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**LA THÈSE A ÉTÉ
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THE TRANSPORT, METABOLISM, AND
REDISTRIBUTION OF XYLEM-BORNE
AMINO ACIDS IN PEA SEEDLINGS.

by

AILEEN ALEXANDER URQUHART, B.Sc.

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

Carleton University
Ottawa, Ontario
February, 1980.

The undersigned hereby recommend to the Faculty of Graduate
Studies acceptance of this thesis, submitted by Aileen Alexander
Urquhart, B.Sc., in partial fulfilment of the requirements for the
degree of Doctor of Philosophy.

.....
Chairman, Department of Biology

.....
Supervisor

.....
External Examiner

For Elsie Urquhart
who would have enjoyed the research
and in memory of
George Urquhart
with love

Wings

We have
a map of the universe
for microbes,
we have
a map of a microbe
for the universe.

We have
a Grand Master of chess
made of electronic circuits.

But above all:
we have
the ability
to sort peas,
to cup water in our hands,
to seek
the right screw
under the sofa
for hours.

This
gives us
wings.

Miroslav Holub, 1967.

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ABSTRACT

Leaves of *Pisum sativum* (L. c.v. Little Marvel) developed through morphologically distinct stages characterized by the unfolding of leaflets and then stipules. In non-nodulated plants grown in nutrient solution (4mM nitrate), the nitrogen levels of the leaves reached a maximum when the leaves were just fully expanded; these levels were maintained for 3 to 4 days. Mature (fully-expanded) and expanding leaves transpired water vigorously, receiving a mixture of nitrate and amino acids (mainly asparagine, glutamine, and homoserine) from the roots in the transpiration stream. Immature leaves (enclosed within the stipules of the older leaves) transpired negligible amounts of water. Nitrogen for these leaves must come from mature leaves, presumably as amino acids in the phloem.

Uptake and redistribution of ^{14}C -amides (asparagine and glutamine) supplied as a pulse via the transpiration stream to detached pea shoots was followed for 12 hours in the light. Labelled carbon from asparagine was taken up rapidly into mature leaves and later exported with little further metabolism or incorporation into protein. Similarly carbon from glutamine was taken up and exported from mature leaves; extensive metabolism to glutamate occurred but there was little further conversions or incorporation into protein. In expanding leaves, labelled carbon rapidly accumulated and the levels of radioactivity remained high, suggesting later influx from older leaves. Asparagine was metabolized in expanding leaves to 2-hydroxysuccinamic acid (HSA); glutamine was metabolized to glutamate and some γ -amino butyric

acid. In these leaves, extensive incorporation of label into protein occurred. Immature leaves (enclosed within stipules) received labelled carbon, levels of radioactivity in soluble and protein fractions rising gradually. Asparagine was the main labelled soluble compound in the early samples of asparagine-tracer experiments; radioactivity was mainly in the amino-acid fraction in early samples of the glutamine-tracer experiments. Data suggested transport of labelled carbon to immature leaves from older leaves as amino acids.

Carbon from asparagine, glutamine, aspartate, glutamate, and HSA (applied as ^{14}C -tracers to the surface of mature leaves) was transported to young (expanding and immature) leaves and to roots. After 2 hours feeding asparagine, carbon recovered in young leaves and roots was mainly (70 to 80%) amino acids, largely asparagine. After glutamine feeding, carbon in young leaves and roots was 70% amino acids, mainly glutamate. With glutamate as tracer, 50% of the label in young leaves was amino acids, two-fifths of this glutamate, and 20 to 30% was organic acids. With aspartate as tracer, however, most of the aspartate in the fed leaf was metabolized in 2 hours; only 5 to 10% of the label remained in aspartate, 40% was recovered in organic acids. Of the radioactivity recovered in young leaves, 4 to 5% was in aspartate, and 30% in organic acids. The data confirmed transport of carbon from glutamine and asparagine to the young leaves mainly as amino acids. Transport of carbon from aspartate or glutamate may involve other compounds in addition to amino acids. When HSA was tracer, most of the label recovered in young leaves was still HSA.

A disodium EDTA solution, pH 7.0, promoted exudation of a phloem sap from cut petioles of detached, mature pea leaves. Amino acids (asparagine, homoserine, glutamate, and also aspartate and serine) were present in the exudate. Carbon from ^{14}C -amino acids applied to the leaf blade was recovered in the exudate. After feeding asparagine in the light, radioactivity in the exudate was only in asparagine and aspartate, despite considerable metabolism of asparagine in the fed leaf-blade. Although little conversion of asparagine took place in dark-treated leaves, labelled asparagine and aspartate were also recovered in the phloem exudate. When aspartate was tracer in the light, 65 to 70% of the label in the exudate was amino acids, mainly aspartate, even though much of the radioactivity in the leaf blade was in organic acids and little labelled aspartate remained. In the dark, aspartate and glutamate were the major labelled amino acids in the exudate. Translocation of carbon from HSA only occurred in the light. HSA was the main labelled compound in the exudate, but the percentage of label in amino acids (aspartate, glutamate, homoserine) was higher in the exudate than in the leaf-blade. Very little HSA was translocated.

These data suggested that amino acids were preferred transport compounds for re-export of xylem-borne amides (and related compounds) which entered mature leaves in the transpiration stream. Thus amino acids from the phloem supplied part of the nitrogen for expanding leaves (also receiving nitrate and amino acids in the transpiration stream) and probably all of the nitrogen for immature (non-transpiring) leaves.

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ABBREVIATIONS

Aaa	α -amino adipic acid
EDTA	ethylenediaminetetraacetic acid,
Gaba	γ -amino butyric acid
HSA	2-hydroxysuccinamate
Hse	homoserine
KSA	2-ketosuccinamate
NCS	NCS Tissue Solubilizer
SSA	5-sulphosalicylic acid
TOX	<u>T</u> riton- <u>O</u> mnifluor- <u>X</u> ylene
Tris	Tris(hydroxymethyl)aminomethane

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Frontispiece - Legend

After the initial supply of amino acids (▲) from the cotyledons (C) is exhausted, the non-nodulated plant is dependent on soil nitrogen for amino acid and protein synthesis. Soil nitrate (▲) is taken up by the roots (R). Some of this nitrate is transferred to the xylem and transported to the shoots in the transpiration stream (▶▶▶▶). The rest of the nitrate is reduced in the roots to ammonia and assimilated into organic nitrogenous compounds (△; amino acids, amides, and ureides) and transferred to the xylem (▶▶▶). Leaves which are transpiring vigorously (↑) such as the mature (M) and expanding (E) leaves, therefore receive a mixture of nitrate and organic nitrogenous compounds via the transpiration stream. Young leaves (Y) which are enclosed in the apical bud and therefore transpiring negligible amounts of water (↓) will receive very little nitrogen directly from the xylem. Nitrogen levels (N) in the leaf increase sigmoidally as the leaf ages. Mature leaves have attained maximum nitrogen levels, yet receive nitrogen from the xylem. This excess nitrogen is transferred to the phloem and exported as organic nitrogenous compounds (◻◻◻) from the mature leaves to the young and expanding leaves and also to the roots. Nitrogen levels in the expanding leaves are increasing rapidly. Nitrogen is supplied via the xylem and also from the mature leaves via the phloem. Young leaves receive nitrogen mainly via the phloem from the mature leaves.

INTRODUCTION

An important aspect of the development of young leaves in the vegetative apices of plants is the supply of nitrogen to these organs for amino-acid synthesis and hence protein synthesis. Nitrogen absorbed by the plant root system enters the shoot, either as nitrate or organically bound nitrogen, via the transpiration stream. Most of this xylem-borne nitrogen will, therefore, be transported initially to strongly transpiring structures such as mature leaves; weakly transpiring organs such as flowering and fruiting structures and immature leaves probably depend largely on translocation of nitrogenous compounds from the mature leaves in the phloem. The transport of amino acids in the transpiration stream to the fully-expanded leaves of *Pisum sativum*, the extent of metabolism there, and the subsequent translocation of amino acids and metabolites to the immature leaves was the scope of this study.

* * * * *

After the limited nitrogen reserves in the seeds have been used up by the rapidly growing seedling, the nitrogen required for the growth of plants must come from the soil (non-nitrogen-fixing plants) or from the atmosphere (nitrogen fixers such as nodulated leguminous plants; non-legumes, for example *Myrica* and *Alnus*; and some tropical grasses). *Pisum sativum* is a legume and can become nodulated, but the three-week-old seedlings used in the present study were non-nodulated and were therefore dependent for their growth on the nitrate supplied in the nutrient solution.

For non-nitrogen-fixing plants, the main source of nitrogen is nitrate. Although some plants will absorb amino acids through the root and transport them to the shoot (Joy, 1962; van Die, 1963), and though pea plants grow almost as well using aspartate or glutamate as the sole nitrogen source as did control plants grown on nitrate (Valle and Virtanen, 1960), nevertheless nitrate is the main form of nitrogen in the soil and is the principal form of nitrogen added. Even when ammonia is supplied or is produced by decomposition of organic nitrogenous material, the ammonia is readily converted to nitrate by nitrifying bacteria.

The fate of nitrate after entering the plant root seems to depend largely on the species. Reduction of nitrate to nitrite and then to ammonia can take place in the root. The enzymes responsible for this reduction (nitrate reductase and nitrite reductase) have been shown to be present in roots (Miflin, 1970; Dalling, Tolbert, and Hageman, 1972b). The ammonia produced by the reduction of nitrate and nitrite is incorporated into amino acids, probably by the glutamine synthetase - glutamate synthase pathway (Lea and Miflin, 1974), the latter enzyme being present in pea roots (Miflin and Lea, 1975). Analyses of xylem bleeding saps from a range of species indicated that some plants such as *Xanthium*, *Stellaria*, and *Trifolium* transported mainly nitrate in the xylem, very little organically bound nitrogen being found in the bleeding sap. Other plants such as *Raphanus* or *Lupinus* transported largely organic nitrogenous compounds. Many plants, however, including *Pisum*, transport a mixture of nitrate and organic nitrogenous compounds (Pate, 1973). In that study, species transporting mainly

nitrate had low root nitrate reductase activity; those transporting amino acids instead of or in addition to nitrate had active root nitrate reductase. Radin (1978) demonstrated that soybean (*Glycine max*) accumulated proportionally more of the plant's nitrate in the roots and exported less in the xylem than did cotton. This suggested that the inability of soybean roots to transport nitrate into the xylem resulted in a build-up of nitrate in the roots which may have been responsible for induction of the active nitrate reductase (non-limited by substrate) in the soybean roots.

In nitrogen-fixing plants, the ammonia resulting from fixation of atmospheric nitrogen has been shown, by the use of ^{13}N -ammonia, to be assimilated in soybean root nodules into glutamine as initial organic product, glutamate being labelled later (Meeks, Wolk, Schilling, Shaffer, Avissar, and Chien, 1978). Glutamine or glutamate may be transported out of the nodule, but asparagine appears to be the preferred compound transported from the nodules of *Vicia* (Pate, Gunning, and Briarty, 1969), and from roots of nodulated *Pisum arvense* (Pate, 1962). Asparagine was the major amino acid in exudates from roots of preflowering soybean, but ureide-N was higher than amino-N in flowering plants (Streeter, 1979). Pate, Walker, and Wallace (1965) noted that asparagine, absent from the bleeding sap of *Pisum arvense* prior to nodulation, became the major component of the bleeding sap as effective nodulation took place.

The organic nitrogenous compounds found in the xylem sap of both nitrogen-fixing and non-fixing plants are often amino acids with high N:C ratios, such as the amides, asparagine and glutamine,

or arginine. In some plants, non-protein amino acids such as homoserine, citrulline, ornithine, and γ -methylene glutamine are transported; ureides such as urea, allantoin, or allantoic acid may also be present in the xylem (Pate, 1973; McLure and Israel, 1979).

The shoots may, therefore, receive nitrate or amino acids or other nitrogenous compounds in the transpiration stream, the mixture depending on the species. Vigorously transpiring organs such as the fully-expanded leaves will receive most of this transported nitrogen. The mature leaves of tomato plants, after 4 hours of feeding ^{14}C -glutamate to the roots, had received more radioactivity than the young leaves or the very old leaves (van Die, 1963). In *Pisum arvense*, the lower, fully-expanded leaves initially received more radioactivity from a ^{14}C -labelled amino-acid mixture (bleeding sap) fed to the cut shoots (Pate, Walker, and Wallace, 1965). Weakly transpiring structures such as young leaves, apices, and fruits will not be main catchment areas for xylem-borne nitrogen. Nitrogen supply to the shoots is shown on page xviii.

Photosynthetically assimilated carbon is translocated from the mature leaves to the fruiting structures and vegetative apices via the phloem, which may also provide a pathway for transport of nitrogen to these parts of the plant. The evidence for the presence of amino acids in the phloem and for the movement of ^{14}C amino acids in the phloem from leaves is described later. Joy (1967) showed that petiole steaming (thereby blocking the phloem) prevented normal growth and nitrogen accumulation in young sugar beet leaves.

In order to investigate the contents of the phloem pathway, several methods for obtaining phloem sap have been used. Certain plants (some Palms and a few Agavaceae) yield an exudate, believed to originate in the phloem, from severed inflorescence stalks (Tammes and van Die, 1964). In addition to sucrose, certain ions (for example, potassium and phosphate), amino acids, and traces of ureides were present. Glutamine was the major amino acid; valine, serine, glycine, and a few others were also present; and asparagine and aspartate were present in trace amounts. Cuts in the bark of trees produce, in some species, large quantities of exudate from the phloem (Ziegler, 1956; Crafts and Crisp, 1971). Milburn (1970) collected an exudate from incisions in the bark of *Ricinus*. Hall and Baker (1972) studied the amino acid composition of this exudate; glutamate, aspartate, threonine, glycine, alanine, serine, and valine were quantitatively the most important. This technique of collecting phloem sap from shallow cuts into the phloem has also been applied to studies with the legume *Lupinus albus* (Pate, Atkins, Hamel, McNeil, and Layzell, 1979).

An interesting approach to collecting phloem sap was initiated by the suggestion of Kennedy and Mittler (1953) that the exudate from severed stylets of aphids (previously feeding on living willow plants) might be used for the study of sieve-tube contents. Mittler (1958) analyzed the aphid stylets exudate from *Salix acutifolia*, recording the presence of several amino acids and noting a variation in their concentrations with the development of the tree. Peel and Weatherley (1959) collected exudate from aphids browsing on *Salix viminalis*, confirming the presence of amino acids

(mainly aspartate, glutamate, and asparagine) and a seasonal variation in their concentration.

These studies used relatively large aphids whose stylets were easily severed. The aphids feeding on herbaceous species tend to be much smaller and more easily disturbed while feeding. Recently, however, Barlow and McCully (1972) used a ruby laser to sever the fine stylets of the small aphid, *Acythrosiphon pisum*, feeding on *Pisum sativum*. The free amino acids accounted for 4.46% (w/w) of this exudate (Barlow and Randolph, 1978). Major obstacles to the routine use of aphid stylets exudate for analyses of phloem contents are the small volumes of exudate obtainable and the difficulty in pre-determining the position of the feeding aphid.

Pate, Sharkey, and Lewis (1974) found that a few leguminous species would yield an exudate from the cut distal, or stylar, end of a developing fruit. Although the quantities collected were small, exudation could be reinitiated from the same fruit by removal of a thin sliver of tissue from the cut end; rates of exudation and sugar levels declined only slightly in subsequent collections. This technique was used to examine the phloem contents during development of pods of *Lupinus albus* (Atkins, Pate, and Sharkey, 1975). It was also used to compare phloem exudate and xylem sap (tracheal extract) in *Lupinus albus* and *Sparticum junceum*, to examine the exchange of nitrogen (^{15}N -labelled) from xylem to phloem of the fruits of *Lupinus*, and to follow the fate of ^{14}C -labelled amino acids in *Sparticum* (Pate, Sharkey, and Lewis, 1975) and *Lupinus* (Sharkey and Pate, 1975).

This technique examines the phloem sap after it has entered the fruit and gives no information on the composition of the phloem sap leaving the leaves. Some leaves will exude through the petiole when detached from the plant (Leonard and Glenn, 1968), but the quantities obtained are small, probably because of callose formation in the damaged sieve tubes and aggregation of the proteinaceous components of the phloem.

King and Zeevaart (1974) have described a technique for enhancing the exudation from the cut petioles of detached leaves; a bathing solution, containing Na_2EDTA at pH 7.0, around the base of the petiole promoted exudation of ^{14}C -labelled stachyose, verbascose, raffinose, and sucrose from *Perilla* leaves previously fed with $^{14}\text{CO}_2$, and of ^{14}C -sucrose from leaves of *Chenopodium* and *Pharbitis*. Dickson (1977) used this technique to obtain a petiolar exudate from *Populus deltoides* and *Phaseolus vulgaris* leaves, previously labelled with $^{14}\text{CO}_2$. Although recovery of radioactivity in the exudate was considerably less from *Populus* than from *Phaseolus*, he nevertheless was able to examine the distribution of radioactivity in amino-acid, sugar, and organic-acid fractions in the phloem exudate from both plants, finding that the distribution of radioactivity in each fraction varied with the age of the exuding leaf. The amino acids were not identified.

Frégeau (1976) used the method to investigate the effect of indole acetic acid and abscisic acid on phloem loading in *Phaseolus vulgaris*. This EDTA-promoted phloem exudation technique was also used to study translocation into developing soybean pods

(Fellows, Egli, and Leggett, 1978). When $^{14}\text{CO}_2$ was fed to a source leaf, most of the radioactivity which leaked into the EDTA solution around the pod tip was in ^{14}C -sucrose; 6.9% of the total labelled carbon exuded was, however, in the amino-acid fraction. Several amino acids were identified in this fraction; only serine and asparagine were labelled.

In a recent study (Tully and Hanson, 1979), the technique was used to study amino acid translocation from turgid and water-stressed barley leaves. In addition to labelled sugars, radioactivity in the exudate from $^{14}\text{CO}_2$ -fed leaves was recovered in aspartate, glutamate, serine, glutamine, and alanine, which were also quantitatively the major amino acids.

Some criticism can be levelled at all of these methods for obtaining phloem sap, but despite the possibility of some contamination from other tissues, the sap or exudate collected is probably representative of phloem sap. The high sucrose (or other transport sugar such as stachyose or verbascose) content, the high pH (8.0, 8.5), low levels or absence of nitrate, and the appearance in the sap or exudate of ^{14}C -label in compounds which are typically labelled from $^{14}\text{CO}_2$ in the leaf blade (e.g. sucrose, serine, glycine) are evidence of the phloem origin of these exudates.

The lack of nitrate in the phloem sap has been noted in many studies. Nitrate was absent in the phloem sap from *Yucca* inflorescences (Tammes and van Die, 1964), and in the phloem exudate from *Ricinus* stem (Hall and Baker, 1972), *Pisum sativum* fruit stalk (Lewis and Pate, 1973), and cut stelar ends of soybean pods (Fellows et al., 1978); no nitrate was detected in the aphid

stylets exudate from *Salix* (Peel and Weatherley, 1959). Pate et al. (1975) did not normally detect nitrate in the phloem exudate from cut pod tips of *Lupinus* even when high levels of nitrate were fed to the intact plants, but they could detect nitrate in the phloem when high levels of nitrate were fed directly to the detached shoots through the cut ends of the stems. These studies suggest that even when nitrate is transported in the xylem to the shoot, little or no nitrate is transferred to the phloem.

The nitrate arriving in the leaves is reduced to ammonia by nitrate reductase and nitrite reductase, these enzymes being present in leaf tissue (Dalling, Tolbert, and Hageman, 1972a; Mifflin, 1974; Magalhaes, Neyra, and Hageman, 1974). Assimilation of ammonia in leaf tissue is believed to proceed (as in the roots) via the glutamine synthetase - glutamate synthase pathway (Lea and Mifflin, 1974). The presence of glutamine synthetase (O'Neal and Joy, 1973a, 1973b; Wallsgrove, Lea, and Mifflin, 1979) was demonstrated in leaves of several species including *Pisum sativum*. Labelling studies with ^{15}N (Yoneyama and Kumazawa, 1974, 1975; Bauer, Urquhart, and Joy, 1977) and with ^{13}N (Skokut, Wolk, Thomas, Meeks, Shaffer, and Chien, 1978), showing initial incorporation of label into glutamine, coupled with inhibitor studies (Arima and Kumazawa, 1977; Skokut et al., 1978), confirm the pathway in leaves.

The absence of nitrate and the presence of amino acids in the phloem emphasizes the role of the mature leaves in processing both the nitrate and the amino acids received by them from the transpiration stream, and their role in redistribution of amino acids to the weakly transpiring parts of the plants. Various

workers have investigated this function of the mature leaves. These studies fall into two major groups; those in which nitrate (^{15}N -labelled) or amino acids (^{15}N - or ^{14}C -labelled, or double-labelled) were supplied to the detached shoot or, via the roots, to the intact plant; and those in which labelled carbon (either as $^{14}\text{CO}_2$ or ^{14}C -labelled amino acids) was fed by some means to the mature leaves.

Some investigators showed that labelled amino acids or nitrate were taken up initially into the older, mature leaves and that subsequent redistribution of the carbon and nitrogen occurred. Initial uptake of labelled glutamate supplied to roots or detached shoots of tomato was mainly by mature leaves (van Die, 1963). Most of this label was in the soluble fraction, glutamate being the most highly labelled amino acid, followed by glutamine and aspartate. Only one harvest was made and, therefore, redistribution of label was not followed in that study. In *Pisum arvense*, however, uptake of ^{14}C -labelled xylem bleeding sap (mainly asparagine, glutamate, aspartate, and homoserine) fed as a short pulse to the base of cut shoots, was initially (4-hour harvest) by the lower leaves and stems; after 24 hours, 25% of the label originally presented to the shoot had been transferred to the upper part of the shoot (Pate et al., 1965).

Preferential accumulation in the young leaves of carbon from ^{14}C -labelled amino acids (glutamine, glutamate, and glycine) fed to the intact roots of sugar beet was demonstrated by Joy (1962). Movement in the phloem was suggested since the transport of label to the young leaves was blocked by petiole steaming (believed to block phloem but not xylem transport). Initial recovery of

radioactivity in older leaves of beet (after 30 minutes of feeding ^{14}C -labelled amino acids) was followed (after a chase period of 150 minutes) by accumulation of label in the younger leaves (Joy and Antcliff, 1966). Selectivity of transport was suggested by the fact that carbon from a non-protein amino acid was taken up and transported more generally to all leaves; petiole steaming did not block this transport. The identity of the labelled carbon in young and old leaves was not further studied in these particular investigations.

The fate of the carbon from asparagine, aspartate, and glutamate was followed in *Spartium junceum* (from which phloem sap can be obtained) by feeding these amino acids (^{14}C -labelled) individually to cut shoots for one hour (Pate et al., 1975). Seeds, stems, and phloem were collected, but only one harvest, at 6 hours, was made. The results indicated, however, that asparagine was transferred to the phloem relatively unchanged, since most of the radioactivity in the phloem exudate was still as asparagine, and over 50% of the radioactivity in the soluble fraction of the seeds was in asparagine. In contrast, carbon from glutamine and from aspartate entered the phloem and the soluble and insoluble fractions of the seeds, but in both cases, the radioactivity in the phloem sap was largely in other amino acids or non-amino compounds, only small amounts of label being recovered in the original compound fed as tracer.

Redistribution of nitrogen from the leaves and stems of *Pisum sativum* to the developing seeds was demonstrated by Lewis and Pate (1973). Nitrate, glutamine, and glutamate, all

^{15}N -labelled, were supplied to cut shoots for 6 to 8 hours, plants being harvested after 10 and 24 hours. The pattern of distribution of ^{15}N -label was essentially the same for all three sources; alanine, glutamate, homoserine, and γ -amino butyric acid showed the greatest ^{15}N enrichment of the soluble amino acids. In the leaf protein, glutamyl residues, serine, aspartyl residues, glycine, alanine, and threonine were the most labelled, suggesting fairly direct incorporation of the leaf soluble amino acids into protein; in the seeds, the ^{15}N -labelling was more evenly distributed through all the seed protein amino acids, suggesting considerable rearrangement of nitrogen from the compounds originally labelled.

In experiments with *Lupinus albus*, from which phloem exudate from cut pods can be obtained, ^{15}N -nitrate was fed to detached shoots; leaves, stems, fruits, and phloem sap were harvested after 4, 8, and 24 hours (Pate et al., 1975). Enriched nitrogen was recovered initially in the leaves and stem, accumulation in fruits occurring later. The specific labelling of the phloem sap increased in the later time samples; after 24 hours of feeding, aspartate, asparagine (amino- and amide-N), glutamate, glutamine (amino- and amide-N), serine, isoleucine, leucine, threonine, alanine, and valine were all labelled.

Asparagine, double-labelled with ^{15}N (amide) and ^{14}C , was fed to detached shoots of *Lupinus* (Atkins et al., 1975) and phloem sap was collected and seeds harvested after 6 hours. For both ^{15}N and ^{14}C , more than 90% of the label in the phloem sap was still in asparagine. In the seed protein, however, 66% of the labelled carbon was in aspartyl residues, and arginine, glutamyl residues,

lysine, and a few other amino acids were also labelled; ^{15}N -label in the seed protein was recovered in the amide groups (39%), arginine, aspartyl residues, leucine, glutamyl residues, glycine, alanine, and a few others. Asparagine, fed via the xylem, had been transferred to the phloem and, although incorporated as such into seed protein, its nitrogen and carbon had undergone considerable reorganisation in the seed.

Sharkey and Pate (1975) further investigated this transfer of amino acids from xylem to phloem in *Lupinus albus*. Several ^{14}C -labelled amino acids were fed individually as a short pulse via the xylem to cut shoots and radioactivity in the phloem sap from a cut fruit pod was monitored and sap collected every hour. Asparagine, valine, and threonine appeared within 45 minutes in the phloem sap as a sharp peak of radioactivity; most of that label was still in the compound originally fed. Other amino acids such as serine, citrulline, and glutamine showed a fairly sharp peak of activity in the phloem sap and a slower decline; 50% of the activity was in the supplied compound, the rest as other compounds. A third group of amino acids, including glutamate, aspartate, and homoserine, gave very broad peaks of activity in the phloem, and very little of this activity was in the originally-labelled compounds. Analyses showed that radioactivity from all three groups of amino acids was spread more evenly among the protein amino acids and non-amino compounds of the seed than in the compounds in the phloem.

In a subsequent investigation (McNeil, Atkins, and Pate, 1979) autoradiography of petioles and leaf blades, after ^{14}C -labelled amino acids had been supplied individually to cut shoots of *Lupinus*,

showed that carbon from asparagine, valine, arginine, serine, and glutamine all had the highest labelling densities in the vascular bundles; label hardly penetrated into the interveinal areas, even 220 minutes after feeding. Label from either ^{14}C -aspartate or glutamate showed much heavier labelling in the interveinal areas, but little retention of label in the veins. The amino acids retained in the vascular bundles (asparagine, valine, etc.) belong to the first two groups of the previous experiment; they showed a sharp peak of label transferred to the phloem. Aspartate and glutamate belong to the third group of amino acids, which were largely metabolized to other compounds, and whose carbon was transferred more slowly to the phloem. The suggestion from these two studies was that the amino acids appearing rapidly in the phloem still in unchanged form, such as asparagine or serine, are not greatly absorbed by the mesophyll tissue of the leaf blade, but transferred directly from the xylem to the phloem. Others like aspartate and glutamate are absorbed by the mesophyll cells, radioactivity appearing more slowly in the phloem exudate, largely in compounds other than the one originally fed to the plant.

