. The compound has an absorption maximum, $\lambda_{\text{max}}=265$ nm which shifts to 267 nm when the solution is raised from pH 2.0 to 10.0. The extinction coefficient of X3 was calculated as 11,700. Another feature of the unknown X3 was its photodegradation by short UV light (254 nm). This photosensitivity is shown in Figure 15. The UV spectrum with X3 was significantly altered the treatment of UV irradiation over successive periods of time.

The UV spectra of BIA and X3 are similar as are their extinction coefficients although the estimated value for X3 is lower. The compounds do share identical features of sensitivity to treatments of short UV light exposure or pH changes. Furthermore these treatments of these compounds can be utilized to compare BIA and X3 in another manner, the identification of degradation products.

CHEMICAL DEGRADATION OF X3

BIA has been shown to be sensitive to treatments of chemical or photochemical hydrolysis (Lambein et al., 1976). After UV light exposure or acid hydrolysis (6N HCl; 100 °C) BIA undergoes water addition and ring cleavage to produce three identifiable compounds (Figure 16). BIA was also reported to be sensitive to alkaline pH (above pH 10) where degradation was rapid and irreversible; however breakdown products were not reported

Figure 14. Ultraviolet (UV) Spectra of X3.

The UV spectrum of X3 (pH 2.0) was obtained with an absorption maximum at 265 nm and extinction coefficient of 11,700. A bathochromic shift was observed when the pH of the X3 solution was raised to pH 10.

Figure 15. Photolytic Degradation of X3

The UV spectra of X3 (aqueous solution) were recorded during a sequence of short-wave UV irradiation ($\lambda=254$ nm) treatments of the sample. Time of irradiation (minutes) is indicated on the figure.

Figure 16. Hypothetical Scheme of
 β -(Isoxazolin-5-one-2-yl)-Alanine
Degradation.

(from Lambein et al., 1976)

B-(ISOXAZOLIN-5-ONE-2-YL)-ALANINE

HCl Hydrolysis
UV Light 254 nm

HYPOTHETICAL H₂O
ADDITION, PRODUCT

α-AMINO-
β-MALONYLAMINO-
PROPIONATE

α-AMINO-
β-ACETYLAMINO-
PROPIONATE

α,β-DIAMINO-
PROPIONATE

for this treatment.

Of the three final products in Figure 16, α,β -diaminopropionic acid (DPA) was readily available in pure form and was also detectable upon amino acid analysis. If BIA and X3 were identical substances, DPA should be a detectable degradation product of treatments of X3.

X3 was treated with short UV light or acid/base hydrolysis. The data is shown in Table 13. X3 is extremely sensitive to alkaline pH. After 10 minutes at pH 12, X3 had almost disappeared (91% degradation). This breakdown occurred without detection of any product (such as DPA) by amino acid analysis. Upon acid hydrolysis, X3 was completely degraded by the treatment and in experiments yielded 7-10% DPA as a recovered breakdown product. The last treatment was UV photolysis which gave only trace amounts of DPA upon analysis of reaction solutions.

INFRARED (IR) SPECTROSCOPY OF X3

Lambein et al. (1969) reported the IR spectrum of BIA where typical features of an amino acid were noted. Absorptions common to amino acid spectra were at 2075 cm^{-1} for the α -amino group and at 1710 cm^{-1} where the C=O stretch with a weak shoulder band was recorded for the carboxylic acid functionality.

Figure 17 shows the IR spectrum of X3 (1.5 mg sample)

Table 13. Degradative Treatments of X3.

Three different chemical treatments of X3 were performed and in each experiment the final reaction solution was analyzed for the presence of a known BIA degradation product α,β -Diaminopropionic Acid, DPA (Figure 16).

Treatment	Original X3 Concentration (total umol)	% of X3 Degradation	% of DPA Detected ^b
NaOH Hydrolysis (pH 12, 10 min)	1) 0.150	100%	ND ^a
	2) 0.987	91%	ND
HCl Hydrolysis (6N HCl, 100 °C)	1) 0.128	100%	7%
	2) 1.20	100%	10%
UV Photolysis (exposure to 254 nm UV light)	1) 3.17	86%	0.3%

ND^a- Not Detected

b-% of total degraded X3 detected as DPA

Figure 17A. Infrared Spectrum of X3,
(Range of 4000-1500 cm^{-1})

IR spectrum of X3 (1.5 mg) was done in a KBr disc.

Figure 17B. Infrared Spectrum of X3.
(Range of 1600-800 cm^{-1})

IR spectrum of X3 (1.5 mg) was done in a KBr disc.

Figure 18. Comparison of IR spectra for BIA and X3.

The IR spectrum of the unknown X3 (Figure 16., upper spectrum) was compared to the published one for β -(Isoxazolin-5-one-2-yl)-alanine (BIA, lower lower spectrum) from Lambein et al., 1969.

in a KBr disc. It exhibits several of the features seen in IR spectra of other amino acids. Broad absorption bands in the region of $2800-3500\text{ cm}^{-1}$ are $-\text{OH}$, $-\text{NH}$ stretches. A shallow band at 2000 cm^{-1} suggests absorption from the α -amino group. The stretches at 1700 and 1600 cm^{-1} are assigned as possible $\text{C}=\text{O}$ or $\text{C}=\text{C}$ bands of absorption.

In Figure 18, a comparison of BIA (lower spectrum) and X3 (upper spectrum) shows similarities in bands of key functional groups, however in the fingerprint region ($1600-900\text{ cm}^{-1}$) the compounds are not identical.

^1H -NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF X3

For this study high resolution proton nuclear magnetic resonance (NMR) spectroscopy was done to compare samples of BIA and X3. The NMR spectrum of BIA (Figure 19) corresponds to its published spectrum (Lambein *et al.*, 1969). However the vinylic protons did not show the splitting found characteristic in the NMR spectra of isoxazolin-5-ones as performed by Lambein *et al.* (1969) and Ván Rompuy *et al.* (1973). This feature was likely due to the greater strength of the experimental magnetic field at 200 MHz than those of the instruments used in the cited reports. The peaks of the vinylic protons were assigned as follows: $\delta 8.1$ (1H, singlet, C-3-H) and $\delta 5.1$ (1H, singlet, C-4-H) while the protons associated with the alanine side chain $\delta 4.1-4.2$ were not distinguished.

The NMR spectrum of X3 is illustrated in Figure 20

Figure 19. ^1H -NMR Spectrum of
 β -(Isoxazolin-5-one-2-yl)-Alanine.

A ^1H -NMR spectrum of β -(Isoxazolin-5-one-2-yl)-Alanine, BIA (15 mg sample) in D_2O was obtained at 200 MHz by Dr. G. Buchanan of Carleton University on a Varian XL200. Tetramethylsilane was used as an external standard.

Figure 20. ^1H -NMR Spectrum of X3.

A NMR spectrum of X3 (15 mg sample) in D_2O obtained at 200 MHz by Dr. G. Buchanan of Carleton University on a Varian XL200. Tetramethylsilane was used as an external standard.

There are no protons present downfield of the DHO exchange peak at δ 4.0 in this spectrum. This is a significant feature as it suggests that X3 does not have vinylic protons as found in the NMR spectrum of BIA. The protons of the alanine side chain are considered to be the signals at δ 3.2 (2H, doublet, $J=4.88$ cps, CH_2) and δ 2.9 (1H, triplet, $J=4.88$ cps, CH).

MASS SPECTROSCOPY (MS) OF X3

To confirm the identity of X3, mass spectroscopy was performed on BIA and X3. Three sets of spectra were obtained, each from a different instrument. In initial analyses, there was a difficulty in detecting the molecular ion. Another problem was the poor reproducibility of spectra from identical samples. Both BIA and X3 decompose at high temperature rather than cleanly volatilize and so detection of m/e fragments by direct probe analysis was made difficult.

In Figures 21 and 22, the molecular weights of BIA and X3 were always found to be 174 instead of 172 which is the calculated mass of the assigned BIA structure. In addition, m/e fragments were not consistent either between sample spectra (BIA versus X3) or duplicate spectra that were obtained on separate instruments (BIA, lower spectra Figures 21 and 22).

The last set of MS spectra (Figures 23-25) were obtained through Dr. L. Weiler of the University of

Figure 21. Mass Spectra of BIA and X3.