Most of the more detailed experiments involving xylem-borne amino acids or nitrate were done with fruiting plants; in those experiments with vegetative plants, transfer of amino acids from mature leaves to young leaves was not studied in detail.

Although these studies show that nitrogen from nitrate or amino acids transported in the xylem can be utilized by fruits or young leaves, much of the carbon for the developing fruits or shoot apices comes from recently assimilated CO_2 , fixed in the fully-

expanded leaves. In sugar beet, carbon from $^{14}\text{CO}_2$ fixed by the mature leaves was transported to the young leaves directly above the treated leaf in the rosette of leaves (Joy, 1964), and contributed more to the insoluble carbohydrate in the young leaves than to protein, but all the amino acids of the protein were labelled (Joy, 1967). When $^{14}\text{CO}_2$ was fed directly to the growing leaves, proportionally more of the assimilated carbon was incorporated into protein than in the mature leaves. Serine was the most highly labelled of the soluble amino acids, glycine, alanine, and aspartate also being labelled; aspartyl residues were the most highly labelled of the protein amino acids in the growing leaves (Joy, 1967).

The transfer of carbon, from CO_2 recently assimilated by mature leaves, to young leaves and roots was shown in *Pisum arvense* (Pate, 1966); carbon fed to upper, recently expanded leaves was transported more to the shoot apex, whereas that fed to older, lower leaves was preferentially transported to the roots. Glycine, serine, and alanine were highly labelled in the protein of the fed leaves; a more even distribution of label in the protein amino acids of the shoot apex was observed.

Similar results were obtained with *Pisum sativum* (Lewis and Pate, 1973). Mature leaves were fed with $^{14}\text{CO}_2$ and the protein amino acids from the fed leaves and from seeds of fruits at that node were analyzed; phloem exudate from the stalk of the fruit at that node was also collected. Serine, glycine, aspartyl residues, alanine, and glutamyl residues had the highest specific activities in the leaf protein; labelling was more general in the seed. In

phloem sap, collected 10 hours after $^{14}\text{CO}_2$ -feeding, serine, glycine, and alanine had the highest specific activities. Ninety-four percent of the radioactivity in the phloem sap was, however, in sugars, mainly sucrose. The recently synthesized amino acids contributed, therefore, only 6% of the carbon in the phloem. They carried about 19% of the nitrogen in the phloem sap compared to the 59% carried by the amides. The lower specific activities of the amides were suggestive of their origin in the root and transport to the shoot in the xylem with subsequent transfer to the phloem.

After feeding $^{14}\text{CO}_2$ to soybean leaves, about 7% of the radioactivity recovered in the phloem exudate from cut, styler ends of pods was in amino acids, mainly serine and asparagine, although several other unlabelled amino acids were present in the exudate; about 90% of the label was sugars, mainly sucrose (Fellows et al., 1978).

These data show that, in some species, a small portion of the recently assimilated carbon may be transported to the young leaves or fruits in the form of amino acids; much of it, however, is transported as sugars. Not all species accumulate recently assimilated carbon into amino acids; hardly any radioactivity was recovered in soluble amino acids in leaves of castor bean or millet fed with $^{14}\text{CO}_2$, but labelled carbon was recovered in soluble amino acids in leaves of tomato, radish, sunflower, and soybean (Hofstra and Nelson, 1969).

A few studies have investigated the transport of amino acids from leaves by applying the labelled compounds to the surface of the leaves. Carbon from ^{14}C -labelled amino acids (glutamate,

glycine, and protein hydrolyzate applied as droplets to the upper surface) was transported from the older leaves of sugar beet to the younger ones, this transport being prevented by petiole steaming (Joy and Antcliff, 1966). Two non-protein amino acids were absorbed but not transported out of the leaves, suggesting selectivity of transport. The identity of the carbon compounds recovered in the young leaves was not examined.

In sunflower, glycine, serine, alanine, and glutamate became labelled in the leaf blade after CO_2 feeding, but only labelled sucrose was detected in the petiole. Direct feeding of ^{14}C -alanine to the leaf blade resulted in some transport of carbon to other parts of the plant but most of the radioactivity in the blade below the treated spot and in the petiole extract was in the form of sucrose, and very little in alanine, except in the treated area (Chopowick and Forward, 1974). In this species, therefore, alanine is largely metabolized in the leaf blade prior to entry of the carbon into the phloem.

Schiller and Martin (1975) supplied ^{14}C -labelled amino acids to the primary leaves of *Phaseolus vulgaris*. They showed that carbon from several amino acids was transported to other parts of the plant. This study was complicated by the fact that vascular connections between primary and apical leaves in *P. vulgaris* are not direct. Asparagine was transported initially to the hypocotyl and later appeared in the upper parts of the shoot; little of this compound was metabolized to other compounds during transport. Other amino acids (valine, lysine, leucine) were also transported from the fed leaves, but greater proportions were metabolized.

Peterson, Houseley, and Schrader (1977) demonstrated transport of applied amino acids from the leaf blade of oats into the sheath. Whereas carbon from lysine was transported into the sheath, still largely in the amino acid fraction, serine was extensively metabolized in the leaf blade, only about 12% remaining as serine, the proportions of radioactivity in the sheath being approximately the same as that in the blade.

Servaites, Schrader, and Jung (1979) applied ¹⁴C-labelled glutamate, γ -amino butyric acid, and leucine to soybean leaves. Distribution of label in the source leaf was not recorded, but both glutamate and γ -amino butyric acid were extensively converted to other soluble compounds and protein in the sink leaf (only 17% and 23% of the total radioactivity in the source leaf remaining as glutamate and γ -amino butyric acid respectively). Leucine, on the other hand, accounted for 54% of the sink leaf radioactivity. In these studies, detailed investigations of the labelled compounds of both fed leaf and young, apical leaves were not made; neither were time courses of labelling in mature and young leaves, nor direct examination of the phloem contents carried out.

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In *Pisum sativum* seedlings, the young, immature leaves of the vegetative apex are enclosed within the stipules of the next oldest leaves (the expanding leaves). These immature leaves are, therefore, unlikely to receive much direct transpirational flow of water and correspondingly little, if any, of the nitrogenous compounds carried in the transpiration stream. Immature leaves of *P. sativum* offer, therefore, a useful system in which to study the

role of the mature leaves in the redistribution of nitrogen to other parts of the plant, in particular to the young leaves of the vegetative apex.

The amides, particularly asparagine, are important in *Pisum* (Lawrence and Grant, 1963; Bauer, Urquhart, and Joy, 1977). The occurrence of several unusual, non-protein amino acids has also been reported in peas (Lawrence and Grant, 1963; van Egeraat, 1975); homoserine is quantitatively a major component of the non-protein amino acids in *Pisum* (Lawrence and Grant, 1963; Larson and Beevers, 1965; Lawrence, 1973). In addition to homoserine, the cultivar "Little Marvel" (used in the present study) has relatively high levels of β -(isoxazolin-5-one-2-yl)-alanine (BIA) (Lambein, Kuo, and van Parijs, 1976) which has been observed in leaf extracts, eluting immediately after aspartate from amino-acid analyzer columns (Mills and Joy, 1980). BIA is the compound "X³" described by Bauer, Urquhart, and Joy (1977).

The xylem sap of *Pisum sativum* contains a mixture of nitrate and organic nitrogenous compounds, mainly asparagine and glutamine, with homoserine, aspartate, and a few other amino acids being present (Bauer, Urquhart, and Joy, 1977). Experiments with ¹⁵N-labelling showed that leaves of pea seedlings utilize not only the nitrogen from nitrate, but also the nitrogen from glutamine and asparagine for further amino acid metabolism (Bauer, Joy, and Urquhart, 1977). Expanding and immature leaves were used in that study but the separate events in young and mature leaves were not followed.

The further metabolism of glutamine by glutamine synthetase in the leaves has already been discussed. In this reaction, demonstrated in pea leaves (Lea and Miflin, 1974), the amide group of glutamine is transferred to α -ketoglutarate, forming two molecules of glutamate. The utilization of asparagine has, however, resisted elucidation for much longer, although possible pathways for asparagine metabolism do exist (Lea and Powden, 1974).

Asparaginase, which catalyzes the deamidation of asparagine to yield aspartate, has been detected in developing lupin seeds (Atkins et al., 1975), but activity was very low in soybean leaf extracts (Streeter, 1977). Transamination, suggested by the redistribution of nitrogen after in vivo ^{15}N -labelling experiments (Bauer, Joy, and Urquhart, 1977) to be occurring in pea leaves, is an alternative route for asparagine utilization. Asparagine transaminase activity was detected in soybean leaves (Streeter, 1977). The product of this reaction, 2-ketosuccinamic acid (KSA) was not, however, detected in soybean leaves after in vivo labelling with ^{14}C -asparagine (Streeter, 1977). Lloyd and Joy (1978) showed that the major product of ^{14}C -asparagine metabolism in peas and soybeans was not KSA but 2-hydroxysuccinamic acid (HSA), which was also rapidly formed if ^{14}C -KSA was fed to pea leaves.

In the following study with *Pisum sativum*, the uptake and redistribution of ^{14}C -labelled asparagine and glutamine (after uptake via the transpiration stream) was followed in mature, expanding, and immature leaves over a 12-hour period in the light. The movement of amino acids from mature leaves to the immature leaves and other parts of the plant were investigated by

application of ^{14}C -labelled compounds to the lower surface of the mature leaves. The export of labelled compounds out of the mature leaves, after foliar application of labelled amino acids and amides to these mature leaves, was investigated by a phloem exudation technique.

METHODS

1. MATERIALS

A. Seeds

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

B. Chemicals

Amino acids, Sigma Chemical Co.

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

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

NCS Tissue Solubilizer, Amersham/Searle.

Omnifluor, New England Nuclear.

o-Phthalaldehyde, Sigma Chemical Co.

Triton-X-114, Rohm & Haas Canada Ltd.

Trizma-base, Sigma Chemical Co.

Trizma-HCl, Sigma Chemical Co.

All other chemicals used were Reagent Grade, supplied by Fisher or Canlab.

C. Enzymes (Supplied by Sigma Chemical Co.)

L-amino acid oxidase Crude Type I *Crotalus adamanteus* venom.

Catalase 2X crystallised from beef liver.

Cellulase Type IV crude *Trichoderma viride*.

Protease Type V *Streptomyces griseus*.

D. Radioisotopes (Supplied by The Radiochemical Centre, Amersham)

L-(U-¹⁴C)Asparagine. 105 mCi/mmol.

L-(U-¹⁴C)Glutamine. 57.3 mCi/mmol.

L-(U-¹⁴C)Aspartic acid. 10 mCi/mmol.

L-(U-¹⁴C)Glutamic acid. 10 mCi/mmol.

E. Miscellaneous

Liquid Silicone Rubber RTVII, Silicone Product, General Electric.

Catalyst (Stannous octoate), Dow Corning Corp.

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, soaked in aerated distilled water for 12 hours, and then germinated in Perlite:Vermiculite (1:1) in the dark for 5 days. The germinated seedlings (roots 8 to 10 cm long; shoots 2 cm long) were transferred to aerated, modified Hoaglands nutrient solution (Appendix A), in growth cabinets with a 12-hour photoperiod (26-28°C) and 12 hours dark (18°C). The light sources were Westinghouse 9GT 12 Daylight, High Output tubes and Westinghouse 100 watt incandescent bulbs, supplying a total of 50 watts per m² at the level of the plants (one meter from the lights).

Cotyledons were removed 15 days after the start of germination when the plants had three fully-expanded leaves. Plants were used about 20 days from the start of germination, by which time they had four fully-expanded leaves and a fifth leaf half-expanded (the "4½ leaf" stage) as shown in Figure 1. Care was taken to select experimental material at uniform stages of development.

The nutrient solution was changed at weekly intervals. Calcium and potassium nitrate (4mM NO₃) were the only external sources of nitrogen. Plants were not nodulated.

The same lighting conditions were used for all experiments as those described above.

3. TRANSPIRATION RATES

Transpiration rates were estimated by measuring water loss from shoots whose stems were cut off under water at the lower end of the epicotyl. The cut end of the stems were inserted, through slits in a covering of Parafilm, into small vials of water. The vials and shoots were weighed at intervals. No water loss was detected from similar vials without shoots.

Transpiration rates of detached leaves were estimated similarly by detaching the leaves (Leaf 4 or Leaf 5) and placing the cut end of the petioles in Parafilm-covered vials.

The effect of removing young leaves at various stages of development was observed by measuring water loss from shoots for one hour, then excising the young leaf, and measuring water loss from the remainder of the shoot for a further hour.

4. XYLEM SAP

Xylem sap was obtained by severing pea shoots at the lower end of the epicotyl and collecting the sap exuding from the roots through the cut stump. The cut end was rinsed and blotted after cutting, and sap was collected from actively exuding roots over a period of 30 minutes. The sap was collected in capillaries and frozen.

5. PHLOEM EXUDATE

Plants at the "4½ leaf" stage were selected. The 4th leaves were detached by cutting the petioles under a solution of 20mM Na_2EDTA , adjusted to pH 7.0 with NaOH and the cut ends of the leaves were placed in small vials (0.25 ml capacity) containing 0.2 ml of 20mM EDTA, pH 7.0 (King and Zeevaart, 1974). Control

leaves were cut under distilled water and transferred to vials containing water. The vials were supported on polystyrene trays in a humid chamber in the dark ($20^{\circ} \pm 1^{\circ}\text{C}$) or the light $27^{\circ} \pm 1^{\circ}\text{C}$ for up to two hours. The pH of the EDTA solution, containing exudate from the petiole, was adjusted to 2.1, and the EDTA was precipitated by centrifugation at top speed (15600 g) in an Eppendorf Micro Centrifuge (model 5412) for 2 minutes. The supernatant was analyzed on a Beckman Amino Acid Analyzer Model 119 BL, using Lithium buffers.

6. PREPARATION OF AMIDES

The amides, asparagine and glutamine, both unlabelled and ^{14}C -labelled, were separated from contaminating aspartic or glutamic acid immediately before use by passing them, at pH 7.0, over Dowex 1 (Cl form) resin. Between pH 6.5 and 8.0, aspartic or glutamic acid is bound to the resin, the amides being recovered in the effluent (Pruisner and Milner, 1970).

7. PREPARATION OF ^{14}C -2-HYDROXYSUCCINAMATE

The method of Meister (1953) was followed to prepare 2-Ketosuccinamate (KSA). Previously purified asparagine, labelled with U- ^{14}C -asparagine, was incubated with L-amino acid oxidase (Type I) and catalase at 30°C for 16 hours. The protein was precipitated with cold ethanol and the supernatant was concentrated by evaporation and passed over Dowex 50 (H^{+}) to separate the resulting KSA from any remaining asparagine. The effluent from the column, containing the KSA, was made alkaline (pH 11.0) to convert KSA to the monomer form (Stephani and Meister, 1971), and

then readjusted to pH 7.5. Sodium borohydride was added in equimolar proportion to the monomer, and the mixture incubated for 30 minutes at room temperature to reduce the KSA to 2-hydroxysuccinamate (HSA). The pH was adjusted to 2.0 to decompose the residual borohydride, and, after neutralization, HSA was absorbed on Dowex 1 (formate) and eluted in 2N formic acid (Lloyd and Joy, 1978).

8. HYDROLYSIS OF HSA TO MALATE

HSA was hydrolyzed in 2N HCl for 1 hour at 100°C. The solution was then evaporated to dryness, redissolved in water, and aliquots spotted on Whatman 3MM paper for ascending chromatography in 1-butanol: acetic acid: water (12:3:5 by volume) for 20 hours. ¹⁴C-HSA and unlabelled malate standards were run on the same chromatogram. Strips of the chromatogram were scanned for radioactivity (see Method 15 B(1)) and the position of the radioactive peaks noted. Malate was detected colourimetrically using potassium permanganate (Methods 16).

9. ANALYTICAL TECHNIQUES

Free ammonia in the soluble fractions was determined by the phenol-hypochlorite method of Bohley (1967); α -amino nitrogen by the Ninhydrin method of Yemm and Cocking (1955); and nitrate by the diphenylamine method of Feller, Bauer, and Erismann (1971).

Total organic nitrogen was estimated by Kjeldahl digestion (Bohley, 1967).

Chlorophyll was determined after extraction of tissue in 80% (v/v) acetone (Arnon, 1949).

Ehrlich's Reagent was used to detect ureides (in Chromatographic Techniques. ed. I. Smith. p. 72. Heineman. London).

10. RADIOTRACER LABELLING EXPERIMENTS

A. Detached Shoots

Shoots of *Pisum sativum* were selected which were at the "4½ leaf" stage, choosing plants in which the 5th leaf was half expanded. At the start of the light period, the stems were cut under water at the lower epicotyl and the cut ends were placed in small vials containing unlabelled feeding solution, consisting of nutrient solution (diluted to give 1.5mM NO_3), 1.5mM glutamine, and 1.5mM asparagine, and the shoots were allowed to adapt for 30 minutes.

The shoots were transferred rapidly to vials containing labelled feeding solution. In experiments with radioactive asparagine, L-(U- ^{14}C)-asparagine (1 uCi per 3 shoots, 0.01 umole asparagine) was added to 0.6 ml of unlabelled feeding solution. In experiments with radioactive glutamine, L-(U- ^{14}C)-glutamine (1 uCi per 3 shoots, 0.019 umole glutamine) was added. This volume (0.2 ml per shoot) was taken up by the cut ends of the shoots over a 30 minute period in the light. The labelled solution was washed in by unlabelled feeding solution (chase solution) and uptake and distribution of radioactivity in the light was followed in individual leaves over a period of 12 hours.

Three shoots were sampled at each time interval and the leaves (including stipules) were separated and frozen in liquid nitrogen.

B. Mature Leaves

Plants were selected with four fully-expanded leaves and the fifth leaf half-expanded. The lower surface of one leaflet of Leaf 4 was lightly touched with fine emery paper to abraid a 2 x 2 mm

area. Small rectangles of coverglass, approximately 5 x 5 mm were sealed on 3 sides over the abraded area with a mixture of Liquid Silicone Rubber and catalyst, forming small chambers between the epidermis and the glass.

After waiting 30 minutes to allow the silicone rubber to polymerize, 1 to 3 ul volumes of radioactive amide or amino acid (carrier free) were introduced into the chambers with a microsyringe. After 2 hours in the light, the plants were removed and the fed leaflet was cut off. The chamber was removed and both chamber and leaflet were rinsed to wash off surface radioactivity. The fed leaflet, the 6th leaf, the 5th leaf, the roots, and in some cases the rest of the 4th leaf were sampled separately. The rest of the shoot was usually taken as one sample or included with the rest of the 4th leaf. All the samples were frozen in liquid nitrogen. Three or four plants were used in each treatment.

In some experiments, the shoots were detached after preparation of the chambers and the cut ends placed in vials of the "chase" solution. Radiotracer feeding of the 4th leaf was then carried out with these detached shoots.

C. Detached Leaves

Plants were selected and leaves and chambers prepared as described above. The petioles of the 4th leaves were severed under 20mM Na₂EDTA, pH 7.0, or distilled water, and the cut ends of the petioles placed in vials containing either 20mM EDTA, pH 7.0, or water, to obtain a phloem exudate as described in Methods 5. Small volumes of carrier-free radiotracer were introduced into the leaf chambers as described above.

After feeding the leaves in the light or the dark for up to 2 hours, the contents of the vials were analyzed. The leaves and chambers were rinsed, and the leaves frozen in liquid nitrogen.

11. EXTRACTION OF ETHANOL-SOLUBLE COMPOUNDS

The plant samples, frozen in liquid nitrogen, were extracted by homogenizing in cold 80% (v/v) ethanol (60 to 80 ml per g freshweight) in a Ten Broeck homogenizer. The homogenate was centrifuged at 12000 g for 10 minutes and the pellet was washed with one or two 5 to 10 ml additions of ethanol, until no chlorophyll remained in the pellet. The supernatant fractions were pooled and concentrated to small volume under vacuum at 30°C. Water and chloroform were added to the concentrated fraction to give a final water: chloroform ratio of 3: 8.5 (by volume). The mixture was agitated vigorously, and then poured into glass centrifuge tubes, allowed to settle in the cold for one hour, and then centrifuged at 17000 g for 10 minutes. The chloroform layer was re-extracted with water and the pooled aqueous fractions made up to known volume. The final aqueous extract contained the equivalent of 0.2 to 0.5 g fresh weight per ml.

In some experiments, water-soluble fractions were prepared by homogenizing the tissue in distilled water containing SSA at 50 mg per ml and centrifuging the mixture at 17000 g for 10 minutes to precipitate the proteins (deWolfe, Baskurt, and Cochrane, 1967). Data in Appendix B shows that there was little difference in amino-acid concentration between leaf extracts prepared by ethanolic extraction or by SSA precipitation.

12. WATER-SOLUBLE COMPOUNDS

Dowex 50 (H^+) and Dowex 1 (formate) resins (X8, 200-400 mesh) in 7 by 80 mm columns were used to separate aqueous extracts into amino-acid, organic-acid, and neutral fractions (Canvin and Beevers, 1961). Extracts were passed first over Dowex 50, and then over Dowex 1. Amino acids were eluted from Dowex 50 with 2 N ammonium hydroxide to avoid hydrolysis of the amides; organic acids were eluted from Dowex 1 with 0.1 to 8.0 N formic acid. The neutral compounds did not absorb to either Dowex 50 or Dowex 1 and were collected in the fraction which passed through both columns. The Dowex 50 and Dowex 1 eluates were air-dried to remove ammonia and formic acid respectively. The dried samples were redissolved in water prior to further analysis. The pH of the Dowex 1 eluate was raised to 9.0-10.0 then neutralized before chromatography in 1-butanol: acetic acid: water to prevent the variable hydrolysis of HSA to malate which can otherwise occur (Lloyd and Joy, 1978).

13. ETHANOL-INSOLUBLE FRACTION

The ethanol-insoluble pellet was resuspended in 1 N NaOH and extracted in the cold overnight. After centrifugation at 12000 g for 10 minutes, the pellet was re-extracted in 1 N NaOH, and the NaOH soluble supernatants pooled and made up to known volume.

14. ENZYMATIC DIGESTION OF ETHANOL INSOLUBLE MATERIAL FOR ESTIMATION OF RADIOACTIVITY

Approximately 10 mg samples of the air-dried ethanol-insoluble pellet were placed in centrifuge tubes with 1 ml of 0.1M Tris buffer, pH 7.7, 5mM $CaCl_2$. Six units of protease were added to

each tube and mixed to dissolve. A lens of toluene was added, and the tubes were covered and incubated at 30°C for 24 hours (M.M. Smith, personal communication).

After evaporation of the toluene, the tubes were boiled to denature the protease, and centrifuged at 17000 g for 10 minutes. The supernatant was decanted and the pellet washed with the Tris buffer. The supernatants were pooled and aliquots taken for estimation of radioactivity.

Cellulase (2.5 units per sample) was weighed out, dissolved in 1 ml of 0.1M sodium acetate buffer, pH 5.0, and added to each protease-treated pellet. The tubes were covered as before and incubated for 48 hours at 30°C. The samples were centrifuged at 17000 g, the pellet washed with sodium acetate buffer, and aliquots of the combined supernatant fraction taken for determination of radioactivity.

The residual material, after solubilization in NaOH or after enzymatic digestion, was solubilized in NCS: water (9: 1 by volume) in tightly capped vials at 50°C overnight. One ml volumes of the resulting solution were mixed with 10 ml of non-aqueous scintillation cocktail (Omnifluor, 4 g per litre of toluene) for estimation of radioactivity.

15. AMINO-ACID ANALYSIS

A. Quantitative amino-acid analysis of ethanol-extracted aqueous fractions or SSA-precipitated water-soluble fractions was carried out with a Beckman Amino Acid Analyzer, Model 119 BL, using lithium buffers.

B. Separation of amino acids for estimation of radioactivity was achieved by several methods.

(i) High-voltage electrophoresis and partition chromatography:

Aqueous extracts or Dowex 50 eluates were subjected to high-voltage electrophoresis at 39 V per cm, 0.8 to 1.0 mAmp per cm, on Whatman 3MM paper for 1 hour with 0.5M sodium acetate buffer, pH 4.1.

Picric acid markers were placed at each edge and between samples.

In this system, aspartic acid ($R_p = \text{picric} + 80$), glutamic acid ($R_p + 50$), neutral amino acids, γ -amino butyric acid (GABA) ($R_p - 55$) and basic amino acids ($R_p - 140$) could be separated.

Organic acids ran beside and ahead of picric.

After drying the paper, 4 to 5 cm-wide strips containing the separated samples were cut, and spots of radioactivity were located with a Packard Radiochromatogram Scanner, Model 7201.

These radioactive spots were cut out and the labelled compounds extracted from the paper by washing 4 times for 1 hour each in 3 ml 60% (v/v) ethanol. After evaporation of the ethanol, radioactivity was estimated by aqueous liquid scintillation counting.

The paper remaining after extraction was oven-dried and residual radioactivity determined by counting the paper, cut into 1 cm squares, in 10 ml non-aqueous scintillation fluid at 70% efficiency.

Neutral amino acids were further separated after elution from the paper in 60% (v/v) ethanol as above, by ascending paper chromatography on Whatman 3MM paper in a mixture of 1-butanol: acetic acid; water (12: 3: 5 by volume) for 20 hours. Radioactive spots were located, and labelled compounds eluted and counted as described above.

(ii) Thin-layer chromatography: Amino acids, after elution from Dowex 50, were separated by 2-dimensional thin-layer chromatography on glass plates, precoated with MN 300 cellulose, 250 microns (Analtech Inc.). Plates were developed twice in the first dimension, initially in methylethyl ketone: acetone: pyridine: water: formic acid (35: 35: 15: 15: 2 by volume), followed by 2-propanol: formic acid: water (20: 1: 5 by volume). In the second dimension, plates were run in 1-butanol: acetone: diethylamine: triethylamine: water (10: 10: 1: 1: 5 by volume) (Lea, Mills, and Mifflin, 1979). The first solvent system of the first dimension allows the separation of a compound present in *Pisum sativum* which otherwise interferes with the resolution of aspartate. (W.R. Mills, personal communication). Amino acids were located with the o-phthalaldehyde reagent described by Davies and Mifflin (1978). The fluorescent spots were scraped off and radioactivity determined by liquid scintillation counting in non-aqueous scintillation fluid (Methods 16).

(iii) Amino-acid analyzer: The Amino Acid Analyzer was also used for separation of amino-acid samples by collecting all of the effluent from the column before it entered the colourimeter, or by using the stream divider and collecting 90% of the effluent unreacted with ninhydrin. Aliquots of the 1 or 2 minute fractions were counted for radioactivity.

Radioactive or unlabelled standards were run with each of these methods.

16. ORGANIC ACIDS

Organic-acid fractions were separated by ascending paper chromatography on Whatman 3MM in 1-butanol: acetic acid: water (12: 3: 5 by volume) for 20 hours.

Radioactive compounds were located with the Radiochromatogram Scanner as described above. Organic acids were visualized colourimetrically with potassium permanganate (Gordon, Thornburg and Werum, 1956), or aniline-xylose (Wood, 1958).

Radioactive compounds suspected of being HSA were eluted from the paper and hydrolyzed as described (Methods 8). The hydrolyzed product was rechromatographed with either ^{14}C -malate or unlabelled malate visualized with potassium permanganate. Appearance of ^{14}C -malate after hydrolysis of suspected HSA confirmed its identity. In the solvent system used, HSA had an R_F of 38 and malate an R_F of 50. KSA (monomer) had R_F of 25; KSA (dimer) R_F of 8.

17. LIQUID SCINTILLATION SPECTROSCOPY

Radioactivity in aqueous samples was estimated by liquid scintillation counting in a Packard Tri-Carb Spectrometer, Model 3003, Automatic Control Mode 574. Acidified samples were mixed with 10 ml of TOX (Triton-X-114, 25% by volume, Omnifluor, 4 g per litre, Xylene, 75% by volume) and counted at 14% gain, window setting 50 - 1000. The optimum water content for this cocktail is 6% (v/v). (Pande, 1976). Estimations of radioactivity were corrected for background fluorescence, and for quenching by means of the external cesium standard. The efficiency of radioactive determinations was estimated by comparing the decrease in radiation of the external standard at 2% gain, window settings 300 - ∞ in the

presence of ^{14}C -labelled standards in 10 ml TOX and 0.6 ml water quenched with nitromethane. Maximum efficiency was 85 to 90% with this scintillation cocktail.

Non-aqueous samples were counted in 10 ml toluene containing Omnifluor, 4 g per litre. Paper and cellulose from thin-layer plates were oven-dried and added to the fluid; NCS-solubilized material was added to the toluene-Omnifluor cocktail in the proportion of 1 ml NCS to 10 ml of cocktail. Efficiency of counting was 70%.

RESULTS

GROWTH OF PEA PLANTS

Pisum sativum (L. c.v. Little Marvel) were grown in nutrient solution under the conditions described in the Methods. By 20 days after the start of germination, they had four fully-expanded leaves and the fifth leaf half-expanded as shown in Figure 1. The leaves, starting with the fourth leaf, were sampled at daily intervals from the 15th day after germination till about the 30th day, when Leaf 7 was fully expanded. Fresh and dry weights, nitrogen content, and chlorophyll content of individual leaves were measured. The length of the leaves was measured and their stage of development described.

The leaves developed through morphologically distinct stages, characterized by the unfolding of the leaf-blades and stipules (Figure 2). At Stage 3, for example, the young leaf becomes visible, partially exposed by the opening of the stipules of the next older leaf; at Stage 4 it is fully exposed; at Stage 5 the leaflets of the young leaf open but its stipules are still closed; at Stage 6 the stipules start to open; and at Stage 7 the stipules have opened and the leaf is fully expanded. When the leaves were fully expanded, i.e. at Stage 7, the next leaf had just fully emerged but its leaflets and stipules were still unfolded.

These developmental stages were one day apart in plants which were undisturbed except for changing of nutrient solutions and removal of cotyledons, and also in plants which were subjected to additional minimal daily handling to remove samples and make measurements. Developmental stages and rates of expansion were

Figure 1. *Pisum sativum* c.v. Little Marvel at the "4½-leaf" stage, 20 days after germination, by which time the plants have 4 fully-expanded leaves, Leaf 1 being the oldest. Leaf 5 is half expanded.

150 mm

100

50

0

Figure 2. Stages of development of the expanding leaves of pea plants, characterized by the unfolding of leaflets (L) and stipules (S). In 3-week-old seedlings grown in nutrient solution, with a 12-hour photoperiod, these stages were one day apart, solid lines represent the expanding leaf; dotted lines represent the next older or younger leaf.

100 mm

L leaflet
S stipule

Developmental stage

the same for the 4th, 5th, 6th, and 7th leaves. Under the growth conditions used, extra leaflets and flower buds appear after Leaf 7.

Although the leaf was fully expanded by Stage 7 and no further visible change took place, its age, in days from Stage 1, could be monitored by the stage of development of the next youngest leaf as shown in Table 1. Thus when Leaf 5 has just fully expanded (Stage 7), Leaf 4 is at Stage 10, and Leaf 6 at Stage 4.

Even when 5-day-old seedlings were selected to be uniform for further growth in nutrient solution, the plants at 20 days from germination showed considerable variability in leaf length and leaf number. Plants were selected by eye for uniformity and the length of Leaves 5 and 6 measured. The mean lengths and error are given in Table 2. It was found that when leaves were selected whose lengths were close to the mean, their fresh weights and dry weights were also uniform and were proportional to the length (Figure 3, A and B). Reasonably uniform material could therefore be selected by stage of development and leaf length.

Fresh and dry weights, organic nitrogen content, and chlorophyll content of the expanding pea leaves (Leaf 5) at each stage of development are shown in Figure 4. Dry weight and total organic nitrogen content reached a maximum by Stage 7, when the leaves were fully expanded. Fresh weight and chlorophyll content continued to increase for a further 3 days.

At Stage 4 to 5, the stage of Leaf 5 selected for the majority of experiments (4½-Leaf plants), the expanding leaf had attained one-half of its maximum dry weight and nitrogen content, and one-third of its maximum fresh weight and chlorophyll content.

TABLE 1

Relationship of Stages of Development of Successive Leaves

Leaf	Stage of Development										
4	4	5	6	7	8	9	10	11	12	13	
5	1	2	3	4	5	6	7	8	9	10	
6				1	2	3	4	5	6	7	
7							1	2	3	4	

TABLE 2

Length of Leaves 5 and 6 During Development

		Stage of Development						
Leaf		1	2	3	4	5	6	7
5	n ^a	4	8	15	34	20	18	15
	length mm	10.8	16.1	26.9	40.5	51.7	71.1	81.5
	σ	± 1.0	± 2.2	± 3.8	± 6.3	± 7.0	± 11.3	± 10.6
6	n	14	22	17	25	14	12	-
	Length mm	12.4	17.9	28.1	41.2	51.7	67.1	-
	σ	± 2.3	± 2.3	± 4.0	± 7.3	± 7.6	± 9.0	-

n^a = Number of leaves in sample

Figure 3. Relationship between fresh and dry weights and leaf length of the 5th and 6th leaves of peas during leaf expansion. A, fresh weight and leaf length; B, dry weight and leaf length.

Figure 4. Fresh weight, dry weight, total organic nitrogen content and chlorophyll content of pea leaves during leaf expansion. ▲, fresh weight; △, dry weight; O, total organic nitrogen; ●, chlorophyll.

The nitrogen content of these leaves reached its maximum when the leaves had just fully expanded (Stage 7). As shown in Figure 5, the nitrogen content of Leaves 4 and 5 remained high for a further 2 to 3 days, and then declined slowly from Stage 10 onwards as shown by the data for ~~Leaf 4~~ in Figure 5.

TRANSPIRATION RATES

Rates of water loss from detached shoots and detached leaves were measured as described (Methods 3) over 2 to 3 hours in the middle of the light period. These rates were used as estimates of the transpiration rates. Transpirational water loss from pea shoots with "4½" leaves was in the range of 0.4 to 0.7 ml per hour; the mean for the detached, half-expanded 5th leaf (Stage 4 - 5) was 0.097 ± 0.25 ml per hour.

The initial transpiration rates of 12 pea shoots were measured for one hour, and the rates were measured for a further hour after excision of the youngest leaf (Leaf 5 or 6). In all of five shoots, in which the young leaf was still enclosed within the stipules of the older leaf (young leaf at Stage 1 or 2), there was no difference between the initial and final transpiration rates of the shoot; the young leaf transpired negligible amounts of water (Figure 6A). When, on the other hand, the excised leaf was partially or fully exposed, the change in transpiration rate after excision of the leaf was considerable in all of seven shoots, (leaves at Stage 5 or 6, two examples shown in Figure 6B).

In summary, the 5th leaf when half expanded contributed about one-fifth of the total transpiration of the shoot; young leaves still enclosed within the stipules of the older leaves showed negligible transpiration.

Figure 5. Organic nitrogen content of Leaves 4 and 5 during their development. O, Leaf 4; ●, Leaf 5.

Figure 6. Transpirational water loss from detached pea shoots before and after removal of the youngest leaf. A, excised leaf at Stage 1 or 2 (○ and ●); B, excised leaf at Stage 5 or 6 (□ or ■).

Transpirational water loss from Leaf 4 when fully expanded was estimated by detaching Leaf 4 and measuring water loss over 1 to 2 hours. The mean water loss from these fully-expanded leaves under the experimental conditions used was 0.14 ± 0.04 ml per hour.

Similar measurements were made on leaves detached and placed in the humid chambers used in the Radiotracer Labelling Experiments III. In the light, leaves with petioles in water or 20mM EDTA lost water at rates of 0.070 ± 0.007 g per hour and 0.038 ± 0.004 g per hour respectively; those in humid chambers in the dark, with petioles in water or EDTA, lost 0.019 ± 0.005 g per hour and 0.005 ± 0.002 g per hour respectively. The high humidity of the chambers reduced transpirational water loss from 0.14 g per hour to 0.07 g per hour in the light and to 0.019 g per hour in the dark. In both light and dark chambers, the EDTA treatment appeared to reduce transpiration from the leaves.

XYLEM BLEEDING SAP

Bleeding sap was collected from the detopped stumps of vigorously bleeding roots. Table 3 shows amino-acid analyses for three samples (from 15 to 20 plants per sample) taken during the light period. Asparagine was the major amino acid, with glutamine, homoserine and aspartate also quantitatively important, although small amounts of other amino acids were measured. The four amino acids, asparagine, glutamine, homoserine, and aspartate supplied, therefore, most of the amino-N transported in the transpiration stream to the shoots. In the sample taken at 13.00 hours, for example, the amino-nitrogen concentration in the bleeding sap was 11.10mM, 70% of this being asparagine plus glutamine and a further

TABLE 3

Amino-Acid Analyses of Xylem Bleeding Saps
from Detopped Pea Roots of Plants Grown in
Nutrient Solution to the 4½-Leaf Stage

Amino Acid	Time (Hours) ^a		
	09.00	11.00	13.00
	n moles per root ^b		
Asp	4.04	11.25	7.59
BIA	2.59	3.22	2.74
Thr	2.00	1.51	0.96
U	tr	tr	0.52
Ser	1.57	1.66	0.69
Asn	36.99	43.40	35.24
Glu	1.07	0.90	0.64
Gln	5.62	7.13	10.66
Hse	14.50	14.23	16.40
Aaa	0.80	0.87	0.70
Gly	0.21	0.34	0.14
Ala	0.41	0.40	0.20
Val	2.42	1.31	1.17
Cys	0.78	1.30	1.08
Met	0.11	0.17	0.22
Ile	1.44	0.54	0.31
Leu	1.21	0.38	0.27
Tyr	0.36	tr	tr
Phe	0.62	0.47	0.17
Gaba	0.84	0.45	0.58
NH ₃	0.61	0.50	0.41
Orn	0.46	0.37	tr
Lys	2.33	1.15	0.68
His	1.59	1.47	1.11
Arg	tr	1.75	tr

^aThe photoperiod started at 06.00 hours, and therefore 09.00, 11.00 and 13.00 hours represent 3, 5, and 7 hours into the light period.

^bBleeding sap was collected for 30 minutes from 15 to 20 vigorously bleeding roots.

18% being homoserine plus aspartate. Nitrate in the bleeding sap was 4 to 5mM N. Low levels (less than 1mM N) of ureides were detected in the sap. About two-thirds of the total nitrogen in the xylem bleeding sap was therefore in the form of amino-N, mainly the amides asparagine and glutamine. The concentration of nitrogen measures in the sap would of course be diluted by the transpirational flow of water through the plant.

RADIOTRACER LABELLING EXPERIMENTS I. UPTAKE AND DISTRIBUTION OF ^{14}C -AMIDES SUPPLIED VIA THE XYLEM TO DETACHED SHOOTS

Radioactive amides, either ^{14}C -asparagine or ^{14}C -glutamine were supplied in a mixture of nitrate, asparagine, and glutamine to the cut ends of detached "4½-Leaf" pea shoots (as described, Methods 10 A), and the shoots were allowed to take up the mixture in the transpiration stream in the light for 30 minutes. The labelled solution was "chased" with unlabelled feeding solution, and the uptake and distribution of labelled compounds into individual leaves in the light was followed by sampling at intervals for a maximum of 12 hours from the start of the experiment. Three shoots were used for each time sample and Leaves 3 to 6 were cut off, frozen, and extracted separately (Methods 11), pooling the extracts from the three replicates.

Figure 7 shows the total radioactivity, ethanol-soluble and insoluble fractions, of the 3rd, 4th, 5th, and 6th leaves of shoots labelled with ^{14}C -asparagine (Figure 7A) and with ^{14}C -glutamine (Figure 7B). In both experiments, the levels of radioactivity in the fully-expanded leaves (Leaves 3 and 4) increased in the first hour of feeding. In Leaves 3 and 4 of the glutamine-labelled and in Leaf 3 of the asparagine-labelled plants,

Figure 7. Total radioactivity in Leaves 3, 4, 5, and 6 of pea shoots fed a mixture of NO_3^- , asparagine and glutamine via the xylem. Radiotracer (A, ^{14}C -asn; B, ^{14}C -gln) was supplied for 30 minutes at the start of the light period and was then chased with unlabelled feeding solution for the rest of the light period. Samples were taken 1, 2, 6, and 12 hours from the start of the experiment. $\circ$, Leaf 3; Δ , Leaf 4; $\bullet$, Leaf 5; $\blacktriangle$, Leaf 6.

the levels of radioactivity were at a maximum in the 1-hour samples and declined during the subsequent time intervals. Radioactivity in Leaf 4 of the asparagine-labelled plants reached a maximum in the 2-hour sample, and then declined.

In the expanding leaves (Leaf 5) in both experiments, the levels of radioactivity increased gradually until 6 hours after the initial labelling period and remained at this high level until the 12-hour sample. Radioactivity in the immature leaves (Leaf 6) of both sets of plants increased slowly over the first 6 hours to a maximum and then remained at that level.

From these data it would appear that the pattern of labelling varied with the stage of development of the leaves. The fully-expanded leaves (3 and 4), which were transpiring vigorously but whose nitrogen levels had reached their maximum, appeared to re-export some of the labelled carbon which they had absorbed during the initial labelling period. The half-expanded leaves (Leaf 5), which were also transpiring, rapidly took up labelled material at first, but the levels of radioactivity continued to rise after this initial labelling period. A small part of this later rise might be due to delay in label reaching the further areas of the stem, but most will be the result of export of radioactively-labelled compounds out of the mature leaves into the young leaves. The immature leaves (Leaf 6) were enclosed in the stipules of the older leaves. Since it was shown that these leaves transpired little water, the major part of the radioactivity recovered in these leaves must have come as exported material from the older leaves.

To test this idea, three groups of shoots of different ages were selected so that leaves 3, 4, 5, and 6 were at different stages of development in the three groups. The ages chosen were 16, 19, and 22 days after the start of germination. At these ages, Leaf 3 was at Stage 7 (fully expanded), 10, and 13 respectively; Leaf 4 was at Stage 4 (half expanded), 7, and 10; Leaf 5 at Stage 1 (enclosed within stipules), 4 and 7; Leaf 6 was too tiny to dissect out at Day 16 but was at Stage 1 at Day 19 and Stage 4 at Day 22.

The shoots were labelled with ^{14}C -asparagine and samples taken exactly as in the previous experiment. The levels of total radioactivity in the individual leaves are shown in Figure 8. Figure 8A shows that as Leaf 3 aged, less ^{14}C -label was taken up initially into the leaf, and a greater proportion of the label was exported in the 6 hours following the labelling period. Figure 8B shows that in Leaf 4 the levels of radioactivity continued to rise during 12 hours when the leaf was expanding (Stage 4), but that as the leaf aged, the radioactivity taken up initially was later exported from the leaf. Leaves 5 and 6 (Figure 8, C and D) show the gradual uptake of radioactivity when these leaves were enclosed within the stipules of the older leaves (Stage 1) and the change in this pattern of uptake when the leaves emerged from the stipules and started rapidly expanding.

The radioactivity in the ethanol-soluble and in the insoluble fractions was measured in the leaves from 19-day shoots (fifth leaf, half expanded) labelled with ^{14}C -asparagine. Figure 9 (A and B) shows that most of the total radioactivity of the fully-expanded

Figure 8. Distribution of total radioactivity, derived from xylem-fed asparagine, in pea leaves at different stages of development. Radiotracer was supplied as described in figure 7. to shoots of 3 different ages (16, 19, and 22 days). Data for the leaves with the same number (3, 4, etc.) at the stages of development indicated (described in Figure 2), are grouped together. S, stage of development; d, day; 0, Stage 1; ●, Stage 4; ▽, Stage 7; ▲, Stage 10; □, Stage 13.

A, Leaf 3: Stages 7 (▽), 10 (▲), and 13 (□).

B, Leaf 4: Stages 4 (●), 7 (▽), and 10 (▲).

C, Leaf 5: Stages 1 (0), 4 (●), and 7 (▽).

D, Leaf 6: Stages 1 (0), and 4 (●).

leaves (3 and 4) was in the ethanol-soluble fraction. A small amount of label was recovered in the insoluble fraction. The patterns of initial rapid uptake of labelled material into the leaves and subsequent export, seen in the values for total radioactivity (Figure 7A) were largely the result of the rapid uptake and later export of soluble radioactivity.

Radioactivity in the soluble fraction of Leaf 5 (Figure 9C) increased rapidly at first and then more gradually for 6 hours. Although the level of soluble radioactivity dropped during the second 6-hour period, the final level was still as high as that 1 hour after the start of the experiment. There was considerable incorporation of radioactivity into the insoluble fraction of Leaf 5, the level rising steadily during the 12 hours.

In the immature leaves (Leaf 6, Figure 9D) the radioactivity present in the first 2 hours was almost entirely in the soluble fraction. Thereafter, the radioactivity in the insoluble fraction increased steadily while that in the soluble fraction declined very slowly until, by 12 hours, the levels of label in soluble and insoluble fractions were equal.

Data for a similar experiment in which 19-day shoots were labelled via the xylem with ^{14}C -glutamine are shown in Figure 10. The patterns of labelling of the soluble and insoluble fractions are similar to those in the experiment with ^{14}C -asparagine. In the fully-expanded leaves (Figure 10, A and B), radioactivity from the labelled amide entered the soluble fraction within 1 hour and was rapidly lost from these fractions. There was only a small incorporation of radioactivity into the insoluble fraction. In

Figure 9. Distribution of radioactivity in the ethanol-soluble and ethanol-insoluble (1N NaOH-soluble) fractions of leaves of pea shoots fed via the xylem with ^{14}C -asparagine for 30 minutes as described in Figure 7.

●, ethanol-soluble; ○, ethanol-insoluble; A, Leaf 3; B, Leaf 4; C, Leaf 5; D, Leaf 6.

Figure 10. Distribution of radioactivity in the ethanol-soluble and ethanol-insoluble (in NaOH-soluble) fractions of leaves of pea shoots fed via the xylem with ^{14}C -glutamine for 30 minutes as described in Figure 7.

●, ethanol-soluble; ○, ethanol-insoluble; A, Leaf 3; B, Leaf 4; C, Leaf 5; D, Leaf 6.

the rapidly expanding leaves (Figure 10C) there was also a rapid flow of label into the soluble fraction, but the level remained high for the subsequent 6 hours. As with the asparagine-labelled plants, there was also considerable incorporation of carbon from glutamine into the insoluble fraction of these leaves. The pattern of labelling in the immature leaves (Figure 10D) was the same in both experiments.

The removal of the ethanol-soluble and NaOH-soluble fractions left a NaOH-insoluble residue. The radioactivity in these residues was measured after solubilization of the material in NCS (Methods 14). In all the samples, the radioactivity found in the NaOH-insoluble residue was less than 1% of the total label recovered in the sample. Data for the longest time sample (12 hours) of ^{14}C -asparagine-labelled plants are shown in Table 4. Very little radioactivity was recovered in these NaOH-insoluble residues.

Aqueous samples were prepared from the ethanol-soluble extracts and passed over Dowex-50 and Dowex-1 resins as described in the Methods (12). The distribution of radioactivity in the amino-acid, neutral, and organic-acid fractions are shown in Figure 11 for ^{14}C -asparagine-labelled plants, and Figure 12 for ^{14}C -glutamine-labelled plants.

In both experiments, the early rise in radioactivity observed in the ethanol-soluble fraction in the mature and the expanding leaves was paralleled in the amino-acid fractions of these leaves. Label from the amides flowed into and later out of the amino-acid fractions of the mature leaves. There was a slow and relatively small incorporation of label into the organic-acid

TABLE 4

Radioactivity in the Ethanol-Soluble, Ethanol-Insoluble,
and NaOH-Insoluble Residues of Leaves of Pea Shoots
Labelled with ^{14}C -Asparagine for 12 Hours

Leaf	Ethanol- Soluble	Ethanol- Insoluble	NaOH-Insoluble Residue	Radioactivity (cpm $\times 10^{-3}$)	% ^a
3	88.70	27.5	0.60		0.5
4	261.60	54.3	1.66		0.5
5	319.90	158.0	4.34		0.9
6	45.30	49.3	0.21		0.2

^aPercent of total radioactivity recovered in leaf.

Figure 11. Distribution of radioactivity in the amino-acid, organic-acid, and neutral fractions of leaves of pea shoots fed via the xylem with ^{14}C -asparagine for 30 minutes as described in Figure 7. O, amino acids; ●, organic acids; □, neutral compounds; A, Leaf 3; B, Leaf 4; C, Leaf 5, D, Leaf 6.

Figure 12. Distribution of radioactivity in the amino-acid, organic-acid, and neutral fractions of leaves of pea shoots fed via the xylem with ^{14}C -glutamine for 30 minutes as described in Figure 7. O, amino acids; ●, organic acids; □, neutral compounds; A, Leaf 3; B, Leaf 4; C, Leaf 5; D, Leaf 6.

fractions of the mature leaves of both asparagine- and glutamine-labelled plants. In the expanding leaves of asparagine-treated shoots (Leaf 5, Figure 11C), the radioactivity of the amino-acid fraction was initially high until 2 hours, and then declined steadily. During the early part of this decline, the level of radioactivity in the organic acids of the same leaves rose sharply. Between 6 and 12 hours, the radioactivity in both amino-acid and organic-acid fractions declined, paralleling the decline in the ethanol-soluble fractions. In the expanding leaves of the glutamine-treated shoots, (Figure 12C), there was some increase in ^{14}C -labelling of the organic-acid fraction, but markedly less than in the asparagine-labelled shoots. In Leaf 5, some incorporation of radioactivity into the neutral fraction occurred, but little labelling of the neutral compounds in any other leaf sample, either asparagine- or glutamine-labelled, was observed.

The distribution of radioactivity in the soluble and insoluble compounds is shown in Figure 13 (asparagine-labelled) and Figure 14 (glutamine-labelled). Considering first the data from the asparagine-labelled shoots, it is apparent that in the mature leaves after 1 hour most of the radioactivity was still in its original form; asparagine amounted to 85% of the total radioactivity recovered in Leaf 3, and 86% in Leaf 4. In the later time samples of these leaves, asparagine was still the major labelled compound; changes in the total radioactivity of the mature leaves were largely the result of changes in the amount of label in asparagine.

In the expanding and immature leaves in the first hour, again most of the radioactivity was in asparagine; 89% in Leaf 5 and 92%

in Leaf 6. In Leaf 5, although asparagine was still the major labelled compound in the later time samples, the increase of label in the insoluble material and in the other soluble compounds was considerable. In Leaf 6, the radioactivity in asparagine decreased in the later time samples concurrent with the rise in labelling in the insoluble fraction and other soluble compounds.

The organic-acid fraction constituted a considerable proportion of the soluble radioactivity in the later time samples of the young leaves, particularly Leaf 5. When this fraction was further analyzed, by high-voltage paper electrophoresis and ascending partition chromatography, this radioactivity was found to be mainly in one major compound. At the time these two experiments were carried out, this compound was believed to be KSA, but a compound having the same characteristics, obtained from ^{14}C -asparagine, was shown by Lloyd and Joy (1978) to be HSA.

In the glutamine-labelled plants, most of the glutamine was converted to glutamate in all of the samples analyzed (no further separation of the amino-acid fraction of Leaf 6 was carried out). In the mature leaves in the first 1 or 2 hours, most of the radioactivity was still as glutamine plus glutamate; 71% of the total label in Leaf 3 and 70% in Leaf 4. In the later time samples of these leaves, although there were slight increases in the label in the insoluble fractions, and small changes in the other soluble compounds, most of the changes which took place in the labelling in the total levels of radioactivity were the result of changes in the labelling of glutamine plus glutamate. Since there was little change in the levels of radioactivity in the rest of the soluble

Figure 13. Distribution of radioactivity in soluble and insoluble compounds of leaves of pea shoots fed with ^{14}C -asparagine via the xylem for 30 minutes as described in Figure 7.

asparagine
aspartate
glutamate
neutrals
organic acids
insoluble

Figure 14. Distribution of radioactivity in soluble and insoluble compounds of leaves of pea shoots fed with ^{14}C -glutamine via the xylem for 30 minutes as described in Figure 7.

glutamine
glutamate
aspartate
γ -amino butyric acid
neutrals
organic acids
insoluble

fraction or in the insoluble fraction, most of this decrease in label in glutamine and glutamate could be a result of export from these leaves.

In Leaf 5, the expanding leaf, the levels of radioactivity in glutamine plus glutamate decreased with time, but the total radioactivity remained high as labelling increased markedly in the insoluble fraction. In Leaf 6, 87% of the total radioactivity in the leaf after 1 hour was in the amino-acid fraction. In the later time samples, very little label was recovered in that fraction, but as the total label in Leaf 6 rose, that in the insolubles increased considerably.

The organic-acid fractions of Leaves 4 and 5 were further analyzed; radioactivity was found in five main compounds, which were not further identified:

The identity of the labelled material in the insoluble fraction was investigated by proteolytic and cellulytic digestion (Methods 14) of the ethanol-insoluble fractions of mature (Leaf 4) and expanding (Leaf 5) asparagine- and glutamine-labelled plants. The data in Table 5 shows that the NaOH-soluble part of the ethanol-insoluble fraction of mature leaves of asparagine- and glutamine-labelled mature leaves, 91.5% and 89.3% respectively, was almost the same as the protease-soluble fraction, 92.7% and 93%. In the young leaves the percentages of radioactivity released by NaOH extraction were 98.4 and 98.8 for asparagine- and glutamine-labelled leaves, while those released by protease digestion were 95.1 and 96.3 respectively. The NaOH solubilization extracted, therefore, the same proportion of the total radioactivity as proteolytic digestion.

TABLE 5

Radioactivity in the Ethanol-Insoluble Fractions of ^{14}C -Asparagine- and ^{14}C -Glutamine-Labelled Leaves. Percentage Radioactivity Released by Enzyme Digestion and NaOH Extraction.

Sample	% Radioactivity in Insoluble Fraction			Residue cpm $\times 10^{-4}$
	Protease-Soluble	Cellulase-Soluble	Residue	
Leaf 4				
Asn	92.7	3.9	3.4	6.48
Gln	93.0	5.0	2.0	7.33
Leaf 5				
Asn	95.1	2.7	2.2	7.28
Gln	96.3	2.6	1.1	9.84

	% Radioactivity in Insoluble Fraction		Residue cpm $\times 10^{-4}$
	NaOH-Soluble	Residue	
Leaf 4			
Asn	91.5	8.5	6.22
Gln	89.3	10.7	7.13
Leaf 5			
Asn	98.4	1.6	8.88
Gln	98.8	1.2	9.68

The labelling patterns in the amino acids of protein digests were not studied.

In summary, it was shown in these experiments that when ^{14}C -labelled amides were supplied to detached shoots via the xylem in the transpiration stream, carbon from the amides entered the mature leaves, and was then exported from them. The large change in levels of radioactivity in these leaves was a result of change in labelling of asparagine (in the asparagine-labelled plants) and glutamine and glutamate (in the glutamine-labelled ones).