Mass spectra were obtained of
 β -(Isoxazolin-5-one-2-yl)-alanine, BIA
(lower spectrum) and X3 (upper spectrum)
by Dr. J. Krauss, University of Ottawa
on an AEI.MS9205 at 60 ev/150-180 °C.

Figure 22. Mass Spectra of BIA and X3.

Mass spectra were obtained of β -(Isoxazolin-5-one-2-yl)-alanine, BIA (lower spectrum) and X3 (upper spectrum) by Judy Lockwood of Environment Canada on a Finnegan 4000 or 4500 at 60 ev/150-180 °C.

Relative Intensity
(Expanded 5X)

Figure 23. Mass Spectrum of
 β -(Isoxazolin-5-one-2-yl)-Alanine.

The mass spectrum of β -(Isoxazolin-5-one-2-yl)-alanine, BIA was obtained by
Dr. L. Weiler, University of British Columbia
on an AEI.MS-9 or MS-50 at 70 ev/150-180 °C.

Relative
Intensity

Figure 24. Mass Spectrum of X3 (Sample 1).

The mass spectrum of X3 was obtained
by Dr. L. Weiler, University of British
Columbia on an AEI.MS-9 or MS-50
at 70ev/150-180 °C.

Relative
Intensity

Figure 25. Mass Spectrum of X3 (Sample 2).

The mass spectrum of X3 was obtained
by Dr. L. Weiler, University of British
Columbia on an AEI. MS-9 or MS-50
at 70ev/150-180 °C.

Relative
Intensity

British Columbia. Weiler and co-workers had reported isoxazolin-5-one mass spectral data with respect to their work, carbanion chemistry (Tischler and Weiler, 1981). It was thought that their experience in handling isoxazolin-5-one derivatives for mass spectral analysis would help to resolve the problems which were encountered by those who performed the previously described spectra. The three samples of BIA (Figure 23) and X3 (Figures 24 and 25) show greater similarity in their patterns of mass fragments than found in the other figures. The spectra of X3 were taken from two different X3 isolation experiments. The difference in the spectra is attributed to a variable degree of purity achieved in X3 separation. Major mass fragments of both compounds are as follows: m/e 172 (molecular ion), 160, 147/148/149 (possible loss of $-C_2H_4$, $-HCN$ or $-CN$), 135 (loss of $-CH_2$ from 149), 128 (loss of CO_2 from 172), 121 (possible loss of $-CH-NH_2$ from 172) 107 (possible loss of $-C_2H_4$ from 135) and 83 (loss of $-COOH$ from 128).

In addition to low resolution mass spectroscopy, the exact mass of BIA and X3 were obtained through high resolution analysis (Table 14.) BIA and X3 (two different purified lots of the unknown) had doublet molecular ions detected at 172. One exact molecular weight corresponds to the calculated value for the molecular formula assigned BIA. The ratio of the doublet was found to change with time which suggests that a mixture of substances is

Table 14. High Resolution Mass Spectroscopy of
 β -(Isoxazolin-5-one-2-yl)-Alanine, BIA and X3.

Analyses of BIA and X3 were performed by Dr L. Weiler,
 University of British Columbia using an Atlas CH-4B
 AEI.MS-9 or MS-50 (operated at 70ev potential).

Experimental Exact Mass Data

BIA (Lambein)	X3 (Sample 1)	X3 (Sample 2)
172.0480	172.0485	172.0485
172.0890	172.0888	172.0888
174.1043	174.1043	174.1038
174.0609		

Calculated Molecular Weights

Note: Initially m/e 172 (doublet) appears;
 m/e 174 appears a few seconds later.

present (Weiler, personal communication). The additional molecular fragment at 174 appears a few seconds after the detection of the doublet in each analyzed sample. This suggests that in early low resolution spectra the respective instrument sensitivity was insufficient to detect the actual molecular weight as compared to the peak at 174. The high resolution mass spectra suggest that BIA and X3 are the same compounds on the basis of molecular formula and mass fragmentation pattern.

DISCUSSION

X3, AN UNCOMMON AMINO ACID

X3 was first detected because of its positive reaction with ninhydrin (Figure 3). This property specifies its α -amino acid functionality. In addition, X3 proved chromatographically distinct from standard protein amino acids and their biosynthetic intermediates (Bauer et al., 1977a). The unknown also appears to be of limited distribution and was found only in peas. Other plant material including tobacco, soybean and sunflower (Joy, unpublished data) did not have detectable levels of X3 when amino acid analyses were done. The variety of pea used initially was Little Marvel; two other varieties (Alaska, Tall Telephone) were analyzed for the presence of X3 which was found in both cultivars (Appendix 2).

The above features suggest that X3 can be categorized as an "uncommon amino acid" (see Introduction). The data for the unknown fits the criteria for these types of substances (Bell, 1976; 1908b). Further investigation was undertaken on this basis.

OCCURENCE AND DISTRIBUTION OF X3

The first plant part examined was the germinating pea seed. Many uncommon amino acids occur primarily in the seed. Examples include 5-hydroxytryptophan in Griffona (Bell et al., 1976) or canavine in Dioclea (Rosenthal et

al., 1976). Both compounds constitute a major proportion of the dry weight of the seed, 14% and 13% respectively.

In pea seeds the major free amino acid component is homoserine, an uncommon amino acid. Although an intermediate in the biosynthesis of threonine or methionine, homoserine is not normally detected in plants. Pisum and a few other legumes show high levels of this compound (Lawrence, 1973; Larson and Beevers, 1965; Lawrence and Grant, 1963). In Figure 5, homoserine is the predominant amino acid during seed germination; absent in the dry seed, its levels increase dramatically in 5 days. X3 has a similar pattern of accumulation (Figure 4.) from initial undetected levels in the dry seed. The variation of the amount of X3 was similar to the pattern of common amino acids such as asparatate and alanine (Figure 5.). At its peak the pool of X3 constituted 3.5% of the predominant amino acids present (those shown in Figure 3. plus glycine and alanine). Other amino acids (emerging later in amino acid analysis) were not included in this calculation; these constitute a small percentage of the total free amino acid pool found in the germinating pea seed (Van Egeraat, 1975).

From seed germination data, two points may be made. First, X3 was formed by de novo synthesis in the germinating seed. This was previously obvious from the concentration pattern of X3 levels during seed germination

as considerable amounts of X3 appeared after a two day period when it was undetectable. Clearly, to account for the lag in production of X3, a metabolic requirement for release of a precursor or formation of biosynthetic enzymes may occur during the first two days of germination.

The second point is the significant amounts of X3 which were present in the seed. Although not as abundant in the seed as homoserine, X3 had comparable levels to aspartate and alanine throughout germination. Like the other amino acids, X3 has a peak in accumulation (8 days) followed by a rapid decline in amount. To account for this change, it is suggested that degradation or mobilization of X3 may increase. Mobilization may be demonstrated by investigating transport and X3 levels in the developing plant.

After establishing that a metabolic pool of X3 was formed in the seed, a range of plant developmental stages were sampled to determine whether there are sites of accumulation or biosynthesis in other parts of the whole plant (Table 1). The roots of the pea plant maintained a stable level of X3 during growth until maturity when X3 concentration decreased. Although the root system is expanding (fresh weight increases), the X3 pool is constant in net level. In fact, the proportion of total X3 in the roots decreases as the plant matures. This is

also true of the percentage of X3 amounts found in the cotyledons. Root and cotyledon pools of X3 are thus at a maximum when synthesis exceeds turnover early in the development of the pea plant.

Only in the whole shoot did X3 continue to accumulate throughout development. The shoot levels of X3 increased from 6 to 20 days growth especially in the growing leaf tissue. The main pools of X3 were in the leaves designated the growing leaf (Leaf 5, Figure 6.) and the maturing leaf immediately below it. Older leaves showed that a loss of X3 was occurring upon leaf maturity.

The old leaf of the 14-day-old plant corresponds to leaf 1 of the mature 20-day plant. The concentration of X3 recovered in the 20-day-old leaf was only 2.2% of the original level detected at 14 days of plant development. Similarly leaf 2 decreased to 6.7% of the original amount during the growth period between 14 to 20 days. This pattern of accumulation and decline of X3 suggests a metabolic relationship of X3 levels to growth development in plant organs. X3 accumulates in all tissues but primarily at sites where growth is active. Upon maturity, X3 turnover either exceeds biosynthesis or the compound is exported from mature tissue to new sites.