Radioactive carbon reaching the immature leaves was initially mainly asparagine in the asparagine-treated plants, and in the amino acid fraction of glutamine-treated plants, suggesting that the labelled amides flowed into and then out of the mature leaves with little metabolism, exchange with stored pools, or incorporation into protein, and that amino acids leaving the mature leaves were transported to the young leaves.

RADIOTRACER LABELLING EXPERIMENTS II. UPTAKE AND TRANSPORT OF LABELLED AMIDES AND AMINO ACIDS SUPPLIED TO THE MATURE LEAVES

Introduction

The results of the last group of experiments suggested that amides arriving in the mature leaves via the transpiration stream underwent a small exchange with existing soluble pools and only a small incorporation into protein, but were mainly re-exported out of the leaf. These findings were consistent with the fact that the mature leaves, by the time they were fully expanded, had reached their maximum nitrogen levels.

The immature leaves, still enclosed within the stipules of the older leaves and therefore not receiving nitrate or organic nitrogenous compounds in the transpiration stream, must depend on nitrogenous compounds (presumed to be mainly amino acids) in the phloem translocate as nitrogen source for protein and amino acid synthesis. It was found that radioactivity entering the immature leaves was almost entirely in the amino acid fraction at first. In the asparagine-labelling experiment, ^{14}C -asparagine accounted for almost all of the radioactivity in the immature leaf after one hour.

Even in the expanding leaf (which does transpire water and therefore is supplied with some nitrogen as nitrate or amino acid, in the transpiration stream) it appeared that after the initial influx of radioactivity into this leaf, there was a secondary rise in level of labelling or maintenance of existing high levels, a result possibly of export from the mature leaves.

Attempts were therefore made to further investigate the transport of amides and amino acids from the mature leaf to the young leaves and other parts of the plant. This was carried out by supplying ^{14}C -labelled compounds to the lower epidermis of mature leaves of both detached shoots and intact plants.

It was necessary to find a method which would allow good uptake of the labelled compounds applied to the leaf, and transport of a measureable quantity of ^{14}C -carbon to other parts of the plant. When application of ^{14}C -asparagine was made to the intact lower surface of the 4th leaf, little radioactivity was recovered in the leaf or other parts of the shoot, most of it being washed off the

leaf surface (data not shown). Several methods were tried to overcome the barrier of the lower epidermis.

In the following experiment, the leaves of four groups of plants were prepared in one of these ways: (1) a small area of the lower epidermis was abraded with carborundum and then rinsed and blotted dry; (2) a small portion of epidermis was peeled off; (3) a small area was gently abraded with fine-grade emery paper; (4) the leaf surface was undisturbed but the radiotracer was applied in a drop of detergent, 0.5% (v/v) Triton X-114.

In all four cases, a small, 5 x 5 mm, square of coverglass was sealed on three sides with Liquid Silicone Polymer over the treated area. Four detached shoots at the "4 $\frac{1}{2}$ -leaf" stage were used per treatment. After preparation of the leaves, 2.5 μ l of 14 C-asparagine (0.125 μ Ci per plant) were introduced with a microsyringe into the chambers formed by the coverglass as described in Methods (10B).

After 2 hours in the light, the treated leaflets were cut off, the glass chamber removed, and both leaflets and chambers rinsed thoroughly to remove unabsorbed surface radioactivity. The other leaves were detached, and treated leaflet and other leaf samples were frozen and extracted in 80% ethanol (v/v) as described.

The radioactivity in the ethanol-soluble fractions of the four groups of shoots is shown in Table 6. Since in most of these preliminary experiments, the insoluble fractions were not analyzed, the levels of radioactivity are expressed as percentages of the total 14 C-asparagine fed to the shoots.

TABLE 6

Radioactivity in the Ethanol-Soluble Fractions of Leaves
from Detached Shoots Labelled with ^{14}C -Asparagine
via the Mature Leaf

	Treatment ^a			
	1	2	3	4
Radioactivity	cpm $\times 10^{-5}$			
Amount Supplied	10.40	10.40	10.40	10.40
Recovered	8.22	8.14	8.58	9.42
	% of Label Supplied			
Total Recovered	79.0	78.3	82.5	90.6
Washed Off	53.0	30.0	14.0	57.0
Recovered in				
Treated Leaflet	21.0	36.0	52.0	26.0
Leaf 6	1.0	3.6	4.5	3.2
Leaf 5	1.4	4.5	3.8	1.3
Rest of Leaf 4	0.7	1.4	2.4	0.7
Leaf 3	0.3	0.2	0.2	0.4
Stem above Leaf 4	0.3	0.7	0.9	0.2
Rest of Stem + lvs	1.4	2.1	5.3	1.5

^aTreatment: (1) abrasion with carborundum; (2) removal of epidermal peel; (3) light abrasion with fine emery paper; (4) application of label in 0.5% (v/v) Triton - X-114.

Less than 50% of the supplied label was taken up into the plant when the treated leaves were abraded with carborundum, or when the ^{14}C -asparagine was supplied in detergent, slightly more than 50% of the label being recovered in the fraction washed off the leaf. More label was recovered in the plant and less was washed off when a small area of epidermis was peeled off prior to addition of the label. The best results were obtained by gentle abrasion of the cuticle with emery paper followed by application of the ^{14}C -labelled compound; only 14% was washed off, and 52% and 17% were recovered in the soluble fractions of the treated leaflet and the other parts of the shoot respectively. This method for applying radioactively labelled compounds was used in the subsequent experiments.

Transport of Labelled Compounds from Mature Leaves of Detached Shoots

Plants were selected and the shoots detached and placed with their cut ends in unlabelled "chase" solution (as used in the previous group of experiments). The lower surface of one leaflet of the mature leaves (Leaf 4) was prepared, and the glass chambers were placed in position. The ^{14}C -asparagine (2.5 μl per plant) was applied 30 minutes later. After two hours in the light, the treated leaflet was detached, the chamber removed, and leaflet and chamber rinsed. The leaf samples were frozen separately and extracted as before.

Table 7 shows that of the total of 9.8×10^5 cpm recovered, 8.4×10^5 cpm were taken up by the plants. Most of this absorbed radioactivity was recovered in the treated leaflet in the soluble

TABLE 7

Distribution of Radioactivity in Detached Shoots Labelled
via the Mature Leaf with ^{14}C -Asparagine

Radioactivity	cpm x 10^{-5}	
Amount Supplied	10.4	
Total Recovered	9.8	
Amount Washed Off	1.4	
Recovered in Plant	8.4	
Loss	6% of total supplied	

	Radioactivity Recovered in Plant	
	cpm x 10^{-4}	% of Total Recovered
Soluble		
Treated Leaflet	53.90	64.2
Leaf 6	4.66	5.5
Leaf 5	3.93	4.7
Rest of Leaf 4	2.54	3.0
Leaf 3	0.23	0.3
Stem above Leaf 4	0.95	1.1
Rest of Stem & Leaves	5.53	6.6
Insoluble		
Treated Leaflet	10.10	12.1
Leaf 6	0.81	1.0
Leaf 5	0.36	0.4
Rest of Leaf 4	0.16	0.2
Leaf 3	0.18	0.2
Stem above Leaf 4	0.03	0
Rest of Stem & Leaves	0.39	0.5

and insoluble fractions, 64.2% of the total taken up by the plant being in the soluble fraction of this sample. However, a total of 11.6% of the radioactivity recovered in the plants was transported to the young leaves (Leaves 5 and 6) and was recovered largely in the soluble fractions (5.5% and 4.7% respectively), but some radioactivity was also recovered in the insoluble fractions of these leaves.

Labelled carbon from ^{14}C -asparagine could therefore be transported from the mature leaflets, where it was applied, to the young leaves and other parts of the detached shoots.

Separation of the aqueous samples on Dowex resin columns into amino-acid, neutral, and organic-acid fractions showed that, in all the samples analyzed, most of the radioactivity was recovered in the amino-acid fraction. Table 8 shows that in three experiments, between 72% and 88% of the radioactivity recovered after separation on Dowex resins were in the amino-acid fraction of the treated leaflet, Leaf 6 and Leaf 5. Less than 2% was recovered in the neutral fractions and the remainder, between 11% and 27% were recovered in the organic-acid fractions.

The same conditions were used here as those in experiments where the radiotracer was supplied via the transpiration stream, and therefore, these experiments, also with detached shoots, show that carbon from ^{14}C -labelled asparagine can be transported from the mature leaves to the young leaves of detached shoots.

TABLE 8

Distribution of Radioactivity in the Amino-Acid, Neutral, and Organic-Acid Fractions of Detached Shoots Labelled with ^{14}C -Asparagine via the Mature Leaves. Data from 3 Experiments are shown.

Sample	Radioactivity in Aqueous Extracts			
	Total in Extract	Amino Acid	Neutral	Organic Acid
	cpm x 10^{-4}	% of Total in Extract		
Treated Leaflet	51.61	82.9	0	17.1
	40.90	86.2	1.2	12.5
	36.60	86.8	1.3	11.9
Leaf 6	4.24	76.0	0.2	23.8
	5.92	76.6	0.6	22.8
	3.44	71.6	1.3	27.1
Leaf 5	3.78	88.4	0.3	11.3
	2.68	83.2	0.8	16.0
	4.40	78.6	0.5	20.8

Transport of Labelled Amides from Mature Leaves of Intact Plants

Having demonstrated that such transport could occur in the detached shoot system, it was then of interest to conduct further studies on whole plants. Table 9 shows the results of experiments in which ^{14}C -asparagine was supplied to mature leaves of detached shoots and whole plants. The plants were selected and prepared as before, and the samples taken and extracted as described. In the detached shoots, the soluble plus insoluble fractions of the young leaves took up 10.1% and 13.6% in two groups of plants; in the whole plants, the percentages of label recovered in the growing regions (young leaves plus roots) were 15.1 and 19.2 in the two experiments. In both cases the young leaves received only a little more radioactivity than the roots; 7.9% (leaves) and 7.2% (roots) in the first experiment; 10.4% (leaves) and 8.8% (roots) in the second.

Similar experiments, using both detached shoots and intact plants, were conducted, labelling the mature leaflets this time with ^{14}C -glutamine. These data are shown in Table 10. As with asparagine-labelled plants, most of the ^{14}C -carbon from glutamine was recovered in the soluble and insoluble fractions of the treated leaflet from both detached shoots and whole plants. Totals of 2.0% and 4.0% (soluble + insoluble) were recovered in the young leaves (Leaves 5 and 6) in the two experiments with detached shoots. In the whole plants, these values were 4.3% and 6.0%. In addition, a further 7.5% and 6.1% were transported to the roots. Carbon from ^{14}C -glutamine was, therefore, transported from the mature to the young leaves in detached shoots and intact plants, and also to the roots in the intact plants.

TABLE 9

Distribution of Radioactivity in Detached Shoots and Intact Plants
Labelled via the Mature Leaf with ^{14}C -Asparagine. Data from 2
Experiments are shown.

	Detached Shoots		Intact Plants	
	1	2	1	2
	cpm $\times 10^{-5}$			
Radioactivity:				
Amount Supplied	10.40	10.40	10.40	10.40
Total Recovered	10.50	10.37	10.22	9.55
Washed Off	2.01	1.29	0.44	0.76
Recovered in Plant	8.495	9.082	9.784	8.784
	% of Total Recovered in Plant			
Soluble				
Treated Leaflet	73.7	67.4	69.3	62.9
Leaf 6	4.2	6.0	1.9	4.6
Leaf 5	5.0	6.1	4.9	4.6
Rest of Shoot	6.0	8.5	6.2	9.9
Root	-	-	6.6	8.0
Insoluble				
Treated Leaflet	10.0	9.9	8.7	9.0
Leaf 6	0.6	0.8	0.3	0.6
Leaf 5	0.3	0.7	0.8	0.6
Rest of Shoot	0.5	0.6	0.5	0.6
Root	-	-	0.6	0.8

TABLE 10

Distribution of Radioactivity in Detached Shoots and Intact Plants
 Labelled via the Mature Leaf with ^{14}C -Glutamine. Data from
 2 Experiments are shown.

	Detached Shoots		Intact Plants	
	1	2	1	2
	cpm x 10 ⁻⁵			
Radioactivity:				
Amount Supplied	9.2	9.2	9.2	9.2
Total Recovered	8.34	8.9	8.12	9.7
Washed Off	0.96	0.11	1.24	1.53
Recovered in Plant	7.37	8.83	6.89	8.17
%				
% of Total Recovered in Plant				
Soluble				
Treated Leaflet	87.5	78.8	76.4	64.5
Leaf 6	0.7	1.1	1.8	1.6
Leaf 5	1.1	1.7	1.8	2.3
Rest of Shoot	3.6	2.4	7.0	6.1
Root	-	-	6.7	4.7
Insoluble				
Treated Leaflet	6.8	14.3	4.4	16.8
Leaf 6	0.1	0.5	0.4	1.1
Leaf 5	0.1	0.7	0.3	1.0
Rest of Shoot	0.04	0.5	0.2	0.5
Root	-	-	0.8	1.4

When the aqueous extracts of treated leaflet and young leaves of the glutamine-labelled, detached shoots were further fractionated by passing over Dowex resins, 83.6%, 73.3%, and 77.3% of the ^{14}C -label were recovered in the amino-acid fractions of treated leaflet, Leaf 6, and Leaf 5 respectively; in the organic-acid fractions, 15.0%, 12.5%, and 22.0% were recovered, the remaining radioactivity being in the neutral fractions.

The distribution of ^{14}C -label in aqueous extracts of whole plants labelled with asparagine or glutamine is shown in Tables 11 and 12. The results of four experiments for each amide are presented. In the asparagine-labelled plants, between 70% and 85% of the radioactivity recovered after separation on Dowex resins were found in the amino-acid fraction of the treated leaflets and the young leaves. There was, however, one exception; the amino-acid fraction of Leaf 5 in the third experiment, where 96.0% of the radioactivity was recovered. In this leaf, only 3.2% of the total radioactivity was recovered in the organic-acid fraction, compared with 12 to 30% recovered in the acid fraction of the other leaf samples. In the root, in all four experiments, over 90% of the ^{14}C -label was found in the amino-acid fraction.

In the glutamine-labelled plants, 65 to 85% of the radioactivity was in the amino-acid fraction of all samples; treated leaflet, young leaves and root. Between 15 and 30% were found in the organic-acid fraction and the rest in the neutrals.

In the whole plants, as in the detached shoots, carbon from the labelled amides supplied to the mature leaflets remained largely in the form of amino acids in the treated leaves, and was transported

TABLE 11

Radioactivity in Amino-Acid, Neutral, and Organic-Acid Fractions of Leaf and Root Samples from Intact Plants Labelled via the Mature Leaf with ^{14}C -Asparagine. Data from 4 Experiments are shown.

Sample	Amino Acid	Neutral	Organic Acid
% of Total Recovered in Sample			
Treated Leaflet	86.3	1.2	12.5
	71.3	2.7	26.0
	85.5	2.2	12.3
	78.0	22.0 ^a	
Leaf 6	76.6	0.6	22.8
	68.9	1.2	29.9
	85.4	3.1	11.5
	71.5	28.5	
Leaf 5	83.2	0.8	16.0
	67.8	1.2	30.9
	96.0	0.8	3.2
	82.8	17.2	
Root	96.2	1.2	2.6
	92.1	1.2	6.7
	97.5	0.8	1.7
	94.5	5.5	

^aThe fraction passing through Dowex-50 was not further separated into neutral and organic-acid fractions in this experiment.

TABLE 12

Radioactivity in the Amino-Acid, Neutral, and Organic-Acid Fraction
of Leaf and Root Samples from Intact Plants Labelled via the
Mature Leaf with ^{14}C -Glutamine.
Data from 4 Experiments are shown.

Sample	Amino Acid	Neutral	Organic Acid
% of Total Recovered in Sample			
Treated Leaflet	84.8	0.1	15.1
	83.0	3.0	14.0
	71.8	4.5	33.8
	78.8	21.2 ^a	
Leaf 6	79.7	1.0	20.0
	67.2	3.8	29.0
	70.0	9.6	20.4
	66.5	33.5	
Leaf 5	74.0	0.7	24.4
	71.8	3.2	25.0
	76.6	4.1	19.3
	76.2	23.8	
Root	79.7	1.0	19.3
	78.1	3.1	18.8
	81.7	3.0	15.2
	79.5	20.5	

^aFractions eluting from Dowex-50 were not further separated
into neutral and organic-acid fractions in this experiment.

to the young leaves and roots, the main proportion of the radioactivity recovered there being in the form of amino acids.

The amino acid and organic-acid fractions were further analyzed in extracts from treated leaflets, young leaves, and roots of ^{14}C -asparagine- and ^{14}C -glutamine-labelled plants.

Tables 13 and 14 show the total radioactivity of the extract and the percentage distributions of label in soluble and insoluble compounds in asparagine-treated plants. In Table 13 the data for two experiments are given, in both of which the amino-acid fractions were separated by a combination of high-voltage electrophoresis and partition chromatography. In the first experiment the organic-acid and neutral fractions were combined, and no special precautions were taken to prevent hydrolysis of HSA; most of the radioactivity in these samples was, therefore, recovered in malate. In the second experiment, the precautions were taken, and radioactivity was found in HSA.

In both experiments, the largest proportion of radioactivity in the treated leaflet (41.7% and 53.4%) was recovered still as asparagine. Some radioactivity was recovered in aspartate, 12.7% and 5.7% in the two experiments, and also in glutamate (7.6% and 6.3%). Homoserine was also labelled in the treated leaflet and a trace of ^{14}C -label was recovered in glutamine in one experiment. About 13% of the total radioactivity was recovered in the protein of the insoluble fraction. In the second experiment, 7.0% of the radioactivity was found in HSA.

In the young leaves, Leaves 5 and 6, most of the label was again still recovered in asparagine; aspartate was also labelled in both young leaves and glutamate in Leaf 6. In both leaf samples,

TABLE 13

Distribution of Radioactivity in the Soluble and Insoluble Compounds of Leaves and Roots, Labelled via the Mature Leaflets with ^{14}C -Asparagine. Separation of Amino Acids by Electrophoresis and Partition Chromatography. Data from 2 Experiments are shown.

	Treated Leaflet		Leaf 6		Leaf 5		Root	
	1 ^a	2 ^b	1	2	1	2	1	2
% of Total Radioactivity								
Amino Acids								
Asp ^c	12.7	5.7	16.4	7.8	20.7	0	12.3	0
Asn	41.7	53.4	38.9	53.3	49.9	65.1	74.9	85.3
Glu	7.6	6.3	4.5	3.9	0	0	0	0
Gln	0	0.7	0	0	0	0	0	0
Hse	3.6	2.2	0	0	0	0	0	0
Neutrals	21.1	1.8	24.0	0.8	14.6	0.8	5.1	0.7
Organic Acids								
HSA		7.0		8.4		9.0		1.9
Others		9.9		11.8		10.6		2.0
Insoluble	13.3	13.1	16.2	14.1	14.8	14.7	7.7	10.2
Total Radioactivity (cpm $\times 10^{-4}$)								
	61.84	62.29	1.23	3.09	2.03	4.54	5.23	6.67

^aNeutral and organic-acid fraction not further separated on Dowex-1, no precaution was taken to prevent hydrolysis of HSA and most of the radioactivity in this combined fraction was recovered in malate.

^bFull precautions were taken to prevent hydrolysis of HSA in the organic acid fraction.

^cThe percentage radioactivity recovered in Asp is much higher in Expt. 1 than in Expt. 2. Although precautions were taken to prevent hydrolysis of the amides, the inconsistency of these data suggest that some hydrolysis of Asn occurred in Expt. 1, probably while the amino acids were absorbed on the Dowex 50 columns.

the radioactivity in HSA accounted for almost half of label in the whole of the organic-acid fraction. Label was also recovered in the insoluble fractions.

In the roots, the largest percentage of label was recovered in asparagine, 74.9% and 85.3% in the two experiments. In one experiment, some label was recovered in aspartate, and in both experiments, some of the transported label was recovered in the protein. Only small amounts of ^{14}C -label were found in the organic-acid fraction (or organic plus neutral fractions) of the root; in the second experiment, about 2% of the total (approximately half that on the organic acids) was recovered in HSA.

Similar data are presented in Table 14. In this experiment, however, the amino-acid fractions were separated on the amino-acid analyzer as outlined in the Methods (15B(iii)). In the treated leaflet, the bulk of the radioactivity was still in asparagine. Again, label was recovered in aspartate and glutamine plus homoserine (not separated in this particular run on the analyzer). No separate glutamate peak was resolved, because of the very large amount of labelled asparagine in the treated leaf sample.

Radioactivity was recovered in small amounts in several other amino acids, a reflection on the greater sensitivity of the analyzer compared to those of the other methods used for separation of the amino acids. Substantial amounts (9.3%) of label were found in HSA in these samples.

In the young leaves, 58.4% of the radioactivity was found in asparagine, 4.9% in aspartate, 15.7% in protein, and 3.9% in HSA. In the roots, almost 50% of the label was in asparagine, 11.3% in

TABLE 14

Distribution of Radioactivity in the Soluble and Insoluble Compounds of Leaves and Roots Labelled via the Mature Leaflet with ^{14}C -Asparagine. Separation of Amino Acids with Amino-Acid Analyzer.

	Treated Leaflet	Young Leaves	Root
% of Total Radioactivity			
Amino Acids			
Asp	4.9	4.9	0.6
Thre	0.3	0	0
Ser	0.3	0	0
Asn	66.9 ^a	58.4	79.7
Glu	-	0	0
Gln	} 3.0	} 0	} 0
Hse			
Pro	0.03	0	0
Ala	0.5	0.4	2.1
Others	4.1	6.1	4.1
Neutral	2.0	2.5	0.7
Organic Acids			
HSA	9.3	3.9	} 1.5
Others	2.2	8.1	
Insoluble	6.4	15.7	11.3
Total Radioactivity (cpm $\times 10^{-4}$)			
	82.51	2.10	6.20

^a The level of radioactivity in the asn peak was so high that a separate glutamate peak was not detected.

the insoluble fraction, and very small amounts in aspartate, alanine, and the organic-acid fraction.

It appeared that in these asparagine-labelled plants the ^{14}C -carbon reaching the young leaves and roots was mainly in the form of asparagine, although some aspartate and HSA were also labelled. Some of the transported radioactivity was incorporated into protein.

The results of equivalent experiments for glutamine-treated plants are shown in Tables 15 (amino acid separation by electrophoresis and partition chromatography) and 16 (separation by amino-acid analyzer).

Most of the radioactivity in the treated leaflet (Table 15) was in glutamate (52.8%) while only 11.3% was recovered in the original glutamine, and a small amount (1.6%) in γ -amino butyric acid. In Leaves 5 and 6, again most of the radioactivity was recovered in glutamate (41.2% and 36.0% respectively). About 13% was recovered in glutamine in each of the young leaves, and in Leaf 5, 8.1% was in γ -amino butyric acid. Considerable amounts of radioactivity were incorporated into the insoluble fraction, particularly in Leaf 6 where 26.3% was recovered.

In the root samples, 18.4% was recovered in glutamine; and 51.1% in glutamate.

In the organic acid plus neutral fractions, five major radioactive compounds were detected in the treated leaflet, the most highly labelled with an R_F of 56 in 1-butanol: acetic acid: water (12: 3: 5 by volume), and two labelled compounds were detected in the young leaves and roots; these had R_F values of 51 to 55, and 71 in the same solvent system. These were not further identified.

TABLE 15

Distribution of Radioactivity in the Soluble and Insoluble Compounds of Leaves and Roots Labelled via the Mature Leaflet with ^{14}C -Glutamine. Separation of Amino Acids by Electrophoresis and Partition Chromatography.

	Treated Leaflet	Leaf 6	Leaf 5	Root
% of Total Radioactivity				
Amino Acid				
Glu	52.8	36.0	41.2	51.1
Gln	11.3	13.0	12.7	18.4
Gaba	1.6	-	8.1	0
Neutrals Organic Acids	} 23.6	} 24.3	} 19.4	} 17.9
Insoluble	10.7	26.3	18.6	12.6
Total Radioactivity (cpm $\times 10^{-4}$)				
	65.315	1.140	2.150	5.571

In the other experiment, data shown in Table 16, again most of the label in the treated leaflet, young leaves, and root was in glutamate. Small amounts (1.4% and 4.7% of the total) were recovered in the glutamine + homoserine peaks in the two leaf samples, but 13.6% was recovered in that peak in the root sample. Gamma-amino butyric acid was labelled in all leaf and root samples. In the treated leaflet, traces of radioactivity were found in several other amino acids, since this method of separation of amino acids (analyzer) allowed better recovery of small peaks.

The organic-acid samples of this experiment were separated further in the same solvent system as the first. Again five major, labelled compounds were detected in the treated leaflet and one or two in the leaf and root samples.

Although differences in distribution among the various labelled compounds occurred between the two experiments in the glutamine-treated plants, it was still apparent that much of the ^{14}C -glutamine supplied to the treated leaflet was metabolized there to glutamate. A large portion of the radioactivity transported to the young leaves and roots was also in the form of glutamate. This carbon that reaches the young leaves and roots, may well be transported as glutamate, but transport as glutamine followed by immediate metabolism on arrival in the leaves or roots cannot be excluded.

Transport of Labelled Aspartate and Glutamate From Mature Leaves to Intact Plants

In the experiments in which ^{14}C -amides were supplied via the transpiration stream to detached shoots, labelled aspartic and

TABLE 16

Distribution of Radioactivity in the Soluble and Insoluble Compounds of Leaves and Roots Labelled via the Mature Leaflet with ^{14}C -Glutamine. Separation of Amino Acids with Amino-Acid Analyzer.

	Treated Leaflet	Young Leaves	Root
% of Total Radioactivity			
Amino Acids			
Asp	0.2	3.5	6.8
BIA	3.6	0	0
Thre	} 0.1	} 0	} 1.0
Ser			
Asn	0.1	0	7.0
Glu	49.2	38.6	29.4
Gln	} 1.4	} 4.7	} 13.6
Hse			
Gly	0.4	0	0
Pro	0.1	0	0
Ala	0.7	0	0
Gaba	6.7	5.1	9.3
Others	2.3	1.1	3.4
Neutral	4.0	7.3	2.6
Organic Acids	21.5	25.3	13.2
Insoluble	9.6	14.3	13.6
Total Radioactivity (cpm $\times 10^{-4}$)			
	66.08	2.10	5.28

glutamic acids were recovered in both the young and mature leaves. These experiments could not distinguish between ^{14}C -carbon transported to the young leaves in the form of aspartate and glutamate and label recovered in these amino acids after metabolism of the amides arriving as such in the young leaves.

In order to see if carbon from aspartic and glutamic acids could be transported from the mature to the young leaves, experiments were conducted in which ^{14}C -labelled aspartate or glutamate were supplied to mature leaves of intact plants. Four groups of four plants each were selected and the leaves prepared as before. Two groups were treated with ^{14}C -aspartate and two with ^{14}C -glutamate. After 2 hours in the light, leaf and root samples were separated, frozen, and extracted. The data for the uptake and distribution of radioactivity are shown in Table 17.

As with the asparagine- and glutamine-labelled plants, most of the radioactivity was recovered in the treated leaflet. In the aspartate-treated plants, however, 4.5% and 4.3% of the total radioactivity recovered in the plants were transported to the young leaves, while 2.8% and 6.1% were transported to the roots in the two experiments. In the glutamate-treated plants, 4.1% and 3.2% were transported to the young leaves and 2.4% and 2.2% to the roots.