The trend for X3 in growing leaves is repeated in the developing pea fruit (Table 2) When the young seed is receiving materials from the mature flowering plant, the

seed X3 level was at a peak (14 day stage). The pod also peaks in X3 amounts at 14 days. It contains the greater proportion of X3 in the fruit (maximum 71.5%). However as the seed and pod metabolisms decline with maturity, X3 again disappears. In this case, degradative turnover is the probable route of X3 decline since it is unlikely that X3 would be re-exported from the mature fruit.

The pattern of X3 accumulation suggests that the unknown was present in all plant organs while the major pools of X3 were maintained at sites of active growth throughout plant development. To account for the decrease of X3 concentration in mature leaves, it is necessary to distinguish whether increased metabolic degradation or export of the substance was responsible. If it is the latter, this increased export of X3 may also account for the amounts which are found in the growing leaves, a metabolic sink for materials transported in the phloem. The transport of X3 and its relation to distribution patterns is discussed below.

TRANSPORT OF X3

From the data on X3 distribution, it was apparent that the immature leaf tissues of the pea plant were the major sites for X3 accumulation. Xylem sap and phloem exudate analyses were performed to establish whether X3 was received by transport of the substance into these

major X3 sinks.

To study the transport of X3, the two techniques of Joy and co-workers were employed. First xylem bleeding sap was used to determine root to shoot movement of substances (Bauer et al., 1977a) while a method of EDTA-promoted phloem exudate analysis was utilized to examine export levels of amino acid compounds from mature leaves (Urquhart and Joy, 1981). The data obtained by these methods estimate the flow of amino acids between plant organs. There are however limitations to the techniques. Disparity in xylem levels of amino acids was primarily due to the fluctuation of water content where change in water flow through the plants varied the amount of substances passed along with it. This degree of water flowthrough varied with time. Volumes of xylem sap were greater during sampling early in the light period (9 a.m. sampling) while at the end of light period, bleeding was less profuse with a greater number of detopped roots unable to exude xylem sap for a prolonged time.

The technique of EDTA-promoted phloem exudation was compared by Urquhart (1980) to other methods of analyses including aphid stylet obtained phloem sap. This EDTA enhanced exudation was originally developed by King and Zeervart (1974) and Urquhart adapted the method for use with detached mature pea leaves. The results of that investigation suggested that EDTA treated samples improved

the recovery of translocated nitrogen metabolites. Although the phloem export system is disturbed by the experiments the technique was considered useful for studying transport of amino acids from leaves. For the experiments reported here, there is variation in level of material exported into the phloem of leaves from replicate experiments. A factor in the estimation of X3 was the disturbance of aspartate and X3 separation upon amino acid analysis. The EDTA remaining in the analyzed solution apparently disturbed the normal analyzer retention of these two substances and overlapping of their elution times may cause an underestimation of their individual amounts. Variation of the total content of amino acids was noted amongsst the replicates and this suggests caution must be used in interpretation of phloem exudate analysis.

Both xylem sap and phloem exudate analyses can be obtained at various stages of plant development. With awareness of their limitations as described above, they can be useful for examining the mobility of particular amino compounds within the plant and to trace the pattern of overall movement with respect to growth.

The balance of import and export of metabolites is important in the growing plant. Webb and Gorham (1964) have demonstrated this relationship of transport pathways in expanding squash leaves. They examined flow of

carbohydrates and found an import/export system to occur within the same maturing leaf. Movement of nitrogenous compounds in pea plants was extensively studied by Urquhart (1980). Urquhart demonstrated that growing leaves (youngest leaf which also encloses the shoot apex) weakly transpire as their leaflets expand. The major proportion of the transpiration stream is received by mature leaves, however these leaves maintain a stable nitrogen level after full expansion (for a three day period). Therefore upon maturity leaves must export material even as they receive metabolites from the plant transpiration stream. Mature leaves were shown to contribute a considerable amount of the required nitrogen for growing leaves by export through the phloem (Urquhart, 1980). Immature pea leaves appeared to obtain the balance of their nitrogenous materials via the phloem. In turn, mature leaves received metabolites from the xylem transpiration stream and may redistribute these to the growing tissue along with products metabolized in the mature leaves themselves. Two studies of Urquhart (1980) are described to explain this phenomenon. First, in ^{14}C -asparagine tracer studies, it was shown that asparagine received by mature leaves via transpiration was re-exported without metabolism. In the other study, ^{14}C -glutamate tracer was taken up by mature leaves. Its carbon redistributed and the label taken up by growing

leaves in different amino forms. Thus these two cases suggest that movement of metabolites may be followed by considering transport analyses.

In xylem sap (Figure 7.), X3 is not a major component as compared to asparagine or homoserine. Its proportion of the xylem amino acid content appears to decline with the maturity of the plant (Table 3). X3 transported out of the roots is ca. 100 nmol per day as estimated from the average of the range of levels (0.82-3.84 nmol per root in 30 minutes) found in the pea xylem sap from 14 to 20 days of growth. The decrease in the level of root X3 may be accounted for in part by the movement of X3 out of the roots via transpiration.

X3 is detectable as a metabolite translocated from mature leaves to growing or young tissue. As a proportion of phloem amino acids, the X3 level is higher when the plant is young than when it is mature. Phloem exudate analysis therefore suggests that X3 may be transported from older leaves and the accumulation of X3 in growing leaves may result from import as well as a potential contribution by biosynthesis.

At the 14-days growth stage, X3 is moved not only from root to shoot but also in the phloem from its older leaves to the growing shoot apex. In the 14-day old plant, the mature leaf immediately below the growing leaf contained the greatest amount X3 in the plant. The mature

leaf was just fully expanded. It would receive the greatest proportion of the xylem sap due to its leaf size (the largest amongst the emerged leaves) and probably increased transpiration rate upon expansion. This leaf would also be changing in its metabolism as it reaches the mature state. As in older leaves which have a stable level of nitrogen flow out (i.e. become net exporters of metabolites) after its maximum growth this leaf must now adjust its use and export of incoming nitrogen materials. Therefore at 14-days growth in the mature leaf the net accumulation of X3 reflects the idea that most of the xylem imported X3 remained in this leaf as the amount re-exported in the phloem does not yet balance with that received by transpiration. This distribution of X3 contrasts with the pattern at 20-days growth. In this plant there was a greater number of leaves that must share the stream of transpiration from the root. The level of X3 found in the mature leaf immediately below the growing leaf was lower. The balance of X3 imported was decreased in proportion to the amount of xylem sap that may be received by this individual leaf. In addition there are more individual mature leaves which now export material to growing or developing tissue. Hence phloem translocation of X3 was greater than at 14 days. The growing leaf of the 20-day plant is thus the major site of X3 accumulation.

METABOLISM OF X3 - ^{14}C INCORPORATION STUDIES

^{14}C -labelling studies were carried out to try to identify potential precursors for X3, in the hope that this would also provide information about the biosynthetic route for this compound. Table 5 lists a number of common metabolites which potentially could have contributed their carbon skeletons to X3. In most cases, there was no significant (i.e. above background levels) incorporation of tracer into X3. Only alanine and pyruvate showed significant ^{14}C -incorporation into the unknown (Figure 8). This flow of carbon label from alanine/pyruvate to X3 was demonstrated not only in the whole shoot but in the seed (a specific site where biosynthesis is known to occur, Figure 9) and also at the main site of accumulation, the growing leaf (Tables 8,9,10). Using ^{14}C -alanine or ^{14}C -pyruvate (Figure 10) as a precursor for X3 formation in leaf tissue, it was demonstrated that X3 could be synthesized in the shoot and not just accumulated upon transfer from other plant parts. This evidence of tracer incorporation into shoot X3 suggests that the germinating seed is not the only site of synthesis.

Alanine/pyruvate tracers were initially used to indicate where biosynthesis of X3 may occur. To further clarify the role of these compounds in X3 formation, it was necessary to distinguish whether the amino acid or its keto acid analogue were both precursors of X3 or if the

incorporation was limited to a step involving only one of the compounds with an indirect flow of label from the second by means of a transamination to produce the precursor. To separate, the potential biosynthetic steps, a transamination inhibitor was used to reduce flow of label between alanine and pyruvate. The flow of carbon from pyruvate and alanine into X3 in control systems was compared with a system in which transamination was inhibited by aminooxyacetate. The elimination of ^{14}C from pyruvate incorporation by this inhibition suggested that pyruvate is initially transaminated before X3 labelling can occur (Table 10). Equilibration of label into alanine by transamination occurs quite rapidly even during short feeding periods (Table 8). Alanine would appear to be a direct precursor into X3 as incorporation was still maintained with the transaminase inhibitor present (Table 9). The inhibitor experiments also suggested that the biosynthetic intermediate steps from alanine to X3 did not involve transamination since incorporation from each precursor was not equally affected by the blocking reaction of that type.