The aqueous extracts of treated leaflets and young leaves were separated into amino-acid, neutral, and organic-acid fractions by passing the extracts over Dowex 50 and Dowex 1 resins. The results are shown in Table 18. In the aspartate-labelled plants, about 40 to 50% of the label remained in the amino-acid fraction of

TABLE 17

Distribution of Radioactivity in Intact Plants Labelled via
the Mature Leaflet with ^{14}C -Aspartate or ^{14}C -Glutamate.
Data from 2 Experiments are shown.

	Aspartate		Glutamate	
	1	2	1	2
	cpm $\times 10^{-5}$			
Radioactivity:				
Amount Supplied	13.01	13.01	9.56	9.56
Total Recovered	9.56	10.58	7.98	7.99
Washed Off	0.14	0.08	0.27	0.39
Recovered in Plant	9.41	10.50	7.71	7.60

% of Total Recovered in Plant				
Soluble:				
Treated Leaflet	77.8	72.8	83.9	84.9
Leaf 6	1.1	0.9	0.7	1.1
Leaf 5	2.4	2.6	3.0	1.5
Rest of Shoot	2.9	4.2	2.5	2.4
Root	2.4	5.5	2.0	2.0
Insoluble:				
Treated Leaflet	11.3	11.6	6.6	6.9
Leaf 6	0.4	0.2	0.2	0.3
Leaf 5	0.6	0.6	0.2	0.3
Rest of Shoot	0.6	1.1	0.3	0.3
Root	0.4	0.6	0.4	0.2

the treated leaflet, and almost 50% was recovered in the organic-acid fraction. In the young leaves, a little over 50% of the total radioactivity remained in the amino-acid fraction, and about 40% was recovered in the organic-acid fraction.

In the treated leaflet of the glutamate-labelled plants, about 65 to 70% of the radioactivity remained in the amino-acid fraction, the remainder being divided almost evenly between the neutral and organic-acid fractions. In the young leaves, 50 to 60% remained in the amino-acid fraction, and about 25 to 30% in the organic acids. The root samples were not analyzed.

It appears that both aspartate and glutamate, but especially the former, were metabolized quite extensively to compounds other than amino acids, particularly in the treated leaflets.

The identity of the compounds in the amino-acid fraction was investigated by separation of these fractions from aspartate- and glutamate-labelled plants by high-voltage electrophoresis into aspartate, glutamate, neutral amino-acid, γ -amino butyric acid, and basic amino-acid fractions (Methods 15B(1)). The neutral amino acids were then separated on the amino-acid analyzer. Quantities of radioactivity in each compound recovered from the analyzer were adjusted to the total neutral amino-acid fraction, and then radioactivity in all compounds recovered was expressed as a percentage of the total in the sample.

Table 19 shows the data for the aspartate-treated plants. Of the total soluble and insoluble radioactivity in the treated leaflet, the largest portion (43.1% and 41.3% in the two experiments) was in the organic-acid fraction. The distribution of label in the

TABLE 18

Radioactivity in Amino-Acid, Neutral, and Organic-Acid Fractions of Treated Leaflet and Young Leaves from Intact Plants Labelled with ^{14}C -Aspartate or ^{14}C -Glutamate. Data from 2 Experiments are shown.

^{14}C -Label	Amino Acid	Neutral	Organic Acid
% of Total in Soluble Fraction			
Aspartate			
Treated Leaflet	39.9	10.7	49.4
"	47.5	4.6	47.9
Young Leaves	54.4	6.4	39.2
"	53.3	4.2	42.5
Glutamate			
Treated Leaflet	68.5	15.0	16.5
"	63.5	19.7	16.8
Young Leaves	60.0	10.6	29.4
"	50.9	22.5	26.6

amino-acid fraction of the treated leaflets varies between the two experiments. In the first experiment, most of the label was in glutamate (14.0%), while 6.1% was in glutamine + homoserine, and 4.7% in aspartate. In the second experiment, 11.6% of the radioactivity was aspartate and only 2.2% in glutamate. In this experiment, 13.9% was recovered in glutamine + homoserine and when an additional sample was run on the analyzer, to separate glutamine and homoserine, it was found that most of the label was in homoserine. Smaller amounts of label were asparagine, alanine, and γ -amino butyric acid.

In the young leaves, a large proportion of the total radioactivity was recovered in the organic-acid fractions (32.0% and 34.5% in the two experiments), some label (13.3% and 20.7%) was recovered in glutamate and smaller amounts (3.9% and 5.3%) in aspartate. No radioactivity was recovered in γ -amino butyric acid in the young leaves. Although a substantial percentage of the total radioactivity was found in the young leaves, the actual radioactivity (7.5 and 5.7×10^3 cpm) was considered too small for further separation on the analyzer.

The organic-acid fractions of the treated leaflet yielded five compounds separable by partition chromatography, the major ones having R_F values of 35, 47 to 51, and 70 to 75 in the 1-butanol: acetic acid: water solvent system already described. In the young leaves, there was only one main labelled compound, which had an R_F value of 56. These compounds were not further identified.

Radioactivity was recovered in the insoluble fractions of treated leaflets and young leaves.

TABLE 19

Distribution of Radioactivity in the Soluble and Insoluble Compounds in Treated Leaflet and Young Leaves of Intact Plants, Labelled via the Mature Leaflet with ^{14}C -Aspartate.

	Treated Leaflet		Young Leaves	
	1	2	1	2
% of Total Radioactivity				
Amino Acids				
Asp	4.7	11.6	3.9	5.3
Thr	* } 0.3	0.5	ND ^a	ND ^a
Ser		0.1	ND	ND
Asn	2.2	1.1	ND	ND
Glu	14.0	2.2	13.3	20.7
Gln	} 6.1	3.7	ND	ND
Hse		10.2	ND	ND
Pro	0.1	0.2	ND	ND
Gly	0.2	0.2	ND	ND
Ala	2.7	2.9	ND	ND
Gaba	2.9	5.5	0 ^b	0 ^b
Others	1.7	2.8	27.0 ^b	16.9 ^b
Neutral	9.3	4.0	5.2	3.4
Organic Acids	43.1	41.3	32.0	34.5
Insoluble	12.7	13.8	18.6	19.1
Total Radioactivity (cpm x 10 ⁻⁴)				
	83.95	88.66	2.80	3.37

^aND: not determined separately.

^bRadioactivity in "other" amino acids in these samples includes all amino acids except Asp, Glu, and Gaba, which were separated by electrophoresis.

Equivalent data for glutamate-treated plants are shown in Table 20. Here, in the treated leaflets, most of the radioactivity was recovered in glutamate (21.9% and 22.9%) and γ -amino butyric acid (29.2% and 23.7%). Small amounts of label (2.2% and 3.0%) were recovered in aspartate, and 5.7% and 7.2% were recovered in glutamine plus homoserine. Separation of glutamine and homoserine peaks showed that, in the second experiment, approximately equal amounts of radioactivity were in each of these two amino acids.

In the young leaves, glutamate, γ -amino butyric acid, and aspartate were labelled in one experiment, but only glutamate in the other, the neutral amino acids again not being further separated.

In both treated leaflet and young leaves, radioactivity was recovered in the organic-acid fractions, but not to the same extent as in the aspartate-labelled plants. Three main labelled compounds were found in these fractions of the treated leaflet, and one major one in the young leaves.

These experiments suggest that, in the aspartate-labelled plants, most of the aspartate is metabolized to other compounds in the mature leaflet, and although a small amount of label transported to the young leaf may be as aspartate, most of the label arriving in the young leaf could be as organic acids of other amino acids. In the glutamate-treated plants, much of the labelled glutamate is metabolized to other compounds in the treated leaflet, in particular, γ -amino butyric acid, but in the young leaves, 15 to 20% of the imported label is in the form of glutamate.

TABLE 20

Distribution of Radioactivity in the Soluble and Insoluble Compounds in Treated Leaflet and Young Leaves of Intact Plants Labelled via the Mature Leaflet with ^{14}C -Glutamate.

	Treated Leaflet		Young Leaves	
	1	2	1	2
	% of Total Radioactivity			
Amino Acid				
Asp	2.2	3.0	3.5	0
Thr	0.8	0.2	ND ^a	ND ^a
Ser		0.6	ND	ND
Asn	0	0.2	ND	ND
Glu	21.9	22.9	21.6	15.3
Gln	5.7	3.5	ND	ND
Hse		3.7	ND	ND
Pro	0.2	0.2	ND	ND
Gly	0.2	0	ND	ND
Ala	0.7	0.4	ND	ND
Gaba	29.2	23.7	11.2	0
Others	2.6	2.8	19.6 ^b	26.3 ^b
Neutral	13.9	19.0	9.9	18.4
Organic Acid	15.3	16.2	27.4	21.7
Insoluble	7.3	7.8	6.7	18.4
	Total Radioactivity (cpm x 10 ⁻⁴)			
	69.79	67.09	2.58	1.40

^aND: not determined separately.

^bRadioactivity in "other" amino acids in these samples includes all amino acids except Asp, Glu, and Gaba, which were separated by electrophoresis.

Transport of Labelled HSA From Mature Leaves of Intact Plants

In plants labelled with ^{14}C -asparagine, either via the xylem or supplied to the mature leaves, radioactivity was consistently found in the organic-acid fraction in one compound which co-chromatographed with authentic HSA, and was, like authentic HSA, hydrolyzed under acid conditions to malate. The characteristics of the compound found in these experiments suggest it is the HSA described by Lloyd and Joy (1978). Not only was it recovered in the mature leaves, but also in the young leaves and, to a lesser extent, in the roots. The HSA in the young leaves or roots could have been synthesized there from asparagine transported unchanged into these parts of the plant. It was, however, still a possibility that some HSA could be transported as such from the mature leaves.

Labelled HSA was therefore prepared from ^{14}C -asparagine as described (Methods 7) and, after purification by partition chromatography, was supplied to mature leaflets of intact "4½-leaf" plants, four plants per experiment. The plants were left for 2 hours in the light, and then leaf and root samples were processed as previously described.

The results of three experiments are shown in Table 21. Most of the radioactivity was recovered in the soluble fraction of the treated leaflet, small percentages being also recovered in the insoluble material of these leaflets. Small amounts of radioactivity were recovered in the young leaves and roots.

Separation of the aqueous extracts of the treated leaflets into amino-acid, neutral and organic-acid fractions showed (Table 22) that most of the ^{14}C -label was still in the organic-acid fraction

TABLE 21

Distribution of Radioactivity in Intact Plants Labelled
via the Mature Leaflet with ^{14}C -HSA. Data from
3 Experiments are shown.

	Experiment		
	1	2	3
	cpm x 10 ⁻⁴		
Radioactivity:			
Amount Supplied	6.1	12.0	12.0
Total Recovered	6.1	12.0	11.0
Washed Off	1.9	1.9	3.2
Recovered in Plant	4.2	10.4	7.8

% of Total Recovered in Plant			
Soluble:			
Treated Leaflet	83.9	83.7	89.7
Leaf 6	3.5	1.4	0.6
Leaf 5	2.8	0.9	1.2
Rest of Shoot	0	8.7	3.2
Root	4.7	1.1	0.5
Insoluble:			
Treated Leaflet	3.5	4.2	4.8
Leaf 6	1.6	0	0
Leaf 5	0	0	0
Rest of Shoot	0	0	0
Root	0	0	0

TABLE 22

Radioactivity in Amino-Acid, Neutral, and Organic-Acid Fractions
of Extracts from Treated Leaflet and Young Leaf of Intact Plants
Labelled via the Mature Leaflet with ^{14}C -HSA.

	Amino Acid	Neutral	Organic Acid
	% of Total Recovered in Sample		
Treated Leaflet			
1 ^a	11.0	0.9	88.1
2	5.6	0.8	93.6
3	4.2	0.7	95.1
Young Leaf			
2 ^b	6.3	0.8	92.9

^aData from 3 experiments.

^bData for young leaf are from Experiment 2.

(the fraction in which HSA is recovered). Although in Experiment 1, 3 to 4% of the total radioactivity was recovered in the young leaves and the roots, the actual amount of radioactivity that this percentage represents was quite low, and only the young leaf sample of Experiment 2 was sufficiently labelled to make further separation on Dowex resins worthwhile. In this sample, most of the label was also in the organic-acid fraction.

The organic-acid fractions of the three treated leaflets and the young leaf of Experiment 2 were further separated by partition chromatography in 1-butanol: acetic acid: water (12: 3: 5 by volume). In the three treated-leaflet samples, 88.4%, 81.6%, and 87.3% of the label in the organic-acid fractions were recovered as HSA. In the organic-acid fraction of the young leaf, 92.4% of the label was HSA.

The amino-acid fractions of the treated leaflet and the young leaf, both from Experiment 2, were separated into individual amino acids on thin-layer plates coated with cellulose. The solvent systems used are described in the methods (15B(11)). The amino-acid spots were visualized by the o-phthalaldehyde reagent of Davies and Mifflin (1978) and the spots scraped off and radioactivity determined. The results are shown in Table 23.1. In the treated leaflet most of the radioactivity was recovered in glutamate and aspartate, while some ¹⁴C-label was also recovered in asparagine. Lysine and asparagine run very close together in the system used and part of the label in lysine could be contamination from overlapping asparagine. In the young leaf, very small amounts of radioactivity were recovered in glutamate and aspartate.

TABLE 23

Radioactivity Recovered in Amino Acids of Treated Leaflet and
Young Leaf of Plants Labelled via the Mature Leaflet with
 ^{14}G -HSA.

Amino Acid ^a	Treated Leaflet	Young Leaf
	cpm x 10 ⁻³	
Asp	5.74	0.11
Thr	0	0
Ser	0	0
Asn	2.13	0
Glu	5.81	1.14
Gln	0.62	0
Hse	0.24	0
Gly	0.06	0
Ala	0.25	0.01
Lys	1.67	0

^aRadioactivity in other amino acids was not determined.

In conclusion, it appears that some carbon from ^{14}C -HSA was transported to the young leaves and roots. Most of the radioactivity in the treated leaves remained as HSA, only a little being metabolized to other compounds, the main ones being glutamate and aspartate. In the young leaves, the radioactivity recovered there was largely still in HSA, suggesting transport in that form to the young leaves.

RADIOTRACER LABELLING EXPERIMENTS III DETACHED LEAVES

Introduction

The previous group of experiments in which ^{14}C -labelled amino acids, amides, and HSA were applied to the surface of mature pea leaves (leaf 4, stage 7), showed that carbon from all of these compounds was transported (in the light) to young leaves and roots. From the labelling pattern of compounds recovered in the young leaves it is possible to draw some conclusions about the identity of the compounds being transported. Asparagine, with some aspartate, appeared to be transported when ^{14}C -asparagine was applied to the leaf; glutamate and glutamine appeared to be the transport compounds of carbon from ^{14}C -glutamine. After foliar application of glutamate, glutamate and possibly γ -amino butyric acid and aspartate appeared to be transported. After labelling with aspartate, amino acids may have been transported but organic acids were heavily labelled in the mature leaves, and were also recovered in young leaves.

The method of King and Zeevaart (1974) for obtaining an EDTA-promoted exudate from the cut ends of petioles of detached leaves

offered a possibility for further investigation of the amino acids in this exudate and of the transport of labelled compounds from pea leaves.

Analyses of Amino Acids in Phloem Exudates from Detached,
Fully-Expanded Pea Leaves

Pea plants at the "4½ leaf" stage were selected and the petioles of the 4th leaves (fully expanded) were cut off under 20mM Na_2EDTA , pH 7.0. The cut ends of the petioles were immersed in 0.2 ml of the EDTA solution in small vials so that about 1 cm of the petiole was covered. The leaves were placed in the dark in a humid chamber as described (Methods 5). After 2 hours, the solution from the vials was collected, the EDTA precipitated by lowering the pH to 2.1, and this "phloem exudate" analyzed on the Beckman amino-acid analyzer.

The results of three experiments, four leaves in each, are shown in Table 24. The analyses, continued to alanine, show that several amino acids were present in the solution into which the leaves exuded. Asparagine, homoserine, and glutamic acid were highest in concentration; aspartic acid and serine were also present in high amounts. Figure 15 shows the analyzer scan of Experiment 1.

To show that the presence of amino acids was not merely leakage from cut or damaged cells at the end of the petiole, the amino-acid content of exudates from leaves with petioles in distilled water or EDTA were analyzed. The results (Figure 16) show that the amino-acid content of the exudate from H_2O -treated petioles after 2 hours was very low, while after only 30 minutes

TABLE 24

Amino-acid analyses of phloem exudate from
EDTA-treated leaves in the dark. Exudate
was collected for 2 hours from 3 groups
of 4 leaves each.

	Expt. 1	Expt. 2	Expt. 3
n moles per leaf in 2 hours			
Asp	3.50	4.22	2.82
BIA	0.57	0.63	0.93
Thr	0.86	0.67	0.42
U ^a	0.70	0.60	0.40
Ser	3.59	4.98	3.05
Asn	12.07	14.65	10.69
Glu	6.25	4.23	4.07
Gln	2.11	1.48	1.53
Hse	8.89	5.10	7.52
Aaa	n.d. ^b	0.93	0.93
Gly	n.d.	0.54	0.39
Ala	n.d.	0.51	0.60

^aU = unknown ninhydrin-positive compound.

^bn.d. = not determined.

Figure 15. Quantitative amino-acid analysis of EDTA-promoted phloem exudate from detached, mature pea leaves, maintained in the dark. Scan from Beckman Analyzer. Absorbance at 570nm (solid line) and 440nm (dotted line).

Figure 16. Amino-acid content of exudate from detached, mature pea leaves maintained in the dark. Ends of petioles were in water for 120 minutes (white bars), EDTA for 30 minutes (stippled bars), or EDTA for 120 minutes (solid bars).

of EDTA-treatment, the amino-acid levels were much higher than the water controls.

Similar experiments were conducted in the light. Identical humid chambers were used, a film of saran wrap covering the chamber in the light. Figure 17 shows the amino-acid analyses of phloem exudates from EDTA-treated and control leaves in the light and in the dark for 2 hours. In both light and dark, the levels of almost all of the amino acids were higher in the exudates from EDTA-treated leaves. Aspartic acid, asparagine, glutamic acid and homoserine were substantially higher in the dark.

Quantitative amino-acid analyses were made of aqueous extracts (SSA-precipitated extracts, Methods 15A) of the leaf blades and petioles of detached, fully-expanded (4th) leaves after 2 hours of EDTA-treatment in both the light and the dark. The phloem exudates were also analyzed. Two groups of three leaves each were used in each treatment, the extracts from each group of three leaves being combined.

The amino-acid levels in leaf blade and petiole were calculated as μ moles per g of fresh weight of tissue and then expressed as percentages of the total in the leaves or petioles. The amino acids in the exudate were calculated as μ moles exuded per leaf in 2 hrs. and the percentage distribution of the amino acids were also calculated.

The results are shown in Table 25 (light) and Table 26 (dark). In the light, the levels of asparagine in the petiole and exudate were highly variable between these two experiments and differed from earlier experiments (data for exudate in Figure 16) while the

Figure 17. Amino-acid content of exudate from detached, mature pea leaves maintained (A) in the dark, and (B) in the light. Ends of petioles were in water (white bars) or EDTA (solid bars) for 2 hours.

TABLE 25

The Percentage Distribution of Amino Acids
in the Leaf Blades, Petioles, and Phloem
Exudates of Detached 4th Leaves after
2 hrs of EDTA-treatment in the Light.
Data from 2 Experiments are shown.

	Leaf Blade		Petiole		Exudate	
	1	2	1	2	1	2
	% Distribution					
Asp	16.1	17.7	24.9	20.2	18.2	5.1
BIA					tr	tr
Thr	1.9	1.7	1.2	2.1	5.7	2.0
U ^a	5.7	4.7	1.2	1.8	2.3	2.0
Ser	7.3	6.5	8.7	8.3	13.6	14.0
Asn	18.3	13.3	1.6	11.7	3.4	tr
Glu	3.7	4.4	24.0	18.4	22.7	24.0
Gln	13.3	12.3	10.0	8.3	8.0	8.0
Hse	18.8	28.5	19.9	21.8	18.2	24.0
Aaa	0	0.1	0	0	0	0
Pro	0.8	0.9	0	0	0	0
Gly	0.7	0.6	2.5	2.5	0.6	14
Ala	13.4	9.4	5.6	4.9	0.2	tr
	μ moles per g F Wt.				n moles per Leaf	
	22.37	28.21	2.87	3.62	8.8	5.0

^aU = unknown ninhydrin-positive compound.

TABLE 26

The Percentage Distribution of Amino Acids
in the Leaf Blades, Petioles, and Phloem
Exudates of Detached 4th Leaves after
2 hours of EDTA-treatment in the Dark.
Data from 2 Experiments are shown.

	Leaf Blade		Petiole		Exudate	
	1	2	1	2	1	2
	% Distribution					
Asp	14.9	31.8	23.5	16.5	16.5	15.6
BIA	17.4				0.8	1.1
Thr	1.4	2.2	2.6	1.1	1.2	1.3
U ^a	6.4	6.7	2.1	3.2	2.3	1.9
Ser	6.9	6.3	7.8	6.2	8.8	8.4
Asn	20.9	15.8	17.6	35.9	14.6	16.2
Glu	5.6	5.6	20.4	11.4	20.0	21.3
Gln	7.5	8.0	8.7	4.1	8.1	8.1
Hse	14.5	18.6	13.3	18.6	23.1	22.1
Aaa	0	0	0	0.6	0	0
Pro	0.8	0.9	0	0	0	0
Gly	0.3	0.2	0.4	0.8	1.5	1.3
Ala	3.4	4.1	3.6	1.6	3.1	2.7
	μ moles per g F. Wt.				n moles per Leaf	
	20.0	20.86	6.42	12.02	26.0	37.1

^aU = unknown ninhydrin-positive compound.

levels of aspartic acid, glycine and alanine in the exudate were also variable. In both light- and dark-treatments, however, the proportions of certain amino acids markedly and consistently differed between the leaf blade and the exudate. Both in the light and the dark, the unknown amino compound (U) running between threonine and serine was present in higher proportions in the leaf blade than those in petiole or exudate, being 5.7 and 4.7% of the total free amino-acid pools in the leaf blades in the light and 6.4 and 6.7% in the dark, compared to 2.3 and 2.0% (light) and 2.3 and 1.9% (dark) in the exudate.

The levels of glutamate, on the other hand increased from 3.7 and 4.4% of the total free amino acid pool in the leaf blade in the light (5.6 and 5.6% in the dark) to 24.0 and 18.4% in the light in the petiole (20.4 and 11.4 in the dark) and 22.7 and 24.0% (light) in the exudate (20.0 and 21.3, dark).

Alanine was higher than glycine in the leaf blade (both light and dark), but the levels of glycine appeared to increase with respect to alanine in the petiole and exudate.

The ratios of certain pairs of amino acids (μ moles per gram fresh weight) is shown in Table 27. The ratios of the unknown (U) to threonine decreased in the petiole and the exudate, compared to those in the leaf blade. Ratios of glutamate to glutamine were higher in the petiole and the exudate than in the leaf blade. The ratios of glycine to alanine were strikingly low in the leaf blades, and were much higher in the petiole and exudate.

From these data it appeared that the amino acid composition of the phloem exudate differed greatly from that of the leaf

TABLE 27

The Ratios of Certain Amino Acids in Leaf Blade
Petiole, and Phloem Exudate of Detached,
EDTA-treated Leaves after 2 hrs in the Light or
in the Dark. Two Experiments of Three Leaves Each.

	LIGHT					
	Leaf Blade		Petiole		Exudate	
	1	2	1	2	1	2
U ^a :Thr	3.0	2.8	1.0	0.9	0.4	1.0
Ser:Thr	3.7	3.8	6.9	3.9	2.4	7.0
Glu:Gln	0.3	0.4	2.4	2.2	2.9	2.9
Gly:Ala	0.05	0.06	0.4	0.5	2.6	>8.0

	DARK					
	Leaf Blade		Petiole		Exudate	
	1	2	1	2	1	2
U ^a :Thr	4.5	3.0	0.8	2.9	1.9	1.4
Ser:Thr	4.8	2.8	3.0	5.7	7.5	6.3
Glu:Gln	0.7	0.7	2.4	2.8	2.5	2.6
Gly:Ala	0.08	0.05	1.0	0.5	0.5	0.5

^aU = unknown ninhydrin-positive compound.

blades in both the light and the dark EDTA-treated leaves.

Amino-acid analyses were also made of aqueous extracts of intact 4th leaves at the time the treated leaves were detached (0 hrs.) and also two hours later when the exudate was collected and the EDTA-treated leaves were extracted (2 hrs.). The amino-acid concentration in μ moles per gram fresh weight of leaf blades and petioles of the intact leaves at 0 and 2 hrs. and of the detached, EDTA-treated leaves, both light and dark, are shown in Figure 18. Data from 2 experiments (3 leaves in each) are shown.

Apart from a slight decrease in the level of asparagine in the leaf blades, and a rise in the level of homoserine in the petioles, very little change in the levels of other amino acids in the intact leaves are observed. Since these experiments were conducted between 12.00 noon and 15.00 hours, during the second half of the light period, little change was expected; Bauer, Urquhart, and Joy (1977) had demonstrated stable levels of amino acids during this period in pea leaves from intact plants or detached shoots grown in similar conditions.

In the detached, EDTA-treated leaves, however, a few changes were observed. In the leaf blade, (compared with the intact, 2-hour controls) aspartate (+ BIA) and glutamate levels were lower in the light; glutamine, homoserine, and alanine were higher in the light; glutamate and alanine levels were lower in the dark. Proline, virtually absent in the control leaf blades, was present in very low concentration in the detached leaves.

Figure 18. Amino-acid content of leaf blade (A) and petiole (B) of mature pea leaves. Control leaves (intact plants) were samples at 0 hours (12.00 noon) (white bars) and 2 hours (solid bars). Detached, EDTA-treated leaves were sampled after 2 hours in the light (stippled bars) or the dark (hatched bars). Two determinations for each treatment.

In the petioles of the detached leaves (compared with the 2-hour controls) aspartate, serine, asparagine, and glutamate were all lower in the light but little changed in the dark; glutamine and alanine were lower in both light and dark. Homoserine was lower than in the 2-hour control, but only slightly less than in the 0-hour control.

¹⁴C-Phloem Exudate from Detached, EDTA-Treated Leaves Fed with Radiotracers

These last experiments showed that it was possible to obtain an exudate, enriched in certain amino acids from cut petioles treated with 20 mM EDTA, pH 7.0, when the leaves were maintained in the light or the dark. If this exudate came from the phloem, then it was of interest to see if exogenously applied ¹⁴C-labelled amino acids (presumed to be translocated in the intact plant to the young leaves and roots via the phloem) would be translocated out of detached leaves into the EDTA solution bathing the cut ends of the petioles.

Plants were therefore selected, and glass chambers attached to the prepared, lower surface of fully-expanded 4th leaves as previously described. After allowing the Silicone Polymer to harden for 30 minutes, the petioles of the 4th leaves were cut under either water or 20 mM EDTA, pH 7.0, and the cut ends of the petioles were placed in 0.2 ml of water or EDTA respectively.

The radiotracers, ¹⁴C-labelled asparagine, glutamine, aspartate, glutamate, and HSA (1 µl per leaf) were introduced into the leaf-chambers with a microsyringe.

After application of the ^{14}C -labelled compounds, the detached leaves with petioles in vials of water or EDTA were placed in the dark, humid chamber for 2 hours. The phloem exudates were then collected, and radioactivity estimated by liquid scintillation counting. The leaves were rinsed and extracted in 80% ethanol as previously described.