Bell (1980b) described four types of pathways for uncommon amino acid biosynthesis (see Introduction). In this study, the survey of precursors into X3 gave insufficient evidence to ascertain which type is involved in X3 biosynthesis. The complexity of X3 structure (to be

discussed later) suggested that it is unlikely to be a modification of a common amino acid. Otherwise it is impossible to distinguish between types of pathways of synthesis.

^{14}C -labelling into X3 provided useful biosynthetic information. To determine the degree of X3 turnover into other compounds, labelled X3 should be fed and traced in plant tissue. Unfortunately attempts to produce ^{14}C -X3 of high specific activity were not successful (Table 11).

$^{14}\text{CO}_2$ fixation was used to attempt to label X3 because a high level of radioactivity was readily introduced to the plants using $^{14}\text{CO}_2$ (100 μCi). A long post-fixation chase was allowed to occur in order that ^{14}C -label from alanine or other undetermined X3 precursors be incorporate into the unknown. Label went predominantly into protein (ethanol-insoluble material) and free carbohydrates (ethanol-soluble neutral components). This distribution of tracer was expected since experimental tissue was young and growth requires these primary metabolites for development, however indirect flow of labelled carbon into X3 was considered to be possible from alanine, a demonstrated precursor. The results of $^{14}\text{CO}_2$ fixation suggest that X3 as a secondary plant metabolite does not rapidly incorporate label or the precursor(s) are imported from the root, which would be less rapidly labelled. Those compounds which were rapidly labelled including

serine, glycine and glutamate (data not shown), are clearly not precursors for X3 (Table 5). Thus yield of ^{14}C -X3 was low in comparison to the concentration of X3 present in the tissue. Specific activity was too low to be useful in subsequent tracer experiments and was insufficient to be detected after redistribution in the various plant parts.

^{14}C -X3 would have been valuable as a metabolic tracer where it could be used as other primary amino acids have been in other studies (Bauer *et al.*, 1977a; Urquhart, 1980). By studying the flow of ^{14}C -X3 carbon, the functional role of X3 and its pattern of turnover (metabolic activity) may be determined in the developing pea plant.

To produce ^{14}C -X3, an improved method of labelling must be used. Although the attempts of this study show inadequate metabolism into X3, sufficient label may trace to the compound if extended post-fixation growth and development was allowed. Labelled plants may upon additional time for growth accumulate labelled X3. Greater direct incorporation into X3 may also be achieved by utilizing alanine/pyruvate as the ^{14}C -precursors for the biosynthesis of X3 tracer. The recovery of labelled X3 was greater with these tracers than by fixation however X3 purification requires considerable amounts of plant material for isolation. On such a large scale, use of

high specific activity alanine/pyruvate may be prohibitive in expense and availability of the initial amount of labelled precursor to be fed to the plants. To develop a source of ^{14}C -X3 it seems likely that further investigation of the method by $^{14}\text{CO}_2$ fixation is most practical.

STRUCTURAL ANALYSIS OF X3

In reviewing the literature, it was suggested from initial experimental properties of X3 that this unknown could be β -(isoxazolin-5-one-2-yl)-alanine (BIA). Comparison of X3 and BIA were thus made in this study.

The results of initial experiments showed that X3 and BIA were identical. First, the substances co-eluted upon chromatographic separation (Figure 13) UV spectroscopy of these compounds produced spectra with similar extinction coefficients and absorption maxima (Figure 14). UV spectroscopy also indicated a sensitivity of X3 to short UV light (254 nm) which had previously been reported for BIA (Figure 16) This lead to the analysis for a specific BIA degradation product, α,β -diaminopropionic acid (DPA) in solutions of X3 after chemical treatments (Table 13) There was difficulty in the UV treatment where degradation was less than complete however acid hydrolysis or UV treatment did produce detectable levels of DPA of similar amounts which were reported for BIA (Lambein et al.,

1969). These three studies strongly suggested that BIA and X3 were the same compound.

On the other hand, mass spectroscopy of BIA and X3 (to be discussed later) raised a question of doubt as to the accuracy of the BIA assignment of structure by Lambein et al. (1969). Part of the structure had been suggested by the detection of α,β -diaminopropionic acid upon chemical treatment of these substances. To further clarify structural features IR and NMR spectroscopy were performed.

Comparison of BIA and X3 IR spectra showed agreement of the absorptions of these compounds however, the fingerprint region which is characteristic of a particular substance was not identical in the two spectra. Strong bands at ca. 1700 and 1600 cm^{-1} indicate the conjugated isoxazolin-5-one ring structure which accounts for the strong UV absorption spectra. Lambein et al. (1969) and Van Rompuy et al. (1973) reported for natural BIA and synthetic isoxazolin-5-ones respectively, bands (C-4-H, C-3-H) in the range of 3000-3200 cm^{-1} . These bands associated with unsubstituted 3,4-isoxazolin-5-ones were not clearly evident in the X3 spectrum. This suggests that the C=C positions have substitutions rather than protons on this part of the ring.

More critically, high resolution ^1H -NMR spectroscopy also showed significant differences between BIA and X3.

The BIA spectrum (Figure 19) was identical to that previously reported by Lambein et al. (1969). Olefinic protons were distinguished at $\delta 8.1$ and $\delta 5.1$ (Figure 19.) while the other peaks were associated with the C-H, C-H₂ of the alanine side chain. In the X3 spectra, there were no signals above the DHO exchange peak (Figure 20). This suggested that there were no olefinic protons which contradicts the study evidence previously discussed. The evidence for the DPA fragment suggested that the substitution of the alanine side chain is the same in BIA and X3. The X3 spectra does show that its structure could include the alanine moiety by the clear triplet and doublet signals below $\delta 5.0$ ($J=4.88$ cps).

To conclusively assign the structure of X3, mass spectroscopy was performed on BIA and X3. Three sets of data are included in this study as it was found that mass spectral data were not reproducible. In early analysis by low resolution mass spectroscopy, the detectable molecular weight was consistently found to be 174 instead of 172 which is the expected formula weight for the given BIA structure (Figures 21 and 22). Prevalent mass fragments in the first set of spectra were at m/e 148, 120/121, 104/105 while in the next pair of analyses m/e 160 and 135 were detected in addition to peaks at 149, 119 and 107. Because the mass spectra were not similar between instruments, the significance of these results

were the critical difference of molecular weight and the difficulty that was encountered in detecting some fragments. The mass spectral data raised a question of accuracy of the BIA structure assignment by Lambein et al. (1969).

The last set of mass spectra however showed agreement in molecular weight (Figures 23 and 24) and pattern of fragmentation between BIA and X3 of the first sample. The second X3 sample did not give as clearly a corresponding spectrum as the first (Figure 25). From all the sets of data, it is evident that problems in the mass spectroscopy of these compounds has to do with the purity of the samples and the general behavior of isoxazolin-5-ones to this type of analysis.

Previous reports of mass spectral analysis of isoxazolin-5-ones give no indication of the difficulty in obtaining these results (Tischler and Weiler, 1981; Van Rompuy et al., 1973). Lambein (personal communication) found that BIA gave a double molecular weight upon mass spectral analysis and so he proceeded with a derivation of BIA to compare natural and derivitized BIA spectral properties (Lambein et al., 1969). The reported melting point of BIA is given as a decomposition range. For direct probe mass spectral analysis, the fact that BIA does not cleanly volatilize may account for the difficulty in detecting fragments where instrument sensitivity cannot

compensate for the poor degradation of the isoxazolin-5-one. This factor also applies to the analysis of X3 which also has a decomposition range (200-205°C) instead of a melting point. Perhaps for BIA and X3 formation of a derivative more suitable to mass spectroscopy may be the best solution to overcome the difficulties encountered in the present study.

The other problem of X3 structural analysis is the question of sample purity. For X3 the differences in all mass spectra can in part be accounted for by the varying degree of purity achieved in each batch of isolated X3 sampled. The possible contaminants of X3 are unknown. The isolation procedure should separate X3 from other amino acids as well as from free neutral and acidic components of plant extracts. To improve purity another chromatographic step may have to be employed. It is possible that thin layer chromatography of the recrystallized X3 may increase the purity of X3 obtained. To assess X3 purity another method of contamination detection must be used other than the one utilized in this study. Amino acid analysis was routinely done to check X3, however this only detects ninhydrin positive compounds. Possibly an high performance liquid chromatographic method could be developed to assess impurities. Detection of X3 could be made by its UV absorption at 265 nm.