The radioactivity recovered in the phloem exudate solution is shown in Table 28. Little or no radioactivity was found in the exudates from petioles treated with water. On the other hand, EDTA treatment enhanced the levels of radioactivity recovered in the exudate, with the exception of HSA. In the glutamate-fed leaves, the ^{14}C -label in the exudate was more than double that in the water controls; in the asparagine-, aspartate-, and glutamine-fed leaves, levels of ^{14}C -label in the exudate were more than six times that in the controls. Radioactivity in the exudate from HSA-fed leaves was however, barely detectable after 2 hours in the dark.

The distance, over which labelled carbon was transported in 2 hours, from the point of application on the leaf blade to the end of the cut petiole was 25 to 30 mm.

The amount of radioactivity exported in 2 hours into the exudate was calculated as a percentage of the total radioactivity taken up by the leaf. These values were 3.85% (asparagine), 4.8% (glutamine), 0.9% (aspartate), and 0.3% (glutamate). Values for radioactivity transported from mature leaves of intact plants to young leaves plus roots (also in 2 hours) were 15 and 19% (asparagine), 11.1 and 11.8% (glutamine), 7.3 and 10.4% (aspartate), and 5.4 and 6.5% (glutamate).

TABLE 28

Radioactivity in the Exudate from Petioles
of Detached Fully-expanded Leaves fed with ^{14}C -labelled
Amino Acids and Amides for 2 hours in
the Dark.

Tracer	Treatment	
	H_2O	EDTA
	cpm per Leaf	
Asn	17 (4) ^a	207 $\pm$ 89 (8)
Gln	0 (2)	2660 $\pm$ 955 (3)
Asp	93 (2)	576 $\pm$ 332 (3)
Glu	65 (2)	146 $\pm$ 99 (3)
HSA	4 (3)	8 $\pm$ 2 (5)

^a Figures in brackets are the numbers of leaves used in each treatment.

These values, from 2 groups of intact plants in the light, are substantially higher than the percentages exported from detached leaves; they are 5, 2.5, 10, and 20 times higher respectively. In the detached leaves, however, the phloem was disrupted by the cutting of petioles and removal of the sinks (young leaves and roots) and transpiration was considerably reduced; the diminished export of ^{14}C -label from these detached leaves (in the dark) compared to that from leaves of intact plants (in the light) is, therefore, hardly surprising.

Phloem Exudate from HSA-Fed, Detached Leaves

The lack of movement of HSA out of the leaves in the dark was of interest. The experiment was, therefore, repeated, supplying ^{14}C -labelled HSA to detached, prepared leaves as before. The exudate (into water or EDTA) was collected after 2 hours from leaves in the light and in the dark.

The radioactivity recovered in the phloem exudate is shown in Table 29. Both light and dark water-treated controls and the EDTA-treated leaves in the dark transported very little ^{14}C -label into the exudate. In the light, however, slightly higher levels of radioactivity (97 ± 35 cpm per leaf in 2 hours) were recovered in the exudate of EDTA-treated leaves. Some component of the export of labelled carbon from HSA out of the leaf blade appeared to require light.

Identification of Labelled Components of the Phloem Exudate from ^{14}C -Asparagine-Labelled Leaves

The EDTA treatment of cut petioles appeared to promote exudation of labelled amino acids and amides from the leaf blade

TABLE 29

Radioactivity in the Exudate from Petioles of
Detached, Fully-Expanded Leaves fed with
 ^{14}C -Labelled HSA for 2 hours in the Light
and in the Dark.

	Treatment	
	H_2O	EDTA
	cpm per Leaf	
Dark	6 (2) ^a	20 $\pm$ 4 (5)
Light	16 (2)	97 $\pm$ 35 (5)

^a Figures in brackets are the numbers of leaves used in each treatment.

through the petiole; therefore this technique was used to examine the nature of the compounds being transported.

Fully-expanded 4th leaves were selected and prepared as before, and the petioles cut under EDTA. Four groups of three leaves each (2 groups in the light; 2 in the dark) were fed with ^{14}C -asparagine as previously described.

After 2 hours, the leaves and coverglass leaf-chambers were rinsed, and the leaves frozen and extracted in 80% (v/v) ethanol as before. Radioactivity was estimated in aliquots of the phloem exudate, the total ethanol-soluble and -insoluble fractions of the leaf, and in the leaf rinse (Table 30).

Most of the radioactivity was still in the ethanol-soluble fractions of the leaves. Although CO_2 release was not measured in these experiments, any loss of label as $^{14}\text{CO}_2$ would be included in the total losses. Some synthesis of insoluble material occurred in the dark, but the incorporation of label into the insoluble fraction was higher in the light. Of the total radioactivity taken up by the leaves, 3.9 and 4.6% were exported into the exudate in the dark and 0.8 and 2.2% in the light.

The phloem exudates and corresponding aqueous leaf extracts were passed over ion exchange resins as previously described, to obtain amino-acid, organic-acid, and neutral fractions. The percentage distribution of radioactivity in these fractions is shown in Table 31.

In the light-treated leaves, 70 to 75% of the soluble radioactivity in the leaf extract was still in the amino-acid fractions, while 20 to 25% was recovered in the organic-acid

TABLE 30

Radioactivity in Phloem Exudate and Soluble
and Insoluble Fractions of Leaf Extracts from
Detached, Fully-expanded Leaves, Treated
with EDTA and Labelled with ^{14}C -Asparagine
for 2 hours in the Light and the Dark.

	Light		Dark	
	1	2	1	2
	cpm x 10 ⁻⁴			
<hr/>				
Radioactivity				
Amount Supplied	31.2	31.2	31.2	31.2
Total Recovered	26.4	22.6	25.5	24.2
Washed Off	3.8	0.4	5.6	5.0
Losses (inc. CO ₂)	4.8	8.6	5.7	7.0
<hr/>				
Radioactivity in Leaf				
Ethanol Soluble	18.0	18.2	18.6	16.2
Insoluble	4.5	4.0	1.3	3.0
Phloem Exudate	0.2	0.5	0.8	0.9
Total Recovered in Leaf + Exudate	22.7	22.7	20.7	20.1
<hr/>				
	% of Total Recovered in Leaf			
Exported	0.83	2.18	3.85	4.55

TABLE 31

Radioactivity in the Amino-Acid, Neutral and Organic-Acid Fractions of Leaf Extracts and Phloem Exudates of ^{14}C -Asparagine-fed, EDTA-treated, Detached Leaves after 2 hours in the Light and in the Dark. Data from 2 Experiments are shown.

		Amino Acid	Neutral	Organic Acid
Leaf Extract		% of Total Radioactivity in Extract		
Light	1	70.1	3.5	26.4
	2	76.2	2.8	21.0
Dark	1	97.6	0.5	1.9
	2	92.0	1.2	6.8
Phloem Exudate		% of Total Radioactivity in Exudate		
Light	1	94.5	4.1	1.4
	2	96.5	2.0	1.5
Dark	1	99.1	0.2	0.7
	2	99.7	0.1	0.2

fractions. Similar distribution of label was observed in aqueous leaf extracts from ^{14}C -asparagine-labelled intact plants (e.g. Table 11). In the dark-treated leaves, however, over 90% of the soluble label in the leaf extract was still in the amino-acid fraction, only a small amount of label, 1.9 and 6.8%, being recovered in the organic-acid fractions.

On the other hand, the radioactivity in the phloem exudates from both light and dark treatments, was found to be almost entirely in the amino-acid fraction (greater than 99% in the dark, and greater than 95% in the light). Regardless of the proportion of radioactivity metabolized to non-amino-acid compounds in the leaf, the radioactivity in the exudate was almost entirely amino acids.

The amino-acid fractions of the phloem exudates and the leaf extracts were further separated on the amino-acid analyzer, estimating the radioactivity in fractions collected from the column by liquid scintillation counting.

Figure 19 shows profiles of the radioactivity in the phloem exudate fractions eluted from the amino-acid analyzer column. Since these exudate samples were too small to permit a second, analytical run on the analyzer, or to split the effluent between the colorimeter and the fraction collector, the elution positions of the radioactive peaks were compared with those of an amino acid standard sample, as shown in the overlay.

Both in the light and the dark, the radioactivity in the amino acids of the phloem exudate was almost all in asparagine or aspartic acid. The results in Table 32, showing detailed distribution of radioactivity in the phloem exudates, indicate that in both light

Figure 19. Separation of amino-acid fractions of EDTA-promoted phloem exudate from ^{14}C -asparagine-fed leaves in the dark or in the light (2 experiments in each treatment). Radioactivity was measured in fractions eluted from the amino-acid analyzer. Transparent overlay shows elution pattern of amino-acid standard (quantitative analysis).

FIGURE 19 IS FIRST FILMED ALONE.
THE TRANSPARENT OVERLAY IS THEN
PLACED ON THE CHART AND
BOTH ARE FILMED TOGETHER.

TABLE 32

Distribution of Radioactivity in the Amino Acids
and Other Fractions of the Phloem Exudates
of ^{14}C -Asparagine-fed Leaves in the Light
and in the Dark. Data for 2 Experiments are shown.

Amino Acid	Light		Dark	
	1.	2	1	2
	% of Total in Exudate			
Asp	22.3	28.3	37.3	36.1
Asn	60.7	58.8	44.6	60.1
Others	11.6	9.4	17.2	3.5
Neutral	4.1	2.0	0.2	0.1
Organic Acid	1.3	1.5	0.7	0.2
Total Radioactivity in Exudate	Radioactivity cpm $\times 10^3$			
	1.9	4.8	7.7	8.8

and dark treatments, asparagine accounted for 45 to 60% of the total, and aspartic acid plus asparagine was 83 and 87.1% of the total label in the light and 81.9 and 96.2% of the total label in the dark.

The radioactivity in aspartate was shown to be not the result of asparagine hydrolysis during concentration of the phloem exudate and precipitation of the EDTA prior to loading the samples on the analyzer: unlabelled, purified asparagine was added to the equivalent volume of 20 mM EDTA, pH 7.0, as that of the phloem exudate (0.6 ml), concentrated and treated in the same way as the phloem exudate, and samples of this solution, after running on the amino-acid analyzer showed no hydrolysis of asparagine to aspartate. Figure 20 shows the analyzer scans of both untreated but purified asparagine (A) and asparagine treated in a similar manner to the phloem exudate. no aspartate appeared in either sample.

The amino-acid fractions of the leaf extracts were also separated on the amino-acid analyzer. Fractions eluting from the column were collected and radioactivity estimated. These leaf extract samples were large enough that either the effluent was divided with 10% of the stream going to the colorimeter, or a separate analytical run was performed.

The distribution of radioactivity in the soluble fractions of the leaf extracts is shown in Table 33. In the dark-treated leaves, most of the radioactivity (77.6 and about 72.5% of the total soluble label) was in asparagine with 14.2 and 12.4% of the total soluble label being recovered in aspartate. In the dark-treated leaves then, the distribution of label between the soluble

Figure 20. Quantitative amino-acid analyses of asparagine (A) untreated and (B) in 20mM EDTA treated in similar manner to phloem exudate. Scan at 570nm.

TABLE 33

Distribution of Radioactivity in the Amino-Acid and Other Soluble Fractions of Leaf Extracts from Detached Leaves, fed with ^{14}C -Asparagine in the Light and in the Dark.
Data for 2 Experiments are shown.

Amino Acid	Light		Dark	
	1	2	1	2
	% of Total in Soluble Fraction			
Asp	8.6	9.9	14.2	12.4
Asn	48.4	45.6	77.6	72.5
Glu		8.0	1.8	
Gln	1.4	4.9	0.4	0.6
Hse	2.3			0
Ala	1.1	0.6	0	0.2
Others	8.3	7.2	3.6	6.4
Neutral	3.5	2.8	0.5	1.2
Organic Acid	26.4	21.0	1.9	6.8
Radioactivity $\text{cpm} \times 10^{-4}$				
Total Soluble Radioactivity	18.02	18.17	18.58	16.24

components of the leaf extract was similar to that in the phloem exudate.

In the light-treated leaves, however, slightly less than 50% of the total label was recovered in asparagine, while aspartate, glutamate, glutamine, and homoserine all had received some ^{14}C -label. The organic-acid fractions accounted for 26.4 and 21.0% of the total, this radioactivity being shown to be almost all as ^{14}C -HSA (after separation of organic-acid fractions by ascending paper chromatography as previously described).

The data showed that in the light, much of the applied ^{14}C -asparagine was metabolized to other amino acids, and other soluble fractions, but only asparagine and aspartic acid were exported into the petiole to be recovered in the phloem exudate. Even though asparagine and aspartate were the most highly labelled compounds in the leaf extract, the proportion of labelled aspartate was higher in the phloem exudate compared to that in the leaf extract, suggesting a selectivity in the transfer of these labelled compounds from the soluble pools in the leaf blade into the petiole.

These results are further evidence that, in the experiments in which ^{14}C -asparagine was fed to mature leaves of intact plants, the radioactive compounds being exported from the mature leaves to the young leaves and roots, were asparagine and aspartate.

Identification of the Labelled Components of the Phloem Exudate from ^{14}C -Aspartate-Labelled Leaves

After feeding intact plants via the mature leaves for 2 hours with ^{14}C -aspartate, 30 to 35% of the label transported to the young leaves was recovered in the organic-acid fraction. These values were

equivalent to about 40% of the soluble radioactivity in these young leaves. Since, in the mature leaves supplying the ^{14}C -label, about 50% of the soluble radioactivity was in the organic-acid fraction, it was possible that transport to the young leaves could be in the form of organic acids. The identity of labelled compounds in the phloem exudate from ^{14}C -aspartate-fed leaves was therefore of interest.

Labelled aspartate (1 μl per leaf) was fed to fully-expanded pea leaves, selected and prepared as before and detached with petioles under EDTA. Exudate was collected after 2 hours from 4 groups of leaves (4 leaves in each), 2 in the light and 2 in the dark.

Radioactivity was estimated in aliquots of the phloem exudate, ethanol-soluble and insoluble fractions of the leaf, and the leaf rinse as before; exudate and soluble leaf-fractions were passed over Dowex resins.

As shown in Table 34, the radioactivity taken up by the leaf (total recovered in leaf extract and phloem exudate) was higher in the light than in the dark; more label was recovered in the leaf washings of the dark-treated leaves. Most of the radioactivity recovered in the leaves was in the ethanol-soluble fractions (both light and dark); the ^{14}C -label incorporated into the insoluble fractions was higher in the light-treatments. The percentages exported from the leaves into the exudates were 0.2 and 0.4% (light) and 0.7 and 3.8% (dark).

Table 35 shows the distribution of radioactivity in amino-acid, neutral, and organic-acid fractions. In the light 35 to 40% of the

TABLE 34

Radioactivity in the Phloem Exudate and Soluble and Insoluble Fractions of Leaf Extracts from Detached, Fully-expanded Leaves, Treated with EDTA and Labelled with ^{14}C -Aspartic Acid for 2 Hours in the Light and in the Dark.

	Light		Dark	
	1	2	1	2
	cpm $\times 10^{-4}$			
Radioactivity				
Amount Supplied	32.9	32.9	32.9	32.9
Total Recovered	28.2	27.7	29.1	27.3
Washed Off	4.3	0.8	18.2	13.1
Losses (inc. CO_2)	4.7	5.2	3.7	5.6
Radioactivity in Leaf				
Ethanol Soluble	20.1	22.7	9.8	12.2
Insoluble	3.8	4.1	1.1	1.5
Phloem Exudate	0.052	0.110	0.073	0.544
Total Recovered in Leaf & Exudate	23.95	26.91	10.97	14.24
	% of Total Recovered in Leaf			
Exported	0.22	0.41	0.67	3.82

TABLE 35

Radioactivity in the Amino-Acid, Neutral
and Organic-Acid Fractions of Leaf Extracts
and Phloem Exudates of Detached, EDTA-treated Leaves
¹⁴C-Aspartate-fed after 2 hours in
the Light and the Dark.

		Amino Acid	Neutral	Organic Acid
% of Total Radioactivity in Soluble Leaf Extract				
Leaf Extract				
Light	1	34.6	7.1	58.3
	2	41.4	6.5	52.1
Dark	1	67.4	0.3	32.3
	2	72.6	0.2	27.2
% of Total Radioactivity in Exudate				
Phloem Exudate				
Light	1	63.7	19.3	17.0
	2	64.1	13.2	22.7
Dark	1	76.6	0	23.4
	2	75.1	0	24.9

soluble radioactivity was in the amino-acid fraction of the leaves, 7.0% in the neutral fraction, and 50 to 60% in the organic-acid fraction; a similar distribution was found in leaves of intact plants (Table 18). In the phloem exudate from these leaves, however, the distribution of label was quite different; over 60% of the radioactivity was in the amino-acid, 13 to 19% in the neutral fractions, and about 20% in the organic acids. As with asparagine-fed leaves, the distribution of label between basic, neutral, and acid fractions in the phloem exudate was not the same as that in the leaf extract in these light-treated leaves. The amino-acid fraction of the phloem exudate had a higher proportion of label than that of the leaf extract.

In the dark-treated leaves, about 70% of the label remained in the amino-acid fraction and about 30% was recovered in the organic-acid fraction; the distribution in the phloem exudate was similar to that in the leaf extracts in these leaves.

The amino-acid fractions from leaf extract and phloem exudate of Experiment 2 were separated on the amino-acid analyzer and the distribution of radioactivity in amino-acid and other soluble fractions is shown in Table 36. In the leaf extracts in the light, only 10.6% of the label remained in aspartate; some label was recovered in glutamate and in glutamine plus homoserine, this peak not being separated in these runs. In the phloem exudate from these leaves, 28.3% was recovered in aspartate and the percentage label in glutamate and glutamine plus homoserine was higher in the phloem than in the leaves.

TABLE 36

Distribution of Radioactivity in the Amino-Acid
and Other Soluble Fractions of Leaf Extracts
and Phloem Exudates from Detached Leaves
fed with ^{14}C -Aspartate in the Light
and in the Dark.

	Phloem Exudate		Leaf Extract	
	Light	Dark	Light	Dark
% of Total Radioactivity in Soluble Fraction ^a				
Amino Acid				
Asp	28.3	36.5	10.6	45.4
BIA	0	0	0	0.6
Thr	0	0	0.2	0
Ser	0	0	0	0
Asn	0	0	0	1.7
Glu	15.1	35.2	5.6	13.8
Gln	17.9	0	13.4	6.0
Hse				
Gly	0	0	0	0
Ala	0	0	1.7	0
Others	2.8	3.4	9.9	5.1
Neutral	13.2	0	6.5	0.2
Organic Acids	22.7	24.9	52.1	27.2
cpm x 10 ⁻⁴				
Total Radio-activity in Soluble Fraction	0.110	0.544	22.809	12.194

^aData from Expt. 2, Table 34.

In the extracts from dark-treated leaves, 45.4% of the radioactivity remained in aspartate. Glutamate and glutamine plus homoserine were also labelled (13.8 and 6.0% respectively) and small amounts of label were also recovered in asparagine. Label was also found in a peak immediately following aspartate and believed to be β -(isoxazolin-5-one-2-yl)-alanine(BIA).

In the phloem exudates from these dark-treated leaves, although the overall distribution of label between amino-acid and organic-acid fractions was similar, the proportion of label in aspartate in the phloem exudate was lower, and that in glutamate much higher than in the leaf extract.

The distribution of label in the amino-acid, neutral, and organic-acid fractions was quite different in the phloem exudates from that in the leaf extracts. In both light and dark, aspartate and glutamate appeared to have been preferentially transported from the leaf blade, but radioactivity was also recovered in organic acids in the phloem exudate. These were not further identified, the quantities being too small.

Identification of the Labelled Components in the Phloem Exudate of ^{14}C -HSA Labelled Leaves

When intact plants were fed via the mature leaves with ^{14}C -HSA, labelled carbon was transported to the young leaves and roots. Since most of the radioactivity was recovered still as HSA in the mature leaves after 2 hrs., and ^{14}C -labelled HSA was also the major component in the young leaves, it was possible that the labelled carbon was being transported as HSA.

Phloem exudates were therefore collected from detached, fully-expanded pea leaves, fed with ^{14}C -HSA. The leaves were prepared and detached with petioles under EDTA, as before, and the leaves were placed with the petioles in 0.2 ml of the EDTA solution.

Since only low levels of radioactivity were obtained previously in phloem exudates of ^{14}C -HSA-fed detached leaves, and only in the light-treated leaves, a total of 32 leaves were fed with labelled HSA for 2 hours in the light in three groups of leaves (8, 12, and 12).

Radioactivity was estimated in phloem exudates and soluble and insoluble fractions of the leaf extracts as described before, and the exudate and soluble leaf fractions were passed over Dowex resins.

Table 37 shows that most of the radioactivity recovered in the leaf was in the ethanol-soluble fractions of all three experiments, (94 to 95%) and between 4 and 6% of the total recovered in the leaf was in the insoluble fractions. Between 0.1 and 0.2% of the total in the leaf was exported in 2 hours in the phloem exudate.

Table 38 shows the distribution of radioactivity in the soluble fractions of the leaf extracts and in the phloem exudates of the three experiments. Most of the radioactivity, 98 to 99%, was still in the organic-acid fractions in the leaf extracts, slightly higher proportions than those found in the leaf extracts of mature leaves of intact plants, where 88 to 95% of the label was in the organic-acid fraction (Table 22).

In the phloem exudate, on the other hand, between 12 and 15% of the radioactivity was recovered in the amino-acid fractions, 8 to 11% in the neutral fraction, and between 74 and 80% in the organic-

TABLE 37

Radioactivity in the Phloem Exudate and Soluble and Insoluble Fractions of Leaf Extracts from Detached, Fully-expanded Leaves, Treated with EDTA and Labelled with ^{14}C -HSA for 2 Hours in the Light.

	Experiment		
	1	2	3
	cpm x 10^{-4}		
Radioactivity			
Amount Supplied	40.0	65.0	65.0
Total Recovered	38.5	65.0	62.2
Washed Off	4.6	11.8	3.4
Losses	1.5	0	2.8
Radioactivity in Leaf			
Ethanol-Soluble	31.94	50.12	55.90
Insoluble	1.91	3.19	2.78
Phloem Exudate	0.044	0.071	0.094
Total Recovered in Leaf & Exudate	33.89	53.38	58.77
	% of Total in Leaf		
Exported	0.13	0.13	0.16

TABLE 38

Radioactivity in the Amino-Acid, Neutral and
Organic-Acid Fractions of Leaf Extracts &
Phloem Exudates of Detached EDTA-treated
Leaves after 2 hours of ^{14}C -HSA Feeding
in the Light.

	Amino Acid	Neutral	Organic Acid
% of Total Radioactivity in Soluble Leaf Extract			
Leaf Extract			
1	0.2	0.9	98.9
2	0.2	1.1	98.7
3	0.2	1.0	98.8
% of Total Radioactivity in Exudate			
Phloem Exudate			
1	14.5	11.2	74.2
2	13.9	9.3	76.8
3	11.9	8.0	80.1

acid fractions. The distribution of ^{14}C -label in the different fractions of the phloem exudates, were, therefore, markedly different from the distribution in the leaf extracts.

The amino-acid fractions of the phloem exudates were combined and further separated on the amino-acid analyzer. Radioactivity was recovered in aspartate (25% of the radioactivity in the amino-acid fraction), threonine (12.5%), glutamate (30%), glutamine (13.7%), and homoserine (18.8%). The combined organic-acid fractions were run on thin-layer plates coated with cellulose in 1-butanol; acetic acid; water (12:3:5 by volume). All of the radioactivity co-chromatographed with authentic ^{14}C -HSA.

The organic-acid fractions of the leaf extracts were found (after separation by ascending paper chromatography as before) to remain almost entirely as ^{14}C -HSA. The label in the amino-acid fractions was too low to permit further separation of those fractions.

In summary, these experiments with detached leaves showed that carbon from ^{14}C -labelled asparagine and aspartic acid appeared to be preferentially loaded as amino acids into the phloem from the site of application on the leaf blade. Carbon from ^{14}C -HSA, applied to the leaf blade, was recovered in the exudate from the petiole mainly still as HSA but labelled amino acids were also recovered in the phloem exudate.

DISCUSSION

NITROGEN STATUS AND DEVELOPMENT OF PEA LEAVES

The clearly recognizeable developmental stages of *Pisum Sativum* leaves, characterized by the stage of unfolding of leaflets and then stipules (as shown in Figure 2) was useful for correlating changes in nitrogen levels and other growth parameters as the leaves expanded. Plants at equivalent stages of development could be selected by the stage of expansion of the leaves.

Fully-expanded and half-expanded leaves were shown to be transpiring vigorously. Since the xylem bleeding sap of pea seedlings of this age contained a mixture of mainly nitrate and amino acids, these transpiring leaves will receive nitrogenous compounds directly in the transpiration stream. The nitrogen levels of expanding leaves were shown in this study to be increasing rapidly (Figure 4). These levels reached a maximum when the leaves were just fully expanded, and were maintained for a few more days, then declined slowly. Thus the nitrogen arriving in leaves via the transpiration stream did not continue to accumulate once the leaves were fully expanded. Very young leaves, on the other hand, were enclosed within the stipules of the older leaves and were shown to transpire negligible amounts of water (Figure 6). It is, therefore, unlikely that the youngest leaves would receive nitrogenous compounds directly from the transpiration stream, but probable that they would receive nitrogen from the mature leaves, presumably as amino acids in the phloem translocate as suggested by the Radiotracer labelling experiments. The diagram on page xviii shows the supply of nitrogen to the shoots and the redistribution from mature leaves via the phloem.

Although $^{14}\text{CO}_2$ -labelling experiments were not carried out in the present study, many other studies show that photosynthetic assimilates would be exported from the mature, fully-expanded leaves to the younger leaves and the roots. The results of the experiments in which ^{14}C -amino acids were applied to leaves of pea plants (radiotracer experiments II, discussed later) showed that carbon (from amino acids) was indeed transported from fully-expanded leaves to the rest of the plant, mainly to the half-expanded and immature leaves and the roots. In these studies, the half-expanded leaves (at Stages 4 to 5 - Figure 2) were importing carbon from the mature leaves (Tables 9, 10 and 17).

From other studies it appears that at some stage during leaf expansion, the leaves start to export photosynthate. *Cucurbita pepo* leaves, for example, start to export carbon when they are 35% expanded (Turgeon, 1973). At certain stages, leaves can both import and export (Webb and Gorham, 1964). The upper (distal) portion of *Cucurbita pepo* leaves started to export photosynthate while the more proximal parts were still importing (Turgeon, 1973). From the present study, it is clear that at the stages of development used, at least part of the half-expanded leaves of *Pisum sativum* were importing.

NITROGEN REQUIREMENTS FOR GROWING PEA LEAVES AND CALCULATIONS OF APPROXIMATE NITROGEN UPTAKE

From the data obtained for nitrogen increments in the expanding leaves, the nitrogen requirements of these leaves can be calculated. Also from the measurements of bleeding sap nitrogen concentration,

and transpiration rates of expanding and mature leaves, approximate values for nitrogen flow into the leaves can be estimated.

From the data in Figure 4, the nitrogen requirements were calculated:

Young leaf (enclosed within stipules)

at stage 1 to 2, 0.15 mg N per day

at stage 2 to 3, 0.30 mg N per day

Expanding leaf

at stage 4 to 5, 1.10 mg N per day

The nitrogen level of the fully-expanded leaf (Leaf 4) had reached a maximum and was constant at that level for about 3 days.

The xylem bleeding sap of these "4½-leaf" plants contained a mixture of amino acids (11 mM N, 70% of which was supplied by the amides) and nitrate (4 to 5 mM N). Similar levels of amino- and nitrate-N were found by Bauer, Urquhart, and Joy (1977). The total nitrogen concentration in the bleeding sap was about 0.015 $\mu\text{mol N}$ per μl . Ureides^a were less than 0.001 μmol per μl .