Table 15 summarizes the structural data for BIA and X3.

Table 15. Summary of BIA and X3 Structural Analysis

	<u>BIA</u>	<u>X3</u>
Amino Acid Chromatography Elution Time (min)	35-37	35-37
Ultraviolet Spectroscopy		
Extinction Coefficient	12,200	11,700
Absorption Maximum	265 nm	265 nm
Infrared Spectroscopy (cm ⁻¹)		
-OH absorption	3100-3500	3100-3500
C-3-H absorption	3060-3080	Not Present
C-4-H absorption	3120-3150	Not Present
C=C absorption	ca.1550	1600
C=O absorption	1710	1700
¹ H-Nuclear Magnetic Resonance Spectroscopy (ppm)		
C-3-H	8.10	Not Present
C-4-H	5.10	Not Present
CH	4.20	3.30
CH	4.10	2.80
High Resolution Mass Spectroscopy	172.0480	172.0485
	172.0890	172.0888
Exact Mass Numbers	174.1043	174.1043

The presented evidence suggests that BIA and X3 are the same substance however some differences in spectral analyses obviously raise questions as to their proposed structure. Initially, it was thought that the isoxazolin-5-one ring may have opened up to change the detected molecular weight from 172 to 174. A similar substance, 3-isoxazolidone (Figure 26A) has been demonstrated to open or close its ring dependant upon pH conditions (Sugi et al., 1981). The high resolution mass spectral data however discounted this possibility as the 174 fragment has a molecular formula without nitrogen and so could not include the isoxazolin-5-one ring. Another possible explanation for the confusing NMR and mass spectral results is the potential internal cyclization of the alanine side chain to the isoxazolin-5-one ring (Figure 26B). In the first proposal, the compound would likely undergo water addition as previously proposed in Figure 16, however instead of ring cleavage, the side chain may cyclize at either the α -amino or carboxylic acid position. In these structures, chemical degradation would yield the same breakdown product, DPA and the molecular weight of X3 would remain 172. The problem with such structures is that the strong UV absorbancy of X3 should be eliminated by its loss of double bond conjugation. The NMR spectrum should have also made evident the additional protons at C-3 and C-4 positions. Both results were not

Figure 26. Hypothetical Schemes for X3 Structure.

(A. from Sugi et al., 1981)

A. Isoxazolidone ring opening.

B. Possible forms of X3.

the case; therefore these structures are not totally satisfactory. Another structural possibility would still be cyclization of the side chain with retention of the double bond. For the X3 NMR spectrum, there should be an olefinic proton peak for the C-4 position but it may be obscured by the DHO exchange peak if rearrangement of the alanine moiety changed the effect of the NMR magnetic field on this signal found at δ 5.1 in BIA. This formation of a double ring structure for X3 would conveniently account for the discrepancies of the different spectral analyses, but then the mass spectral molecular weight would not be 172. It would seem that regardless of the possible changes made to the proposed BIA structure, the data of X3 experiments will not all correspond to subsequent analysis.

In this study, the structural analysis of X3 has raised some interesting question with respect to isoxazolin-5-one chemistry. BIA and X3 appear to be the same compound. To proceed to clarify the forms of this substance two procedures are suggested. First the purity of X3 must be improved to give a consistent analysis by NMR and mass spectroscopy. Subsequent chemical analysis of X3 may then be possible without the problems of assessing potential contaminant behavior in these treatments. Chemical analysis would mean further treatments of X3 as an isoxazolin-5-one compound to see if

the substance is internally cyclizing. For example BIA has been derivitized to an N-acetylated compound where the substitution was on the amino group of the alanine moiety (Lambein et al., 1969). If X3 was treated in this manner, the ease of formation of the BIA derivative would suggest whether the nitrogen of the amino group was involved in a bridge to the C-3 position.

With a cleaner X3 preparation, continued structural analysis should also be attempted. ^{13}C -NMR spectra of BIA and X3 may help to elucidate their form as the carbons of the assigned BIA structure are individually distinct. A preliminary attempt at obtaining a ^{13}C -spectra of X3 was made (data not shown) however the amount of X3 in the sample was insufficient to give good peak resolution. This form of analysis is however promising in potentially providing information as to the behavior of the side chain and the ring.

BIOLOGICAL ASPECTS OF BIA AND X3

Structural determination of X3 suggests that it is the same substance as BIA, the isoxazolin-5-one derivative found in legumes by Lambein and his co-workers (for review, Lambein et al., 1976). Physiological similarities of X3 and BIA are briefly discussed here.

With respect to BIA occurrence, Lambein found that isoxazolin-5-ones were detectable in a variety of Lathyrus

and Pisum species as well as in Lens culinaris and Vicia sativa. The maximum concentration of BIA was found after 6-8 days of germination on a dry weight basis while the maximum level was 3 μmol per seedling (without cotyledons and 2 μmol in a pair of cotyledons. Comparing green and etiolated seedlings, no differences in BIA levels were noted which suggested that BIA biosynthesis did not involve or require photosynthetic activity. Possible formation of BIA occurs with the breakdown of seed protein (globulins) carbon and nitrogen.

This distribution information of BIA is similar to the data of X3 levels where both substances show the same trends in occurrence during germination. Varietal differences can account for the fact that BIA and X3 quantities are not identical as even among cultivars of Pisum sativum the levels are not the same. Etiolated pea seedlings were also examined for X3 accumulation (see Appendix 3.) and it was found that even in the dark the levels of X3 were similar to those of data in Table 1. This feature suggests that the metabolism of plant X3 may not be affected by changes in photosynthetic activity. The concentration of X3 ($\mu\text{mol/g}$ fresh weight) was highest in the elongated internodes of the shoot which may mean that X3 levels were high in the tissue xylem sap or that X3 may have been stored in this part of the plant as the normal site (growing leaves) are underdeveloped. X3 is

thus present in the etiolated tissue in identical levels as the normal photosynthetic plant. This feature of X3 in the etiolated plants may be useful for the consideration of X3 isolation. The etiolated seedling does not have normal metabolism and the number of potential contaminants of purified X3 may be reduced from use of this type of starting material.

With respect to BIA biosynthesis, the groups headed by Lambein and Murakoshi have made extensive investigations. Among the ^{14}C -precursors tested for BIA incorporation, labelled alanine/pyruvate have been traced into the isoxazolin-5-one. Other organic acids including malonate, succinate and malate have been shown to have greater specific activity found in BIA (Kuo *et al.*, 1981a; Kuo and Lambein, 1981). Murakoshi and co-workers have found that O-acetylserine (OAS) to be a BIA precursor. The isoxazolin-5-one derivative seems to be formed by a common side chain transfer (alanine) to the free ring via OAS. With X3, studies (data not shown) were attempted to synthesize increased levels of X3 by incubating crude seed or leaf extracts with a high concentration of OAS. However it was obvious that a labelled OAS source was required as no net X3 production was observed. Tracer studies using ^{14}C -OAS would clearly demonstrate if X3 received its alanine moiety via OAS transfer through the specific detection of labelled DPA. Besides the work as

described here (also see Introduction), little information has been reported about the origin of the isoxazolin-5-one ring. Tracer studies have suggested the possible precursors of the ring. Detection of labelled DPA is used to infer whether the ring may or may not contain labelling. For example the total radioactivity of BIA can be separated into the levels found in DPA and the carbon of the ring where the difference of percentages is taken as a measure of site with the greater level of incorporation. This type of structural information was not done in X3 labelling experiments. The amount of plant material used in the experiments was insufficient to extract and subsequently produce a reasonable (detectable) concentration of DPA by which to estimate radioactivity. DPA is only 7-10% of the produced degradation products. To pursue the carbon site of precursor incorporation labelling experiments of X3 must be done on a larger scale such that sufficient material can be processed for X3 isolation and chemical treatment to produce DPA.

Transport of BIA has not been previously reported although it would be useful to study its movement in the species of legumes studied by Lambein. Identifying the mobility of the substance and its catabolism would give perspective to the manner of distribution of BIA and its potential role within the plant.