The young, or immature, leaf (Stages 1 to 3) did not transpire, but there was a net increase in water content of 10 μl per day between Stages 1 and 2, and 38 μl per day between Stages 2 and 3 (calculated from Fresh and Dry Weight increases shown in Figure 4). The maximum nitrogen that the xylem could contribute (if all of this net water increase came from this source) would be 0.15 $\mu\text{mol N}$ and 0.57 $\mu\text{mol N}$ respectively, or 2 to 4 μgN and 8 to 15 μgN respectively depending on the proportion of amino- to amide-N available. These values for possible xylem contribution are therefore far below the nitrogen requirements of the immature leaf.

^aUreides, since they comprised such a small portion of the xylem-borne nitrogen, were not included in the following calculations.

The transpiration rates for expanding leaves (Leaf 5 at Stages 4 to 5) were approximately 0.1 ml per hour. Even if the transpirational xylem sap maintained the same nitrogen concentration as the slowly exuding, bleeding sap, it could only supply 1.1 μmol amino acids and 0.4 μmol NO_3 per hour, or 13.2 μmol amino acid and 4.8 μmol NO_3 per day (12-hour light period). Since the bleeding sap was about 70% amide, and assuming no selective absorption of amino acids, amides, or nitrate from the xylem pathway during ascent of the sap, the supply of nitrogen to the expanding leaf would be approximately 259 $\mu\text{g N}$ (amide), 55 $\mu\text{g N}$ (amino acid) and 67 $\mu\text{g N}$ (nitrate) per day, a total of 381 $\mu\text{g N}$ per day. Since the actual nitrogen increase between Stages 4 and 5 was 1.1 mg N per day, transpiration alone from this leaf would therefore be insufficient to provide the required nitrogen.

The mature leaf (Leaf 4) transpiring approximately 0.14 ml per hour would, based on the same assumptions as for Leaf 5, receive 362 $\mu\text{g N}$ (amide), 78 $\mu\text{g N}$ (amino acid), and 94 $\mu\text{g N}$ (nitrate), a total of 534 $\mu\text{g N}$ per day. Since the fully-expanded leaves of Stage 7 had reached the maximum nitrogen content, all of this nitrogen was therefore available for export. Similar calculations showed that Leaves 1 to 3 could receive and export 1182 $\mu\text{g N}$ per day.

A balance-sheet of the nitrogen requirements and supply for leaves of pea shoots is shown in Table 39. These estimations show that almost all of the nitrogen required by the immature leaves must come from the mature leaves. A little over one-third of the nitrogen requirements of the expanding leaf (at Stages 4 to 5)

TABLE 39

Balance Sheet of Nitrogen Requirements and Nitrogen Supply for Immature, Expanding, and Mature Leaves of Pea Shoots.

Number	Leaf Stage	Nitrogen (ug N per day)		
		Requirements	Supply ^a	Balance
6	1-2 (immature)	-150	+2 to +4	-146 to -148
5	4-5 (expanding)	-1100	+381	-719
4	7 (mature)	0	+534	+534
1 to 3	(mature)	0 ^b	+1182	+1182

^aby transpiration or absorption.

^bolder leaves may show net decrease in nitrogen content and hence would contribute additional exported nitrogen.

could be supplied by transpiration of that leaf, the remainder would have to be supplied by the mature leaves. The mature leaves received in the transpiration stream a total of 1716 $\mu\text{g N}$, considerably more than that still required by the immature and expanding leaves together (866 $\mu\text{g N}$).

These calculations, however, did not allow for dilution of the amino-N and nitrate by the through-flow of water or absorption of nitrogenous compounds from the pathway. Such dilution of the xylem contents by 40% can in fact occur, before the supply of nitrogen to the immature and expanding leaves by transpiration (229 $\mu\text{g N}$ per day, Leaf 5) or re-export (1030 $\mu\text{g N}$ per day, Leaves 1 to 4) just equals their total requirements (1250 $\mu\text{g N}$ per day). No allowance was made in constructing the balance-sheet for redistribution of nitrogen to the roots via the phloem which may normally occur (discussed later).

The calculations for the nitrogen balance of pea leaves can only be crude estimations. Nevertheless the results of radiotracer-labelling experiments confirmed that such flow of amino acids in the transpiration stream and subsequent redistribution from the mature leaves to immature and expanding leaves did occur.

UPTAKE AND REDISTRIBUTION OF AMIDES MOVING IN THE TRANSPIRATION STREAM

The amides asparagine and glutamine, the two major xylem-borne nitrogenous compounds, were shown in the first radiotracer labelling experiments, when they were supplied as a pulse via the transpiration stream to cut shoots, to move rapidly into transpiring leaves. In the fully-expanded leaves, the levels of radioactivity rose rapidly

within 1 hour, and then fell during the next few hours (Figure 7, A and B). Although chased with unlabelled solution, the labelled carbon was not completely exported from these leaves. Some soluble radioactivity remained indicating mixing of carbon from the amides with existing soluble pools in the leaf cells, and a little carbon was incorporated into protein indicating that some protein turnover occurred in the mature leaves.

The results of these experiments showed that the flow into and later export from the mature leaves of labelled carbon was due largely to changes in levels of soluble amino acids: asparagine in the asparagine-tracer experiments, and glutamate and glutamine in the glutamine-tracer experiments.

In the expanding leaves, levels of radioactivity also rose rapidly initially as expected, since these leaves would receive the ^{14}C -amides in the transpiration stream. The levels of radioactivity, however, remained high and increased in the later samples after the pulse of ^{14}C -label had been absorbed and at a time when the older leaves were exporting label. The data suggested import of carbon from the mature leaves, and the radiotracer experiments II confirmed that the expanding leaves did indeed import carbon from amino acids from mature leaves.

Considerable incorporation of carbon from asparagine and glutamine into protein occurred in the expanding leaves (Figures 13 and 14). Such protein synthesis was expected since the nitrogen content was increasing most rapidly at these stages of leaf expansion.

Immature leaves, which were not transpiring, accumulated radioactivity gradually and incorporated a high proportion of this label into protein (Figures 13 and 14). Levels of radioactivity were very low in the first sample (1 hour) and there was no sign of the initial sharp rise of radioactivity observed in the transpiring leaves. The gradual accumulation of label in the immature leaves occurred at the time when labelled carbon was being exported from the mature leaves.

These patterns of initial arrival of amino acids (supplied via the transpiration stream) in transpiring leaves of pea shoots and subsequent redistribution of carbon from these amino acids are similar to patterns observed in other studies. Van Die (1963), for example, showed that most of the carbon from ^{14}C -glutamate (supplied to the roots of tomato) was recovered in the soluble fraction of the mature leaves but of the label in the young leaves most was in the insoluble fraction. Uptake of ^{15}N -nitrogenous compounds (supplied to detached shoots via the transpiration stream) was initially into leaves and was followed by later redistribution of nitrogen to seeds and pods of lupin (Pate et al., 1975) and to seeds of *Pisum sativum* (Lewis and Pate, 1973).

The present study used non-fruiting pea seedlings and showed that the initial uptake of ^{14}C -labelled amides through the xylem was followed by redistribution of label to the expanding and immature leaves of the vegetative apex. The data from the first radiotracer labelling experiment suggested that this redistribution of carbon from amides occurred mainly as amino acids (Figures 11, 12, 13 and 14); the major changes of levels of radioactivity in

the mature leaves was shown to be due mainly to changes in levels of labelled asparagine (asparagine-tracer) and labelled glutamate and glutamine (glutamine-tracer); the label in the early samples of the immature leaves was almost entirely asparagine (asparagine-tracer) or amino acids (glutamine-tracer).

ROLE OF THE MATURE LEAVES

Although one of the most important functions of fully-expanded leaves is photosynthetic fixation of CO_2 and subsequent export of assimilate to the growing leaves, fruits, and roots, the mature leaves have also a major role in processing transpirationally-derived compounds, including nitrogenous compounds. Nitrate is reduced in the leaves of many plants (Dalling et al., 1972a; Mifflin, 1974) and nitrite reductase has been located in chloroplasts of spinach (Magalhaes et al., 1974; Mifflin, 1974) and *Pisum sativum* (Wallsgrave et al., 1979). The overall reduction of nitrate to amino-nitrogen is strongly light dependent (Canvin and Atkins, 1974). Amino acids transported to the mature leaves may be metabolized there or be re-exported without further metabolism. Even if metabolism of imported nitrogenous compounds takes place, export of some form of nitrogen must occur in leaves which have attained their maximum nitrogen levels.

In the present study with pea plants, ^{14}C -asparagine, fed via the xylem, was taken up into the mature leaves, and though some conversion (to HSA and aspartate) and some incorporation into protein occurred, most of the asparagine was re-exported. The early appearance of labelled asparagine in the immature leaves in

the first radiotracer experiments (I) the heavy labelling in asparagine in the young leaves 2 hours after foliar application of ^{14}C -asparagine to mature leaves (radiotracer experiments II), and the presence of ^{14}C -asparagine and -aspartate in the phloem exudate from ^{14}C -asparagine applied to the leaf (radiotracer experiments III) supported the idea that much of the xylem-borne asparagine was directly re-exported from the mature leaves.

In contrast, glutamine supplied to pea shoots in the transpiration stream was extensively metabolized to glutamate in the mature leaves, probably by glutamate synthase (Lea and Mifflin, 1974, discussed later). Export of label from these leaves was probably mainly as glutamate; the changes in levels of radioactivity in the mature leaves was due principally to changes in the amounts of ^{14}C -labelled glutamate (Figure 14). Import of label into the young leaves of these shoots was mainly as labelled amino acids (Figure 14), when ^{14}C -glutamine was supplied to the shoots via the transpiration stream. Also the second radiotracer labelling experiments showed that 2 hours after feeding ^{14}C -glutamine or -glutamate to mature leaves, radioactivity recovered in the young leaves was mainly in amino acids, largely glutamate (Tables 15, 16 and 20).

The nitrogen levels of the fully-expanded leaves were constant for 3 days after maximum expansion had been reached; they then declined gradually (Figures 4 and 5). During this period of constant nitrogen content, the mature leaves must process and export as much nitrogen as is imported in the transpiration stream. For Leaves 1 to 4, this transpirationally-derived nitrogen could be as much as 1.716 mg N per day (based on calculations discussed

earlier). The gradual loss of nitrogen from the older leaves could be a result of (a) decreased influx of transpirationally-derived amino acids, (b) hydrolysis of leaf protein and export of these amino acids, (c) export of the soluble storage pools of amino acids or a combination of any of those. There is considerable mobilization of nitrogen from the older leaves of cereals to the grain (Tully and Hanson, 1979), and also withdrawal of nitrogen from water-stressed leaves of barley (Tully, Hanson, and Nelsen, 1979). Chopowick and Forward (1974) demonstrated that whereas young sunflower leaves do not export amino acids labelled from recently assimilated CO_2 into the petiole, old sunflower leaves do export recently synthesized amino acids.

Superimposed upon the day-by-day changes of the expanding leaf and constant levels of nitrogen in the mature leaves, are the diurnal changes in leaf soluble amino-acid levels (Bauer, Urquhart, and Joy, 1977). Root exudation of xylem bleeding sap exhibits diurnal rhythm of volume of exudate and amino-nitrogen concentration; maxima were in the middle of the light period (Pate, 1962). In addition, transpiration occurring during the light period will increase the flow of nitrate and amino acids into the leaves. Sharkey and Pate (1976), using phloem bleeding from cut fruit, have shown a diurnal rhythm of sugars and amino acids in the phloem of *Lupinus albus*. Levels were highest shortly after noon, but the amino-acid composition did not change through the day.

Because of these diurnal changes, the experiments described in this study were always done at the same time of day; the 2-hour feeding periods in radiotracer experiments II and III were carried

out between 12.00 and 14.00 hours, the middle of the 12-hour photoperiod.

IDENTITY OF AMINO ACIDS EXPORTED IN THE PHLOEM

The experiments in which the amides were supplied via the transpiration stream to detached shoots (radiotracer experiments I) and those in which labelled amides and amino acids were applied to mature leaves (radiotracer experiments II) suggested that much of the carbon from asparagine was directly re-exported, and that carbon from glutamine and glutamate was re-exported largely as glutamate. When labelled HSA was applied to mature leaves, labelled HSA was recovered in young leaves and roots. When labelled aspartate was applied to mature leaves, however, extensive metabolism, mainly to organic acids, occurred. Although labelled aspartate was recovered in the young leaves, most of the soluble label was recovered in organic acids and other amino acids, particularly glutamate (Table 19), transport of these compounds, derived from aspartate, was a possibility.

In these two groups of labelling experiments, the identity of the transported compounds was suggested, but by no means proved, by the labelling patterns observed in the young leaves and roots. The pathway for the transport from the mature leaves was presumed to be in the phloem.

The third group of labelling experiments, in which phloem exudate was collected from leaves fed with ^{14}C -compounds, was designed to investigate the identities of the pathway and the transported compounds.

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Amino-acid analyses of the EDTA-promoted phloem sap from mature pea leaves (Table 24 and Figure 15) showed that asparagine, homoserine, glutamate, aspartate and serine were the principal amino acids found in the exudate. Similar amino-acid concentrations were measured in phloem sap obtained from cut fruit stalks in another variety of *Pisum sativum* (Lewis and Pate, 1973). The evidence for the general occurrence of amino acids in phloem exudates obtained by a variety of methods has been described in the Introduction. Phloem-blocking techniques were not used in the present study, but petiole-steaming inhibited the movement of foliar applied amino acids from the older leaves to the young leaves of sugar-beet (Joy and Antcliff, 1966), soybean (Houseley, Peterson, and Schrader, 1977) and oats (Peterson et al., 1977).

When labelled asparagine was fed to mature, detached leaves in the light, asparagine (60% of the radioactivity) and aspartate (25%) were the only major, labelled compounds present in the phloem exudate after 2 hours (Table 32, Figure 19), although some metabolism to other amino acids and to HSA occurred in the leaf blade (Table 33). There was much less metabolism of asparagine in the leaf blade in the dark, but still only labelled asparagine and aspartate were recovered in the exudate from these leaves, the proportion of labelled aspartate to asparagine being much higher in the exudate than in the leaf extract. In both the light and the dark, the proportions of labelled asparagine to aspartate in the exudate were the same.

The recovery of only labelled asparagine and aspartate in the phloem exudate, when other compounds were also labelled in the leaf extract, suggested a selectivity of phloem loading of asparagine and aspartate. These data provide further evidence that much of the asparagine transported into the leaves in the transpiration stream is loaded directly into the phloem and re-exported from the mature leaves. Some of the asparagine (supplied in the transpiration stream) was taken up into the mature leaf cells and metabolized (Figure 13) and so aspartate produced in the leaf cells could then be loaded into the phloem.

When ^{14}C -asparagine is applied to the leaf surface the route to the phloem may be quite different from that when the labelled asparagine is supplied via the transpiration stream. In the latter case, direct xylem to phloem transfer of some of the label could occur in the petiole or the major veins. This could allow transfer of some transpirationally-derived nitrogen directly to young leaves without necessarily entering the mature leaves. When asparagine is applied directly to the leaf, the probability for uptake of labelled asparagine into mesophyll cells and metabolism there is perhaps higher, although some movement of labelled asparagine through cell walls and extra-cellular space, followed by direct uptake into the phloem from the apoplast is possible. In addition to labelled aspartate loaded into the phloem after metabolism in the leaf cells, some of the aspartate recovered in the phloem exudate could also be the result of metabolism of asparagine to aspartate in the sieve-tubes or adjacent phloem tissue.

When labelled aspartate was fed to the detached leaves in the light, a little over 50% of the total soluble radioactivity in the leaf blade was recovered in the organic-acid fraction, and 35 to 40% in the amino-acid fraction. In the phloem exudate from these leaves, however, only about 20% of the radioactivity was in the organic-acid fraction and 64% was in amino acids, principally aspartate, glutamate, and glutamine plus homoserine (Table 36). Again, there appeared to be selectivity for the compounds taken up into the transport pathway, and although much of the radioactivity from aspartate was recovered in organic acids in the leaf blade, amino acids, including aspartate, appeared to be preferentially loaded into the phloem. Comparing these results to those of the experiment in which ^{14}C -aspartate was fed to the leaves of intact plants (data from Tables 18 and 19), the distribution of label in the leaf extract of both experiments was very similar. Expressed as percentages of the soluble radioactivity, the label in aspartate in the leaf blade was 10.6% (detached leaf) and 13.5% (intact plant, data from Table 19, Expt. 2), in the phloem extract label in aspartate was 28.3%, and in the young leaves 6.6% (data from Table 19); the percentages of radioactivity in the organic-acid fractions were 52.1% and 47.9% (leaves), 22.7% (phloem) and 42.6% (young leaves). Thus although extensively metabolized in the leaf blade in the light, aspartate appeared to be selectively loaded into the phloem with other amino acids (glutamate, glutamine and homoserine).

In the dark, there was less metabolism of aspartate in the leaf blade, 45.4% of the label being recovered (after 2 hours) still in aspartate, and only 27.2% in the organic acids (Table 36). Although the percentage of radioactivity in amino acids and organic acids was the same in the leaf extract and phloem exudate from dark-treated leaves, the distribution of label among the amino acids was different. In the leaf extract, 45.4% was in aspartate, 13.8% in glutamate, and 6.0% in glutamine plus homoersine; in the phloem exudate, the equivalent values were 36.5, 35.2, and 0% respectively. Selectivity of amino acid uptake into the phloem was again suggested by these data.

Labelled organic acids were recovered in the phloem exudate from both light- and dark-treated leaves, the proportion of the total label being about the same in both case, but it was not possible to tell from the present experiments if these were loaded into the phloem from the mesophyll cells or if they were the product of amino acid metabolism within the phloem cells. Peterson et al (1977) detected label in organic acids in the sheath of oat leaves, the upper portion of which had been fed with $^{14}\text{CO}_2$, ^{14}C -sucrose, or ^{14}C -amino acids. Houseley et al. (1979) recovered labelled organic acids in the petioles of soybean after feeding $^{14}\text{CO}_2$ to the leaf blade. Organic acids are present in aphid stylets exudate (Peel and Weatherley, 1959), phloem sap from inflorescences of *Yucca* (Tammes and van Die, 1964), and in *Ricinus* bark exudate (Hall and Baker, 1972). But again it is not possible to say in any of these experiments whether these organic acids are loaded from the leaf cells or are the result of metabolism in the sieve cells.

When ^{14}C -HSA was applied to detached leaves in the light most of the transported radioactivity was in HSA, but the proportion of label in amino acids was higher in the exudate than in the leaf blade. These results suggested a preferential transport of the amino acids (mainly ^{14}C -labelled aspartate, glutamate, and homoserine), although ^{14}C -HSA was recovered in the phloem and was the main labelled compound in the exudate. Since there is as yet no sensitive method to detect unlabelled HSA (Lloyd and Joy, 1978) it is not known if HSA is normally present in large amounts in the phloem exudate.

EDTA-PROMOTED PHLOEM EXUDATION.

The true phloem origin of the EDTA-promoted exudate can, of course, be questioned. King and Zeevaart (1974) argued that the presence of sucrose, verbascose, raffinose and stachyose as only labelled compounds in the exudate from $^{14}\text{CO}_2$ -labelled *Perilla* leaves was evidence for the phloem origin since those sugars are typical of the transport pathway; very little labelled glucose or fructose was found in the exudate. The petiole extract contained the labelled transport sugars but again little labelled glucose or fructose although these were labelled in the leaf blade. These workers had, however, fed CO_2 to the whole plant, and then detached the leaves. The labelled compounds need not, therefore, have come from the leaf blade, but could have leached out of cells in the petiole since these would have been exposed to CO_2 and could have assimilated carbon. Although the amount of radioactivity exuded was calculated as a percentage of the total label in the leaf after CO_2 -exposure, no data for distribution of radioactivity between

leaf blade and petiole was given. It was not stated if, during the 6-hour exudation period, label was actually mobilized from the blade, through the petiole, and into the exudate.

Dickson (1977) exposed individual leaves of *Populus deltoides* and *Phaseolus vulgaris* to $^{14}\text{CO}_2$. The distribution of radioactivity among basic, neutral, and organic-acid fractions from phloem exudate and petiole extract was different in the two species. A higher percentage of radioactivity was recovered in sugars (mainly sucrose) in the phloem exudate than in the petiole extract. His data suggested that some metabolism of the transported compounds had taken place in the petiole with radial movement out of the phloem, but that the phloem exudate fairly closely resembled the probable contents of the sieve-tubes.

When an EDTA-promoted exudate was collected from soybean pod tips (Fellows et al., 1978) the radioactivity recovered in the exudate was label which had been translocated from the source leaf (flushed with CO_2) some distance into and through the pod. Since the exudate was collected from the distal end of the pod, metabolites could be extracted from the phloem sap during its passage through the pod. Pate et al., (1974), on the other hand, have stated that there is little difference in phloem exudate from cut fruit stalks or from cut pod tips.

King and Zeevaart (1974) suggested that EDTA may promote exudation by chelating calcium involved in callose formation; they give evidence in the form of micrographs, that indicate less callose formation in the EDTA-treated petioles. EDTA could, however, have

caused disruption of cell membranes, not only of the sieve-tubes but also of the adjacent companion cells, phloem parenchyma and other tissues in the petiole.

Tully and Hanson (1979) argue that the optimal EDTA concentration reported by them for phloem exudation in barley and by King and Zeevaart in *Perilla* is consistent with a specific process rather than a general degradative one such as increased tissue permeability or wound-induced autolysis. However it is possible that two independent processes, both affected by the EDTA concentration, could occur concurrently. If one of these, for example membrane permeability, was affected at low EDTA concentration to give increased leakage as the EDTA concentration increased, and the other, for example severe tissue disruption, resulted in complete blockage of the phloem at high EDTA concentrations, then an apparent optimum for EDTA would be observed.

A more convincing argument is the increased EDTA-promoted exudation from the leaf sheath at higher light intensities and increased CO₂ concentration (Tully and Hanson, 1979). They suggested that exudation (from the phloem) was increased as a result of increased CO₂ assimilation and light-dependent loading of photosynthate; such light dependence would not be expected if general degradative processes in the leaf sheaths immersed in EDTA were the cause of the exudation.

They also compared the amounts of ¹⁴C-assimilate present in the blade and sheath immediately after ¹⁴CO₂ feeding. More ¹⁴C-assimilate was exuded out of the sheaths during the subsequent 6 hours than was present in the sheath immediately after feeding.

There was in fact an increase in label in the leaf sheath, indicating movement of radioactivity out of the leaf blade, through the sheath and into the exudate.

Tully and Hanson did observe some general leakage from the sheath tissue. Nitrate exuded into the EDTA solution in the first two hours but whereas the sugar and amino acid exudation continued at a constant rate for several hours, the nitrate exudation slowed and almost ceased after 2 hours. The specific activities of sucrose, glutamate and glutamine were higher in the exudate from $^{14}\text{CO}_2$ -fed leaves than in the leaf-sheath extracts. Their results collectively suggested that the sucrose and amino acids recovered in the EDTA-promoted exudate had come from recent photosynthate exported from the leaf in the phloem.

In the present experiments with *Pisum sativum*, EDTA promoted the exudation from detached leaves. This exudate contained amino acids, the major ones being asparagine, homoserine, aspartate, glutamate, and serine. The amino-acid composition of the exudate resembled closely that of the petiole, but was quite different from that of the leaf blade (Tables 25 to 27). Label from ^{14}C -labelled amino acids applied to the surface of detached, mature leaves was transported from the blade to the ends of the petioles in the EDTA solution, a distance of 3 to 4 cm (Table 28). This distance is too great for transport solely by diffusion. Loading of the labelled compounds into the phloem and translocation out of the leaf blade is therefore indicated.

When amino acids (asparagine, glutamine, aspartate, and glutamate) were applied to the leaf blade, translocation out of the

leaf occurred both in the light and in the dark. When ^{14}C -HSA was fed to the leaves, however, export of labelled material out of the leaf only occurred in the light. This light-dependent step, either in the metabolism of HSA or in the phloem loading process itself, was further evidence for the phloem origin of the exudate.

Ideally the EDTA-promoted exudate should be compared with phloem sap obtained by other means. Pate et al. (1974) stated that phloem exudate from cut tips of *Lupinus albus* fruits did not differ significantly from sap from the severed stylets of aphids previously feeding on the fruit of this species of plant. In *Pisum sativum* c.v. Meteor, data are given for amino acid concentration in the phloem sap collected from cut fruit stalks (Lewis and Pate, 1973). The same major amino acids (asn, hse, gln, serine) are present in "Meteor" as in "Little Marvel", but the relative concentrations are different. Since varietal differences in amino acid composition of leaf and root tissue may occur, differences in amino acid composition of phloem exudates can also be expected.

Barlow and Randolph (1978) reported amino-acid concentrations in aphid stylet exudate from *Pisum sativum* c.v. Alaska. Here, however, not only was the cultivar different, but also the amino-acid analyses were carried out in sodium buffers. Neither the amides nor homoserine was recorded as being present (these amino acids are normally important in *Pisum*) and large quantities of threonine were recorded. In sodium buffers, asparagine, glutamine, and homoserine co-chromatograph with threonine and (or) serine, making identification of these compounds difficult, whereas in lithium

buffers, the amides and homoserine can all be separated from each other and from the other amino acids. It is possible, therefore, that Barlow and Randolph have not correctly identified all of the amino-acid components. Their methionine sulfone is possibly β -(isoxazolin-5-one-2-yl)-alanine. A direct comparison of aphid stylet sap and phloem exudate was not therefore possible.

EDTA-promoted phloem exudate was collected from "Alaska" leaves and run in both sodium and lithium buffers (Appendix C). Lithium buffers showed the presence of amides and homoserine, yet analyses of exudates run with sodium buffers were similar in appearance to the data for aphid stylets sap published by Barlow and Randolph (1978) clearly suggesting mis-identification. Amino-acid profiles from "Little Marvel" and "Alaska" exudates were quite different. Ideally aphid stylets sap and phloem exudate, both from "Little Marvel" should be compared.

Data from the work of others and from the present study suggest that the EDTA-promoted exudate is largely of phloem origin. The information provided by analyses of such exudates should still be used with caution since some contamination from tissue other than the sieve-tubes may occur, and the reduced export (compared to that from intact leaves) show that the phloem pathway has been somewhat disturbed.

UTILIZATION OF AMINO ACIDS IN YOUNG LEAVES

In the first radiotracer experiments, there was extensive incorporation of label from asparagine and glutamine into the insoluble fraction of the young leaves (expanding and immature) as

shown in Figure 9. This ethanol-insoluble label was almost entirely soluble in NaOH and was shown by protease digestion to be protein (Table 5). Label from the amides, aspartate, and glutamate (applied to the mature leaves) was also incorporated into protein in the young leaves during the 2-hour labelling period of the second radiotracer experiments (Tables 9, 10 and 17).

In addition to incorporation into protein, the amino acids transported to the young leaves also appeared to be involved in transamination, thereby providing amino-nitrogen for synthesis of those amino acids which were not carried to the young leaves either in the xylem or in the phloem. Evidence for amino acid transaminase activity was provided by the accumulation of label in organic acids, which would result from transamination. In particular ^{14}C -HSA (the product of asparagine transamination, discussed later) was found in asparagine feeding experiments (Leaf 5, Figure 13; Tables 13 and 14). Label from glutamate, glutamine, and in particular from aspartate was also recovered in the organic acid fractions of young leaves after 2 hours of feeding to the mature leaves. Although some of this label could have been transported as organic acids (phloem exudate from aspartate-labelled leaves, Table 36), the higher percentage of labelled aspartate in the phloem exudate compared to that in either the fed leaf (Table 36, Leaf Extract, Light) or in the young leaves (Table 19) suggested transport, at least in part, as aspartate and subsequent metabolism in the young leaves. In the present study, organic acids other than HSA were not positively identified.