CONCLUSIONS

X3 was an unusual nitrogen metabolite found in pea. Initially its occurrence was determined in pea leaf extracts, however X3 was found throughout the plant upon examination of its distribution. The major proportion of X3 was accumulated in the whole shoot especially the growing leaves. Throughout development the unknown was readily transported not only from root to shoot via transpiration, but it was also exported by mature leaves which translocated it by the phloem to active metabolic sinks. The biosynthesis of X3 was demonstrated in seeds and growing leaves by labelling studies where alanine and pyruvate were found to be possible precursors as they showed significant ^{14}C -incorporation into X3.

In addition to the metabolic study of X3, structural analysis was done to identify the unknown. Spectroscopic data suggested that X3 was a member of a class of heterocyclic compounds, isoxazolin-5-ones. It appeared to be a specific substance, β -(isoxazolin-5-one-2-yl)-alanine, BIA. This conclusion was made on the basis of UV spectroscopy, results of X3 chemical degradation and especially the mass spectral data. Mass spectroscopy assigned BIA and X3 exact molecular weights of 172 which suggested identical molecular formulae while their fragmentation patterns were also similar. The ^1H -NMR and IR were the two analyses which did not indicate that BIA

and X3 were the same compounds. BIA was shown to have two olefinic protons by NMR spectroscopy, however these signals were absent in the X3 results. IR spectra were also not comparable in the fingerprint region. Since all evidence did not indicate that BIA and X3 were identical, further work is suggested to clarify what structural differences account for the changes in spectral properties.

The structural analysis of X3 indicated that it was likely the compound BIA. Comparison of their biological properties was then made and it was found that the two compounds are similar in their trends and levels of accumulation in plants. Alanine and pyruvate were also shown to be precursors for both compounds although more extensive work has been done with organic acid precursors in the reports of BIA biosynthesis. Thus it would appear from the metabolic and structural study of the compound X3 that this unknown is the substance previously reported as BIA.

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SUMMARY

1. X3 was initially detected in pea leaf extracts. It was not identified as any standard amino compound upon comparison with known substances by amino acid analysis. It was also not evident in a variety of other types of plants such as tobacco or sunflower.

2. The occurrence of X3 was first studied in the cotyledons during seed germination. Absent in the dry seed, X3 was newly synthesized after two days of imbibition which suggested the possible release of biosynthetic enzymes or precursors was required for its formation. X3 levels in the seed peaked at 8 days germination (1.20 μmol per seed) and was comparable in its trend of accumulation to alanine, aspartate and asparagine. It composed a minor percentage (ca. 3.5%) of the total free amino acid pool of the seed during germination.

3. X3 was found to be distributed amongst all other parts of the pea plant. The whole shoot accumulated the major proportion of X3 especially in the growing leaves where a maximum level of 1.43 μmol per leaf (38.8% of total plant X3) was reached after 20-days growth. In addition, a decrease of X3 levels appeared to be the trend in fully-expanded leaves as they become mature. The cotyledons and roots peak in X3 accumulation after 6-8

days of growth. Small amounts of the unknown were also detected in the seed (maximum 0.25 μmol per seed) and pod (maximum 0.63 per pod) of developing pea fruit.

4. The unknown appeared to be readily transported as indicated by xylem sap and phloem exudate analyses. In both studies it was found that X3 composed 3-5% of the pool of translocated amino acids when the plants were 14-days old. This proportion decreased as the plant matured (1-3%) as indicated by sampling times at 17 and 20-days. In the xylem sap the major components were asparagine, aspartate, glutamine and homoserine at all stages of development sampled. In the transpiration stream, the maximum detected level of X3 was 3.25 nmol per root during a 30 minute sampling period. The EDTA-promoted method of phloem exudate analysis was used to look at amino acids translocated from the mature leaves. The major components of this exudate were also found to be asparagine, aspartate, glutamate and homoserine although their relative proportions differ from those found in the xylem sap. The sampling period was for two hours, in replicates of exudate treatments it was found that the maximum level of X3 detected was 0.624 nmol per detached leaf (14-day old plants).

5. ^{14}C -labelling studies were done with a number of amino and organic acid tracers which were thought to be

potential precursors of X3. Of those tracers, significant levels of incorporation were found only with labelled alanine or pyruvate where 2-3% of the total radioactivity recovered was detected in X3. Labelling studies were also performed with a transamination inhibitor, aminooxyacetate to determine if incorporation of alanine/pyruvate was dependant upon equilibration of label from one to the other before X3 incorporation occurred. Alanine was found to be the more direct precursor of X3 as label was still traced into X3 after transamination was inhibited while in pyruvate experiments incorporation was eliminated by this treatment.

6. An attempt was made to produce ^{14}C -X3 by $^{14}\text{CO}_2$ - fixation experiments. It was thought that a large quantity of plant material could be easily labelled by $^{14}\text{CO}_2$ -fixation and with a prolonged chase period subsequent plant metabolism would incorporate carbon label into X3 through its precursors such as alanine. Although X3 did incorporate label (4.4% of total recovered radioactivity), its specific activity was insufficient for the ^{14}C -X3 to be useful for further tracer studies.

7. Pure X3 was obtained in a three step isolation procedure. Initially, a crude X3 fraction was obtained from plant extracts by ion exchange chromatography with a liner HCl gradient (0.5-2.0N). Further purification was

achieved by 1-dimensional paper chromatography using a solvent system of isopropanol:formic acid:water (20:1:5). The band of X3 from the previous step was then recrystallized from ethanol: water (80:20). Pure X3 was a creamy white crystal. A low yield, 20-30 mg of pure X3 was obtained from 30-50 g fresh weight of starting plant material.

8. Co-chromatography of X3 and β -(isoxaolin-5-one-2-yl)-alanine, BIA was shown by amino acid analysis. In subsequent structural analyses of X3 comparison of its properties was made with respect to those reported or experimentally determined for BIA.

9. X3 was found to be a strongly UV absorbing material. It had an extinction coefficient of 11,700 and an absorption maximum of 265 nm. This was similar to BIA which had an extinction coefficient of 12,200 and the same absorption maximum of 265 nm. In addition to UV absorbancy, X3 was found to be sensitive to short wave UV irradiation (254 nm). This feature was also reported for BIA.

10. X3 was found to be sensitive to chemical treatment of acid or base hydrolysis as well as UV photolysis. BIA had been shown to be labile to these treatments with the detection of specific breakdown products as a result of ring cleavage of its structure.

One of these products, α,β -diaminopropionic acid, DPA was analysed for in X3 solutions after degradative treatments. After acid hydrolysis, DPA was detected as 7-10% of the amount of degraded X3. A small amount of DPA (0.3%) was also detected after UV photolysis of X3.

11. The IR spectra of BIA and X3 were comparable in the region of $1600-4000\text{ cm}^{-1}$ however in the fingerprint region ($800-1600\text{ cm}^{-1}$) they did not correspond.

Absorptions of X3 were noted for key functional groups: -OH, -NH ($2800-3500\text{ cm}^{-1}$); α -amino group (2000 cm^{-1}); C=O (1700 cm^{-1}); C=C (1600 cm^{-1}).

12. ^1H -NMR spectroscopy of BIA and X3 further indicated differences between the two compounds. The BIA spectrum had signals for two olefinic protons of its isoxazolin-5-one ring at $\delta 8.1$ (C-3-H, singlet) and $\delta 5.1$ (C-4-H, singlet) with the protons associated with its alanine sidechain at $\delta 4.1-4.2$. The X3 spectrum did not indicate the presence of any olefinic protons in the region $\delta 5.0-8.0$, although the protons of the alanine sidechain were considered to be the signals at $\delta 3.2$ (2H, doublet, $J=4.88\text{ cps}$) and $\delta 2.9$ (1H, triplet, $J=4.88\text{ cps}$).

13. To confirm the structural identity of X3 mass spectral analysis of X3 and BIA were done. High resolution mass spectral data conclusively showed that BIA and X3 had the same exact mass, 172.0480 and therefore

molecular formula, $C_6H_8N_2O_4$. Low resolution mass spectral fragmentation patterns were also similar between samples of BIA and X3. Although these results strongly suggest that these compounds are identical there was difficulty in obtaining reproducible data for both samples. A number of mass spectra were obtained since initially the calculated molecular weight of 172 was not detected, but rather 174 was the given molecular weight. Even among replicates of a sample (different instruments) the spectra were not the identical.

14. It was concluded that the substances BIA and X3 were the same compound, however different isolation methods may have resulted in the compounds being in non-identical forms which may account for their different spectral properties of NMR and IR. It was recommended that the isolation of X3 be modified to improve purity of the preparation. With clean BIA and X3 preparations further work is suggested that would make use of ^{13}C -NMR spectroscopy or derivitization of the substances for continued structural analysis.

15. The comparison of biological aspects of BIA and X3 were made last. These compounds were found to have similar levels and trends in their accumulation in peas as found in this study and reported by Lambein et al. (1976). Varietal differences were considered to account for the

fact that the features were not identical in terms of detected amounts of BIA and X3 in pea plants. Of the potential precursors of X3, this study found that pyruvate and alanine were the only tracers to have been significantly incorporated. These compounds were also reported to be incorporated by BIA and further studies by Lambein and co-workers also indicated that several organic acids such as malate and malonate were precursors as well. Lambein and co-workers also used the detection of labelled DPA to give an indication of the position of ^{14}C -incorporation in BIA. This technique was not utilized in this study as the scale of the experiments did not make it feasible, however it would be useful in further work to analyze for labelled DPA to identify the part of X3 structure where incorporation of label occurred.

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LITERATURE CITED

ADRIAENS, P., MEESCHAERT, B., WUYTS, W., VANDERHAEGHE, H., and EYSEN, H. (1977). Separation of ninhydrin positive compounds on a single column amino acid analyzer using lithium buffers. J. Chromatog. 140: 103-108.

BAUER, A., URQUHART, A., and JOY, K.W. (1977a). Amino acid metabolism of pea leaves: I Diurnal changes and amino acid synthesis from ^{15}N -nitrate. Plant Physiol. 59: 915-919.

BAUER, A., URQUHART, A., and JOY, K.W. (1977b). Amino acid metabolism of pea leaves: II Labelling studies on utilization of amides. Plant Physiol. 59: 920-924.

BELL, E.A. (1976). Uncommon amino acids in plants. FEBS Lett. 64: 29-35.

BELL, E.A. (1980a). In "Secondary Plant Products" (E.A. Bell and B.V. Charlwood, eds.) pp. 11-22. Springer-Verlag, New York.

BELL, E.A. (1980b). In "Secondary Plant Products" (E.A. Bell and B.V. Charlwood, eds.) pp. 403-432. Springer-Verlag, New York.

BELL, E.A., FELLOWS, L.E. and QURESHI, M.F. (1976). 5-Hydroxy-L-tryptophan; taxonomic character and chemical defence in *Griffonia*. Phytochem. 15: 823.

BROWN, E.G. and MANGAT, B.S. (1969). Structure of a pyrimidine amino acid from pea seedlings. Biochim. Biophys. Acta 177: 427-433.

CALLEBAUT, A., LAMBEIN, F. and VAN PARIJS, R. (1977). Biosynthesis of isoxazolinone amino acids: Incorporation studies on the synthesis of 2-(2-amino-3-carboxypropyl)-isoxazolin-5-one in *Lathyrus odoratus* seedlings. Arch. Internat. Physiol. Biochem. 85: 157-188.

CANVIN, D.T. and BEEVERS, H. (1961). Sucrose synthesis from acetate in the germinating castor bean: Kinetics and pathway. J. Biol. Chem. 236: 988-995.

DENT, C.E. (1948). A study of the behavior of some 60 amino acids and other ninhydrin reacting substances on phenol-collidine filter paper chromatograms. Biochem. J. 43: 168-180.

DE SARLO, F., Fabbrini, L. and Renzi, G. (1966a). 2-Methylisoxazolin-5-ones: I. Tetrahedron 22: 2989-2994.

DE SARLO, F., Fabbrini, L. and Renzi, G. (1966b). 2-Methylisoxazolin-5-ones: II. Tetrathedron 22: 2995-2999.

DE SARLO, F., DINI, G. AND LACRIMINI, P. (1971). Isoxazolin-5-one. J. Chem. Soc. (C). 86.

FOWDEN, F., LEWIS, D. and TRISTRAM, H. (1967). Toxic amino acids and their action as antimetabolites. Adv. Enzymol. 29: 89-164.

FOWDEN, L., LEA, P.J. and Bell, E.A. (1979). The non-protein amino acids of plants. Adv. Enzymol. 50: 118-175.

GRANT, D.R. and VOELKERT, E. (1970). The effect of temperature and light on the accumulation of homoserine in pea seedlings. Phytochem. 9: 985-990.

GRANT, D.R. and VOELKERT, E. (1972). The fate of homoserine-¹⁴C in germinating peas. Phytochem. 11: 911-916.

IKEGAMI, F., DE BLAUWE, M. and LAMBEIN, F. (1981). Distribution of isoxazolin-5-one derivatives in some Lathyrus species. Arch. Internat. Physiol. Biochim. 89: B174-175.

JOY, K.W., SHAY, C. and MCLIMONT, M.J. (1980). Effect of some organic buffers on the estimation of aspartic acid and resolution in amino acid analysis. J. Chromatog. 194: 76-79.

KING, R.W. and ZEEVAART, J.A.D. (1974). Enhancement of phloem exudation from cut petioles by chelating agents. *Plant Physiol.* 53: 96-103.

KUO, Y.H. and LAMBEIN, F. (1981a). Biosynthesis of isoxazolin-5-one amino acids and uracil derivatives from ^{14}C -labelled precursors in *Pisum sativum*. *Arch. Internat. Physiol. Biochim.* 89: B181-182.

KUO, Y.H. and LAMBEIN, F. (1981b). Biosynthesis in vivo of isoxazolin-5-one derivatives from ^{14}C -labelled precursors in *Lathyrus odoratus*. *Arch. Internat. Physiol. Biochim.* 89: B182-183. LAMBEIN, F. and VAN PARIJS, R. (1968a). Identification of 1-alanyl-5-ribosyl-4-amino-uracil in pea seedlings. *Arch. Internat. Physiol. Biochem.* 76: 188-189.

LAMBEIN, F. and VAN PARIJS, R. (1968b). Isolation and characterization of 1-alanyl-uracil and 3-alanyl-uracil from *Pisum sativum*. *Biochem. Biophys. Res. Comm.* 32: 474-479.

LAMBEIN, F. and VAN PARIJS, R. (1970). Alanyl-3-isoxazolin-5-one: A second UV sensitive heterocyclic α -amino acid from *Pisum sativum* L. *Biochem. Biophys. Res. Comm.* 40: 557-564.

LAMBEIN, F. and DE VOS, B. (1981). Lathyrism in young chicks induced by isoxazolinones from *Lathyrus odoratus* seedlings. *Arch. Internat. Physiol. Biochem.* 89: B66.

LAMBEIN, F., SCHAMP, N., VANDENDRIESSCHE, L. and VAN PARIJS, R. (1969). A new UV sensitive heterocyclic amino acids from pea seedlings: 2-Alanyl-3-isoxazolin-5-one. *Biochem. Biophys. Res. Comm.* 37: 375-382.

LAMBEIN, F., KUO, Y.H. and VAN PARIJS, R. (1976). Isoxazolin-5-ones. Chemistry and biology of a new class of plant products. *Heterocycles* 4: 567-577.

LARSON, L.A. and BEEVERS, H. (1965). Amino acid metabolism in young pea seedlings. *Plant Physiol.* 40: 424-432.

LAWRENCE, J.M. (1973). Homoserine in seedlings of the tribe Vicieae of the Leguminosae. *Phytochem.* 12: 2207-2209.

LAWRENCE, J.M. and GRANT, D.R. (1963). Nitrogen metabolism in pea seedlings: II. Free amino acids. *Plant Physiol.* 38: 561-566.

MILLS, R.W. and JOY, K.W. (1980). A rapid method for isolation of purified physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. *Planta* 148: 75-83.

MURAKOSHI, I., KURAMOTO, H., HAGINIWA, J. and FOWDEN, L. (1972). The enzymic synthesis of β -substituted alanines. *Phytochem.* 11: 177-182.

MURAKOSHI, I., KATO, F., HAGINAWA, J. and Fowden, L. (1973). Enzymic synthesis of 2-alanyl-3-isoxazolin-5-one from O-acetylserine and isoxazolin-5-one by *Pisum* seedling extracts. *Chem. Pharm. Bulletin* 21: 918-920.