In many plants $^{14}\text{CO}_2$ is incorporated into amino acids such as serine, glycine, and alanine (Pate, 1966; Joy, 1967; Fellows et al., 1978; Tully and Hanson, 1979) and transported in this form to the young leaves (Lewis and Pate, 1973). Various studies on phloem content composition show that carbon from this group of amino acids constitutes a small percentage of the total translocate, and $^{14}\text{CO}_2$ -labelling studies show that only a low percentage of the label is channelled into these amino acids (Lewis and Pate, 1973; Fellows et al., 1978).

TRANSLOCATION TO THE ROOT

When asparagine, glutamine, aspartate and glutamate were applied to the mature leaves of intact plants (radiotracer experiments II), label was recovered in the roots (Tables 9, 10 and 17). The radioactivity in the roots of aspartate- and glutamate-fed plants was not analysed. In asparagine-labelled plants, 10 to 12% of the radioactivity in the roots was recovered in protein, and 80 to 85% of the total label was still in asparagine (Tables 13 and 14). In glutamine-labelled plants, 12 to 14% of the label was in protein, and 60 to 70% in amino acids, mainly glutamate (Table 16).

It would seem odd that the roots, which synthesised large quantities of these amino acids and exported them in the xylem, should import these same amino acids from the mature leaves. Pate (1973) found that nitrogen, fed as ^{15}N -nitrate to fully-expanded lupin leaves, was also transported to the roots, particularly the fine, lateral roots. He suggested that nitrogen entering the roots and assimilated there is preferentially channelled into the vascular

system and exported in the xylem, and that the non-vascular cells of the roots do not retain enough nitrogen for their own requirements. Amino acids translocated concurrently with the photosynthate from the leaves to the roots (Pate, 1966) would therefore fulfill the nitrogen requirements of these tissues. Some transport of amino acids concurrently with the sugars which are transported in the phloem to the root may be unavoidable unless a mechanism exists to distinguish apex-bound from root-bound phloem tissue.

Examination of the amino-acid content of phloem exudate from leaves of different ages or in different positions on the stem with respect to root and shoot apex would therefore be of interest. Some information on this topic is provided by Dickson (1977) who demonstrated a higher percentage of radioactivity (from $^{14}\text{CO}_2$ -fixation) in amino-acid and organic-acid than in sugar fractions of petiole exudates from younger leaves of both *Phaseolus vulgaris* and *Populus deltoides*.

METABOLISM OF ASPARAGINE

Although much of the asparagine which was transported to the mature leaves in the transpiration stream was directly re-exported with little further metabolism, the asparagine transported to the expanding leaves underwent considerable conversions.

The metabolism of asparagine was not well understood until recently. As reviewed in the Introduction, asparagine deamidase activity, resulting in aspartate formation with release of ammonia, is present in lupin seeds (Atkins et al., 1975) and soybean root nodules (Streeter, 1977), but its activity was low in soybean

leaves (Streeter, 1977). Aspartate was not a major metabolite of ^{14}C -asparagine in the present study with *Pisum sativum*. Other work in this laboratory (Shingles, 1979) showed low activity of asparagine deamidase in pea leaves, although activities were high in developing pea seeds. Levels of activity in mature and immature leaves were not compared.

In all the radiotracer experiments in these studies with peas, the major metabolite from ^{14}C -asparagine was not aspartate, but was a compound identical in properties to HSA, found by Lloyd and Joy (1978) in peas and soybean after in vivo feeding of ^{14}C -asparagine and ^{14}C -ketosuccinamic acid (KSA). Streeter (1977) measured an active asparagine aminotransferase with pyruvate and glyoxylate by monitoring the formation of the dinitrophenyl hydrazone derivatives of KSA. Similarly, activities of an asparagine: pyruvate aminotransferase were measured in pea leaves using a spectrophotometric assay (Nishikawa, 1979); asparagine: α -ketoglutarate transamination was also measured. Asparagine transamination activity was found in roots and developing pods in addition to leaves, but not in old pods or in seeds. The ^{15}N -labelling experiments of Bauer, Joy, and Urquhart (1977) using asparagine as tracer are consistent with the presence of asparagine: pyruvate transamination in peas; some nitrogen from asparagine was transferred to alanine.

KSA, the product of asparagine transamination, did not accumulate in ^{14}C -asparagine-fed plants (Lloyd and Joy, 1978) and ^{14}C -KSA was rapidly metabolized in pea plants, mainly to HSA. In the present experiments, no KSA (either monomer or dimer) was detected, and HSA was the main labelled compound from asparagine.

HSA accumulated in the expanding leaves of peas and was present in mature leaves, roots, and young leaves.

ASPARTATE AND HOMOSERINE

Most of the ^{14}C -labelled aspartate fed to mature leaves of both detached shoots and intact plants (in the light, was metabolized, much of the label being recovered in the organic-acid fractions (Tables 19 and 36). Similar distributions of label were observed in the soluble fractions of leaves of lupin fed with ^{14}C -aspartate via the transpiration stream (McNeil et al., 1979). The extensive transfer of label from aspartate to organic acids is evidence for aspartate transamination.

In experiments where label in glutamine and homoserine were measured separately, radioactivity from aspartate was recovered in homoserine (e.g. Table 19). Homoserine is a major, non-protein amino acid in *Pisum*, particularly in young seedlings. Label from ^{14}C -aspartate injected into pea cotyledons was incorporated into homoserine, and labelled homoserine was transported into the shoot of the embryo (Larson and Beevers, 1965). Aarnes and Rognes (1974) partially purified aspartate kinase and homoserine dehydrogenase from *Pisum sativum*. Lea, Mills, and Mifflin (1979) demonstrated light-dependent incorporation of ^{14}C -aspartate into homoserine in isolated chloroplasts from pea leaves.

The results of the present experiments are consistent with metabolism of aspartate to homoserine via aspartate kinase, but in ^{15}N -labelling studies in peas (Bauer, Joy, and Urquhart, 1977) the incorporation of nitrogen into homoserine seemed to be more closely related to the amide-N of asparagine than the amino-N of aspartate.

Mitchell and Bidwell (1970) showed that in pea roots, carbon from ^{14}C -aspartate did not flow directly into homoserine, but appeared to enter the organic-acid pool first.

METABOLISM OF GLUTAMINE

In radiotracer experiments I (glutamine fed via the transpiration stream) and radiotracer experiments II and III (glutamine applied to mature leaves), most of the labelled glutamine was metabolized mainly to glutamate. Glutamine can be metabolized by glutamate synthase (glutamine: α -oxoglutarate amido transferase) to form glutamate. Glutaminase (glutamine deamidase) would also result in glutamate formation. Glutamine has been shown to donate amide-N to aspartate in a glutamine-dependent asparagine synthetase, which is thus another possibility for glutamine utilization.

Lea and Mifflin (1974) demonstrated the presence of a ferredoxin-dependent glutamate synthase in leaf tissue and this enzyme has been shown to be located in chloroplasts of *Pisum sativum* (Wallsgrave et al., 1979). As described in the Introduction, most of the evidence for the glutamine synthetase - glutamate synthase pathway (in addition to demonstrating the presence of the enzymes) comes from use of $^{15}\text{N-NO}_3$ or $^{13}\text{N-NO}_3$ and the use of inhibitors. For the reduction of nitrate and the assimilation of the resulting ammonia into amino-N, the evidence favours the glutamine synthetase - glutamate synthase pathway. Glutamine transported to the leaves in the transpiration stream, however, could be metabolized prior to entering the chloroplast where glutamate synthase is located. Glutaminase activity has in

fact been demonstrated in pea leaves (Shingles, 1979) and under conditions of asparagine accumulation glutamine could be the amide donor (Lea and Fowdon, 1975). Asparagine levels in pea leaves stabilize, however, during the second half of the photoperiod (Bauer, Urquhart, and Joy, 1977). Moreover, the experiments of Lewis and Pate (1973) in *Pisum sativum* showed that nitrogen (^{15}N -labelled) from three sources, nitrate, glutamine (amide-N), and glutamate, was metabolized in an essentially similar fashion. The utilization of glutamine in the present experiments in pea via glutamate synthase is therefore highly probable.

METABOLISM OF HSA

In the present study, ^{14}C -asparagine was metabolized to HSA in growing leaves of pea. When ^{14}C -asparagine was fed via the xylem to pea shoots, the levels of ^{14}C -HSA increased in the growing leaves (Figure 13). Labelled HSA was also recovered in mature leaves, young leaves, and in roots of plants which had been fed ^{14}C -asparagine ~~via the mature leaves~~ (Tables 13 and 14). Although labelled HSA was recovered in leaf-blade extracts of detached leaf blades fed with ^{14}C -asparagine, no labelled HSA was detected in the phloem exudate. When ^{14}C -HSA was applied to the leaf surface as a carrier-free tracer of high specific activity HSA was recovered in the phloem exudate (Table 29) and in young leaves and roots (Table 21). No labelled HSA was recovered in the phloem after asparagine feeding to the leaf even though HSA was synthesized in the leaf blade. HSA, however, was recovered in the phloem if applied directly to the leaf surface. This suggested that a non-selective uptake of

exogenously applied HSA was occurring, perhaps from the apoplast, but that HSA synthesized within the mesophyll cells was not loaded into the phloem. The light-dependence of HSA transport into the phloem exudate, however, argues against such a possibility. Also the percentage transport of applied HSA out of the leaf was low. If the equivalent proportion of HSA synthesized from labelled asparagine was transported, the levels could be sufficiently low in the phloem to be virtually undetectable. When HSA was supplied either to leaves of intact plants or detached leaves, some metabolism of HSA occurred (Tables 22 and 38). Label from HSA was recovered in aspartate, glutamate, asparagine, glutamine and homoserine in the leaves. Some label was incorporated into protein, but very little label was recovered in the neutral fraction of the soluble extracts. Label was recovered in aspartate, glutamate and homoserine in the phloem exudate. Lloyd and Joy (1978) reported a slow utilization of HSA in peas in the light, mainly to amino acids and insoluble material.

CONCLUSIONS

Transpiring leaves of "4½-leaf" pea shoots received a mixture of amino acids and nitrate via the xylem in the transpiration stream. Mature leaves were shown to have constant nitrogen levels for 3 to 4 days after they had fully expanded, and therefore the nitrogen received by these leaves was presumed to be exported since it did not accumulate. Very young (immature) leaves were shown to have negligible transpiration and therefore received little, if any, nitrogen directly from the xylem. Calculations based on water uptake and transpiration rates of the immature and expanding leaves,

and on amino acid and nitrate concentrations in the bleeding sap, suggested that almost all of the nitrogen required by the immature leaves, and about two-thirds of that required by the half-expanded leaf would have to be supplied by redistribution.

Labelling experiments showed that asparagine and glutamine (the two major xylem-borne amino acids) when supplied as ^{14}C -tracers via the transpiration stream rapidly entered the mature leaves. Much of the radioactivity from asparagine was directly re-exported, and most of the radioactivity from glutamine was exported as glutamate. Although most of the radioactivity was exported largely as amino acids, metabolism of the amides in these mature leaves occurred. Nitrogen from this metabolized portion of the amides could be used for amino acids such as serine and glycine, which are synthesized from recent assimilate and can be exported in the phloem.

Redistribution of the amino acids from the mature leaves to the growing leaves was suggested (radiotracer experiments I) by the high levels of radioactivity maintained in the later samples of the expanding leaves and the gradual increase in radioactivity in the immature leaves at the time when the mature leaves were exporting. Results of the other radiotracer experiments (II and III) confirmed, by the pattern of labelling of amino acids in the young leaves and roots and in the phloem exudate, that carbon from the amides or their major metabolites (aspartate, glutamate) were exported from the mature leaf mainly in the form of amino acids, and there appeared to be selective loading into the phloem of amino acids labelled from HSA.

Thus, taken collectively, the data suggested that much of the xylem-borne amino acids (largely asparagine and glutamine) transported to the mature fully-expanded leaves was directly exported either unaltered or as other amino acids. The nitrogen required for growth of the immature and expanding leaves could therefore be supplied by the mature in the form of amino acids transported in the phloem.

SUMMARY

1. The leaves of *Pisum sativum* (L. c.v. Little Marvel) seedlings (2 to 4 weeks old) developed through a series of morphologically distinct stages characterized by the unfolding of the leaflets then the stipules; each stage was about one day apart for plants grown in nutrient solution in growth cabinets with a 12-hour photoperiod.

2. The nitrogen levels of these leaves increased rapidly as the leaves were expanding and reached a maximum at the time when they were just fully expanded. These maximum levels were maintained for the next 3 to 4 days and then declined slowly. Nitrate in the nutrient solution (4mM NO_3^-) was the only source of nitrogen for the plants; at this stage of growth the plants were not nodulated.

3. The expanding leaves and the fully-expanded leaves transpired water vigorously, $0.097 \pm 0.025 \text{ g per leaf. h}^{-1}$ and $0.140 \pm 0.040 \text{ g per leaf. h}^{-1}$ respectively. The immature leaves, still enclosed within the stipules of the next older leaves, transpired negligible quantities of water. Transpiring leaves received a mixture of nitrate and amino acids (mainly asparagine, glutamine, and homoserine) from the roots via the xylem. Traces of ureides were present in the xylem sap of these young plants.

4. Asparagine and glutamine, supplied as ^{14}C -labelled tracers in a 30 minute pulse in the light via the transpiration stream to the cut base of "4½-Leaf" shoots, were taken up rapidly (within 1 hour) into the expanding and fully-expanded, mature leaves. Levels of radioactivity in the mature leaves subsequently

declined, mainly due to a decline in the levels of ^{14}C -asparagine (in the asparagine-tracer experiments) and ^{14}C -glutamate plus ^{14}C -glutamine (glutamine-tracer experiments). In the expanding leaves, the initial influx of radiotracer was followed by continued high levels of radioactivity, largely a result of high levels of ^{14}C -label in the amino acids (asparagine in the asparagine-tracer experiments and glutamate in the glutamine-tracer experiments). In the immature leaves, which were not transpiring and therefore not receiving compounds directly from the transpiration stream, levels of radioactivity increased gradually during the experiment. In the early samples, almost all of this radioactivity was in asparagine (asparagine-tracer experiments) or amino acids (glutamine-tracer experiments). The influx of ^{14}C -label into the immature leaves and the later influx in expanding leaves was presumed to be from labelled amino acids exported from the mature leaves.

5. Considerable incorporation of label into protein from ^{14}C -labelled asparagine and glutamine (supplied via the xylem) occurred in the expanding leaves (which were at the stage of most rapid increase in nitrogen levels) and also in the immature leaves. A small incorporation of label into protein occurred in the mature leaves, showing that some turnover of protein was occurring while the leaves maintained maximum nitrogen levels.

6. After foliar application (to the 4th leaf, fully-expanded) of ^{14}C -labelled amides (asparagine, glutamine, or HSA) or ^{14}C -amino acids (aspartate or glutamate), uptake into the cells of the treated leaflet and metabolism of the applied compounds occurred.

Carbon was also transported (within 2 hours in the light) to the immature leaves (Leaf 6) and the expanding leaves (Leaf 5), and also to the roots. In detached shoots, carbon from ^{14}C -asparagine was transported (in 2 hours in the light) to the immature and expanding leaves of these shoots.

7. After foliar application of asparagine, 70 to 85% of the label remained (in 2 hours) in the amino acid fraction, mostly still in asparagine. Of the metabolized carbon, the major soluble compounds in the fed leaf were HSA, aspartate, and glutamate. The distribution of radioactivity transported to the young leaves was similar, the major labelled compound being asparagine; HSA and aspartate were also labelled. Ninety percent of the label transported to the roots was amino acids, mainly asparagine. The data suggested that transport of carbon to the young leaves and roots was in the form of amino acids, largely asparagine.

8. When labelled glutamine was applied to the mature leaf, 70 to 80% of the radioactivity remained in the amino acid fraction of that leaf, mainly as glutamate and glutamine. Carbon was transported to young leaves and roots; the distribution of radioactivity was similar to that in the mature leaves, most of the label being recovered in glutamate and glutamine. In some samples, label was recovered in γ -amino butyric acid. In these experiments, transport to young leaves and roots appeared to be as amino acids, mainly glutamate and glutamine.

9. In the mature leaves, after foliar application of aspartate, considerable metabolism to other compounds occurred in 2 hours; 40% of the radioactivity was recovered in organic acids,

10% in neutral compounds and the remainder in insolubles and various amino acid compounds, largely homoserine, glutamate, and γ -amino butyric acid, only 5 to 10% remaining as aspartate. Of the radioactive carbon transported to the young leaves, about 30% was in organic acids, 20% in protein, 5% in neutral compounds, and the rest amino acids, of which only 4 to 5% was aspartate. Transport of label to the young leaves could have been as amino acids (aspartate, glutamate, homoserine) but transport in the form of organic acids was also a strong possibility.

10. Glutamate underwent extensive metabolism in the treated leaves, mainly to γ -amino butyric acid, but 20% of the label remained in glutamate, a few percent of the radioactivity was recovered in glutamine, aspartate, and homoserine, 15 to 16% was in the organic acid fraction, and 8% was in protein. Of the radioactivity recovered in the young leaves, 15 to 20% was in glutamate; γ -amino butyric acid and aspartate were also labelled. A total of 40 to 50% of the recovered label was in the amino acid fraction in these young leaves; 20 to 30% in the organic acids, and 10 to 20% in protein after 2 hours of feeding. Transport as amino acids, largely glutamate, was suggested by these results, though other compounds may have been transported.

11. HSA, applied to mature leaves in the light, was taken up and metabolized. Of the radioactivity recovered in the fed leaflet, 5 to 6% was in amino acids, mainly glutamate and aspartate, 3 to 5% was in protein, and the remainder in these leaflets was still mainly HSA. Between 5 and 12% of the total label recovered in the plant was transported (within 2 hours) to

the young leaves and roots. The label in the young leaves was mainly HSA, suggesting transport in that form.

12. A solution of Na_2EDTA , pH 7.0, bathing the cut ends of petioles of detached, fully-expanded pea leaves, promoted the exudation of a phloem sap containing amino acids, the major ones being asparagine, homoserine, and glutamate, with aspartate and serine also present in high amounts. EDTA-promoted exudation of amino acids occurred with the leaves in the light or in the dark.

13. Carbon from asparagine, glutamine, aspartate, and glutamate (applied as ^{14}C -tracers to the detached leaves) was transported from the leaf blades through the petioles and into the EDTA solution, when the leaves were either in the light or the dark. Transport of label from HSA into the EDTA solution occurred only when the leaves were in the light.

14. Analyses of leaf extract and EDTA-promoted phloem exudate of ^{14}C -asparagine-fed leaves showed that while asparagine underwent metabolism in the leaf blade (similar to that in the mature leaf of intact plants) asparagine (60%) and aspartate (30%) were the main labelled components recovered in the phloem exudate. These data confirmed the results of the previous experiments (feeding labelled amino acids to mature leaves of intact plants) which suggested that much of the asparagine arriving in the fully-expanded leaves from the transpiration stream was directly re-exported, although there was some uptake into the leaf cells with some further metabolism. With leaves in the dark, little metabolism of asparagine occurred in the leaf blade, but asparagine (60%) and aspartate (30%) were recovered in the phloem exudate.

15. Radioactivity in the phloem exudate from ^{14}C -aspartate-fed leaves (in the light or the dark) was mainly (65 to 75%) in the amino acid fractions, and about 25% was in the organic acid fraction. Aspartate was the main labelled compound in the exudate. Glutamate was also highly labelled in the exudate from dark-treated leaves, and glutamate and glutamine + homoserine were highly labelled in the light. The distribution of radioactivity in the exudate from light-treated leaves was very different from that in the leaf-blades; aspartate was more highly labelled in the exudate, whereas the organic acids were more highly labelled in the leaf-blades. Distributions of radioactivity in exudate and leaf-blade were more similar in the dark than in the light, but the ratio of label, glutamate : aspartate, was higher in the exudate than in the leaf-blade. The data showed that some labelled carbon from aspartate was transported via the phloem in the form of organic acids, but most was as amino acids, mainly aspartate and glutamate, and also glutamine + homoserine in the light.

16. When HSA was applied to the leaf-blade of detached leaves, 75 to 80% of the radioactivity recovered in the phloem exudate was in the organic acid fraction, almost entirely as HSA. About 12 to 15% was in the amino acid fraction of the exudate; aspartate, glutamate, homoserine, glutamine, and threonine were labelled. The data suggested that carbon from HSA applied to the mature leaves was, therefore, transported mainly as HSA to the young leaves and roots. No translocation of HSA occurred in the dark; very little was translocated in the light (less than 0.1% of HSA supplied to leaves was recovered in exudate).

17. Although the percentage of applied radioactivity transported out of the detached leaf was considerably less than that from leaves of intact plants, presumably because of disruption of the phloem and removal of sinks, nevertheless translocation of labelled amino acids and other compounds occurred from the leaf-blade through the petiole and into the exudate. Selectivity in transfer of labelled compounds from soluble pools in the leaf-blade to the transport pathway was exhibited. Translocation of at least one compound (HSA) was light-dependent.

18. The results indicated that both xylem and phloem are involved in distribution of nitrogen to developing leaves of pea plants, the contribution via the xylem increasing as the leaves expand and transpire vigorously. The mature leaves are involved in the circulation of nitrogen arriving from the root; amino-N is transferred to the phloem and redistributed to the developing leaves. The distribution of xylem-borne nitrogen in the shoot is summarized on page xviii.

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APPENDIX A

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
$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	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
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.000025

Element	uM
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 B

Comparison of Soluble Amino-Acid Levels in Leaf Extracts

Prepared by Ethanolic Extraction and SSA-Precipitation

Methods

The effects of two extraction methods on amino-acid levels in pea leaves were examined. Leaves were removed from several plants, and divided so that one leaflet and one stipule from each leaf were in one tissue sample, and the other leaflet and stipule in the second sample. Samples were weighed and immediately frozen in liquid N₂.

Samples were extracted by homogenizing in 80% ethanol (details in Methods 11). The final aqueous extract was passed over Dowex 50 (H⁺) resin and amino acids were eluted from the resin with 2N ammonium hydroxide. The sample was dried to remove ammonia and redissolved in lithium dilution buffer at pH 2.1.

The other samples were homogenized in distilled water containing 5-sulphosalicylic acid (SSA) at 50mg per ml and then centrifuged to precipitate the proteins (deWolfe, Baskurt, and Cochrane, 1967). The pH of the supernatant was adjusted to 2.1, and lithium dilution buffer added.

Amino-acid levels in both samples were determined using a Beckman Amino-Acid Analyzer, Model 119 BL using lithium buffers.

Results

See Table 1.

Table 1

Amino-Acid Concentrations in Pea Leaf Extracts Prepared
by Ethanolic Extraction or SSA-Precipitation

	SSA-ppid μ moles amino acid	ETOH-extracted per g tissue	% Recovery ^a
asp	2,600	2.49	96
asn	14.86	13.72	92
glu	5.02	4.58	91
gln	1.07	3.31	93
hse	2.49		

^a Amino acid recovery in ETOH-extract as percentage of amino-acid levels in SSA-precipitated extract.

Discussion

The results show that amino-acid levels were very similar in extracts prepared by both methods. The slightly higher levels in the SSA-precipitated extracts were expected since fewer steps are involved in preparing extracts by this method compared to the many steps in ethanol extraction. In all cases, however, the amino-acid levels recovered from ethanol extracts were better than 90% of those from SSA-precipitated samples.

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APPENDIX C

Amino-Acid Analyses of Phloem Exudate from "Alaska" Peas. Effect of Lithium and Sodium Buffers and Comparison of Phloem Exudate and Aphid Stylets Sap.

EDTA-promoted phloem exudates (King and Zeevaart, 1974) were collected from fully-expanded leaves of *Pisum sativum* c.v. Alaska grown in nutrient solution in growth cabinets with a 12-hour photoperiod. Amino-acid levels in the exudates were determined using a Beckman Amino-Acid Analyzer, Model 119 BL. Samples were run in lithium buffers (physiological mode) and sodium buffers (hydrolyzate mode). Comparisons were made with amino-acid contents of phloem exudate from cultivar Little Marvel (Table 1). The data for aphid stylets exudate from Alaska (Table 2), obtained by Barlow and Randolph (1978) was compared with the EDTA-promoted phloem exudate from Alaska analyzed in sodium buffers.

Results

See Tables 1 and 2.

Discussion

The data in Table 1 shows some differences in amino-acid composition of phloem exudate from the two cultivars. BIA(β -(isoxazolin-5-one-2-yl)alanine) was present in both cultivars, but Alaska had none of U, the unknown compound running between threonine and serine, in Little Marvel samples, and had much higher levels of the compound eluting after homoserine and tentatively identified as α -amino adipic acid. Little Marvel

samples showed slightly lower levels of the amides and homoserine. These data were obtained from analyses in lithium buffers.

For a comparison with aphid stylet sap analysis, phloem exudate samples (from Alaska) were analyzed in sodium buffers since it was suspected that amides, if present, were not recognised in the data of Barlow and Randolph (1978) which were obtained from analyses in sodium buffers.

Table 2 shows that, in the hydrolyzate mode, BIA is separated from aspartate, but no separate peaks of the amides or homoserine were detected. These compounds elute, in sodium buffers, at about the same time as threonine or serine and this may explain the lack of separation of these compounds in the phloem exudate analyses. The results show that there is an overall similarity in amino-acid profile between phloem exudate from Alaska leaves and aphid stylets sap from this cultivar when the analyses were run with sodium buffers. In the aphid stylets sap a compound eluting just after aspartate was identified by Barlow and Randolph (1978) as methionine sulfene. The compound may, however, be β -(isoxazolin-5-one-2-yl)alanine (Lambeck, Kuo, and van Parijs, 1976).

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Table 1

Amino-Acid Analyses (Lithium Buffers) of Phloem Exudate from Fully-Expanded Leaves of "Alaska" and "Little Marvel" Peas

	Alaska		Little Marvel
	Light	Dark	Dark
	n moles exuded per leaf in 2 hours		
Asp	1.39	3.81	4.22
BIA	0.74	1.22	0.63
Thr	0.94	1.74	0.67
U	0	0	0.60
Ser	4.56	8.70	4.98
Asn	17.05	26.50	14.65
Glu	4.69	9.81	4.23
Gln	3.73	6.55	1.48
Hse	9.89	16.47	5.10
Aaa	7.14	8.75	0.93
Pro	tr	tr	0
Gly	1.60	0.69	0.54
Ala	0.99	2.11	0.51

Table 2

Amino-Acid Analyses (Sodium Buffers) of Phloem Exudate from Fully-Expanded Leaves as "Alaska" and Comparison with Data for Aphid Stylets Sap

	Phloem Exudate		Aphid Stylets Sap ^a
	Light	Dark	n mol
	n mol/leaf in 2 hours		
Asp	0.93	0.69	0.29 ^b
BIA	0.36	0.17	0.22 ^b
"Thr" ^c	4.65	3.70	3.16
"Ser" ^c			1.42
"Glu" ^c	2.72	2.06	3.21
Pro	0	0	0
Cys	0	0	0
Gly	0.96	0.34	0.07
Ala	0.53	0.19	0.50

^aData of Barlow and Randolph (1978) recalculated to give amino-acid levels relative to alanine 0.50 n moles.

^bIdentified by Barlow and Randolph (1978) as methionine sulfene, but probably BIA.

^cThis region will contain amides and homoserine if present.

END

29 | 1 | 2 | 8 | 1 |

FIN