MURAKOSHI, I., IKEGAMI, F., KATO, F., HAGINAWA, J., LAMBEIN, F., VAN ROMPUY, L. and VAN PARIJS, R. (1975a). Enzymic alanylation of an isoxazolinone glucoside by legume seedling extracts. *Phytochem.* 14: 1515-1517.

MURAKOSHI, I., IKEGAMI, F., KATO, F., HAGINIWA, J., LAMBEIN, F., VAN ROMPUY, L. AND VAN PARIJS, R. (1975b). Enzymatic synthesis of isoxazolin-5-one glucoside by extracts from *Pisum* and *Lathyrus* species. *Phytochem.* 14: 1269-1270.

MURAKOSHI, I., IKEGAMI, F., OOKAWA, N., ARIKI, T., HAGINAWA, J., KUO, Y.H. and LAMBEIN, F. (1978). Biosynthesis of the uracil alanines, willardine and isowillardine in higher plants. *Phytochem.* 17: 1571-1576.

PANDE, S.V. (1976). Liquid scintillation counting of aqueous samples using Triton-containing scintillants. *Anal. Biochem.* 74: 25-34.

PRUISNER, S. and MILNER, L. (1970). A rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthase and asparaginase. *Anal. Biochem.* 37: 429-438.

ROSENTHAL, G.A., DAHLMAN, D.L. and JANZEN, D.H. (1976). A novel means for dealing with L-canavanine, a toxic metabolite. *Science* 192:256-258.

STEIN, W.H. and MOORE (1954). Procedures for the chromatographic determination of amino acids on 4% cross-linked sulfonated polystyrene resins. *J. Biol. Chem.* 211: 893-906.

SUGI, M., MIURA, H., and NAGATA, K. (1981). 3-Isoxazolindone from jack bean seedlings. *Phytochem.* 20:4451-453.

TISCHLER, S. and WEILER, L. (1979). Alkylation of Carbanions from Isoxazolin-5-ones. *Tet. Lett.* 52: 4903-4906.

URQUHART, A. (1980). The transport, metabolism and redistribution of xylem-borne amino acids in pea seedlings. Ph.D. Dissertation. Carleton University, Ottawa Ontario, Canada.

URQUHART, A. and JOY, K.W. (1981). Use of phloem exudate technique in the study of amino acid transport in pea plants. *Plant Physiol.* 68: 750-754.

VAN EGERAAT, A.W.S.M. (1975). Changes in free ninhydrin-positive compounds of young pea plants as effected by different nutritional and environmental conditions. *Plant and Soil* 47: 15-36.

VAN EGERAAT, A.W.S.M. (1976). Exudation of γ -glutamyl-alanine by root tips of pea plants. *Plant and Soil.* 44: 501-503.

VAN EGERAAT, A.W.S.M. (1977). Enzymic studies on ninhydrin-positive compounds exuded by root tips of pea seedlings. *Plant and Soil* 47: 645-651.

VAN ROMPUY, N., SCHAMP, N., DE KIMPE, N. and VAN PARIJS, R. (1973). 2-Substituted-3,4-unsubstituted isoxazolin-5-ones synthesis and comparison with the natural compounds. *J. Chem. Soc. Perkin I*: 2503-2506.

VAN ROMPUY, N., LAMBEIN, F., DE GUSSEM, R. and VAN PARIJS, R. (1974). The isolation and structural analysis of 2-(2-cyanoethyl)-3-isoxazolinone, 2-(β -D-glucopyranosyl)-3-isoxazolinone and 2-carboxy-3-isoxazolinone from Lathyrus odoratus. Biochem. Biophys. Res. Commun. 56:199-205.

VIRTANEN, A., BERG, A.M. and KARI, S. (1953). Formation of homoserine in germinating pea seeds. Acta Chem. Scand. 7: 1423-1424.

VIRTANEN, A. and BERG, A.M. (1954). γ -Glutamyl-alanine in pea seedlings. Acta Chem. Scand. 8: 1089-1090.

WEBB, J.A. and GORHAM, P.M. (1964). Translocation of photosynthetically assimilated C₁₄ in straight-necked squash. Plant Physiol. 39: 663-672.

WIGFIELD, D.C. (1974). The determination of the radioactivity of ¹⁴C samples adsorbed on glass vials by direct liquid scintillation counting. Anal. Biochem. 59: 11-15.

APPENDIX 1

Composition of Nutrient Solution used for Hydroponic
Culture of Pisum sativum

Compound	g/L,
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.354
$\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$	0.246
KNO_3	0.101
KH_2PO_4	0.136
$\text{Fe}(\text{NH}_4)\text{SO}_4 \cdot 6\text{H}_2\text{O}$	0.0036
versenol	0.0076
H_3BO_4	0.00125
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.00075
ZnCl	0.00005
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.0000025
Element	μM
N as NO_3^-	4000
N as NH_4^+	9
K	2000
Ca	1500
P	1000
S	1000
Mg	1000
Fe	9
B	20
Mn	3.8
Zn	0.4
Mo	0.02
Cl	4.2

APPENDIX 2

The Detection of X3 in the Plant Organs
of Two Different Pea Cultivars.

Lambein and co-workers have compared a number of *Lathyrus* species to determine the distribution of isoxazolinone compounds among them (Ikegami et al., 1981). Since *Pisum sativum* was the original plant material in which X3 was initially detected, other pea cultivars were examined to determine if comparable levels of X3 could be found elsewhere. Alaska and Tall Telephone varieties were available and sampled at the three growth periods as in Table 1.

Plant tissue was sampled at various growth stages and were homogenized in distilled water with 5-sulphosalicylic acid, SSA (50 mg per ml extract). The extract was then centrifuged to remove precipitated proteins. Samples were adjusted to pH 2.0 and analysis done using a Beckman Amino Acid Analyzer, Model 119L B L (lithium citrate buffer system).

Results

See Table 1.

Discussion

The data in Table 1 shows that some difference in levels of X3 and its distribution occurs in the different

Table 1. Levels of X3 in Tall Telephone and Alaska
Pea Cultivars.

Stage of Growth	Tall Telephone Whole Shoot	Root	Seed	Whole Shoot	Alaska Root	Seed
(μmol per organ)						
6 Day	2.07	1.65	1.28	1.10	0.128	1.10
14 Day	3.55	1.13	0.560	3.51	0.272	0.142
20 Day	4.56	0.781	ND	3.51	0.884	ND

ND - Not Determined

cultivars. The trend of X3 accumulation appears to be directed to growing leaves as the whole shoot continues to increase its levels of X3 during development while the root and germinating seed pools of X3 decline. Of the three available cultivars, Little Marvel, the study pea material was the best choice for investigation of X3. Tall varieties would have been more difficult to grow and handle while their metabolism and development are not as well documented as for Little Marvel.

Reference

IKEGAMI, F., deBLAUWE, M. and LAMBEIN, F.
(1981) Distribution of isoxazolin-5-one amino acids and derivatives in some Lathyrus species. Arch. Internat. Physiol. Biochem. 89: B174-175.

APPENDIX 3

Detection of X3 in Etoilated Pea Seedlings.

Etoilated pea seedlings were examined to determine if photosynthetic activity affected the formation and accumulation of X3. If a greater accumulation of the compound occurred it was thought that etoilated tissue could be used as examine biosynthesis by labelling studies. This material could have also been useful in X3 purification as less crude material would be required for processing when its concentration in the tissue had increased.

For this study the plants were grown in perlite:vermiculite under conditions of continous darkness for 14 days. The plants were sampled by aqueous extraction using 5-sulphosalicylic acid, SSA to precipitate proteins (50 mg per ml extract). The extract was prepared and adjusted to pH 2.0 before amino acid analysis by Beckman Model 119L BL (lithium citrate buffer system).

Results

See Table 1.

Discussion

Although the plant material was treated to continual darkness, there was no apparent effect on X3 metabolism.

Table 1. The Detection of X3 in Etoilated Pea Seedlings.

	Days Growth in Continual Darkness		
	8 Days	10 Days	12 Days
	Level of X3 (μ mol per organ)		
Whole Shoot	1.86	1.05	3.24
Roots	1.08	1.81	1.76
Cotyledons	0.542	ND	ND

ND - Not Determined

The levels of X3 in the etoilated tissues were comparable to the organs when normal development occurs. This suggests that the formation of X3 is not dependent on the energy or metabolism of the plant normally associated with photosynthetic activity. However some amino acids (data not shown) did change in concentration such as asparagine and glutamine which increased upon etoilation of the plants.

END

1	0	0	9	8	4
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FIN