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Distribution and Metabolism of Xylem-Borne Ureido and Amino Compounds in Developing Soybean Shoots[1]

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ABSTRACT

Pulse-chase feeding (30–120 minutes) of ^{14}C-labeled nitrogenous compounds to cut transpiring shoots was used to investigate the early fate of the major xylem-borne solutes in N_2-fixing soybean (*Glycine max*) plants at the V_4 growth stage. By comparison with the foliar distribution of [^{14}C]inulin (a xylem marker), it was determined that the phloem supply of allantoin, allantoic acid, asparagine, glutamine, aspartate, and arginine, respectively, provided about 20, 10, three, two, five, and 20 times the ^{14}C delivered to the developing trifoliolate in the xylem stream. Recovery of unmetabolized asparagine, aspartate, and arginine in this indicator trifoliolate, and significant declines in the percentage of ^{14}C from allantoic acid and allantoin recovered in the first trifoliolate, provided some support for the direct xylem-to-phloem transfer of these compounds, but did not preclude the involvement of indirect transfer. Data on stem retention and foliar distribution, expressed as a function of the relative xylem sap composition, indicated that ureides provide the major sources of nitrogen to all plant parts. There was no consistent distinction in distribution patterns between pairs of similar anionic and neutral compounds. The extent of xylem-to-phloem transfer among the ureido or the amino compounds was inversely related to its prominence in xylem sap.

Evidence from lupin, a legume species that exports amides as major products of N_2-fixation, indicates that several processes are involved in the initial distribution of xylem-borne organic N (7, 10, 13, 14). In the lower stem, the negatively charged cell walls largely abstract cationic forms of N (*e.g.* Arg), whereas anionic forms of N (dicarboxylic amino acids) are prevented from doing so by co-ion exclusion. Neutral N forms, such as Asn and Gln, are not abstracted to the same degree as Arg. Therefore, the amides and their dicarboxylic amino acids provide the major sources of N for growth of the foliage, with the dicarboxylic amino acids predominantly supplying mature leaves via the transpiration stream, and the amides extensively undergoing direct xylem-to-phloem transfer to supply young developing leaves. In general, studies on pea (19) and cottonwood (5, 23) support these conclusions. It has been suggested that similar partitioning of organic N may occur in the shoot of ureide-producing legumes, with the anionic form ALA[2] predominantly supplying the mature leaves, and the neutral form ALN supplying the developing organs after xylem-to-phloem transfer (1, 3).

Recently, using a soybean plant at the V_4 growth stage, we identified the transfer of xylem-borne organic N to the phloem stream from the difference in the percentage foliar distribution of [^{14}C]inulin, a xylem marker, and [^{14}C]AIB a synthetic amino acid (4). The developing third trifoliolate consistently received more AIB than inulin, and this process was sensitive to the rate of water flow through the xylem stream, and to stem and petiole girdling.

In this report, the early distribution and metabolism of xylem-borne ureido and amino compounds are investigated using cut transpiring shoots of soybean, a legume species that exports ureides rather than amides from N_2-fixing root nodules as the major forms of N available for shoot growth (6, 8, 9, 17, 18). The foliar distribution of these compounds is compared with that of inulin providing an indication of the extent of xylem-to-phloem transfer.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* (L.) Merr. cv Maple Arrow) plants, effectively nodulated with *Bradyrhizobium japonicum* (The Nitragin Co., Milwaukee, WI), were grown in the greenhouse in a N-free vermiculite culture as previously described (4). Plants at the V_4 stage were used for all experiments. At this stage the plant has two unifoliolates, one mature trifoliolate, one almost fully expanded trifoliolate, one immature trifoliolate leaf about one-third developed, and a vegetative bud at the apex.

Xylem Sap Collection and Solute Analysis

Xylem sap was collected for 1 h at 11 AM from freshly cut, blotted root stumps with tygon tubing placed over the stump. The sap was stored in micro test tubes at −20°C.

Amino acids in xylem sap were derivatized with *o*-pthalaldehyde/2-mercaptoethanol, and were separated with a Waters HPLC gradient system using reverse-phase chromatography as described previously (11). With this method, it was difficult to consistently separate His from Gln. Since it has been shown that the concentration of His in xylem sap is only about 20%

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[2] Abbreviations: ALA, allantoic acid; AIB, α-aminoisobutyric acid; ALN, allantoin.

of the Gln (6, 8), we have assumed here that no His was present. A Waters HPLC system coupled with a UV detector operating at 210 nm was employed to isocratically elute organic bases from an Aminex HPX-72S column (300 × 7.8 mm; Bio-Rad) using 0.065 M ammonium sulfate at ambient temperature and a flow rate of 0.6 mL min^{-1}. Data processing was accomplished with a Ramona Radio-Chromatography system (Raytest Instruments, Quebec, Canada) in conjunction with an IBM personal computer.

Preparation of [^{14}C]ALN and [^{14}C]ALA

The [4,5-^{14}C]ALN was prepared by incubating 0.5 mCi of [4,5-^{14}C]uric acid (132 mCi/mmol; Amersham) with 10 units of uricase (Sigma type IV) for 1 h, followed by purification using ion exclusion chromatography as described above. The radioactive peaks were identified by passing the column eluant into the solid cell of a Ramona D (Raytest Instruments), a flow-through isotope detector. A portion of [4,5-^{14}C]ALN was hydrolyzed to [4,5-^{14}C]ALA (24) and then purified as above. Both ^{14}C-labeled compounds were collected after HPLC separation, evaporated to dryness, and then resuspended in 100 mM Tris-HCl (pH 8.5) and frozen at −20°C until further use.

Feeding and Assay of ^{14}C

The roots were removed under water and the cut transpiring shoots were placed in xylem sap (diluted fivefold with distilled water to simulate the concentrations of nitrogenous solutes in xylem sap of intact plants) containing [U-^{14}C]Asn, [U-^{14}C]Asp, [U-^{14}C]Arg, [U-^{14}C]Gln, [4,5-^{14}C]Aln, or [4,5-^{14}C]ALA (0.25 or 0.5 μCi ml^{-1}) for 0.5 h followed by a chase period of 2 h in unlabeled diluted xylem sap (4). The plants were then harvested for determination of ^{14}C distribution between the sulphosalicylic acid soluble and insoluble (protein) fractions of the various plant parts, identification of ^{14}C-labeled amino compounds in the soluble fraction using reverse-phase HPLC separation of phenylthiocarbamate-amino acid derivatives (11), or whole plant autoradiography. For autoradiography, the shoots were harvested by cutting the petioles, and dried for 3 d in a plant press at 70°C. The shoots were mounted on bristol board and exposed to Kodak X-Omat RP-1 x-ray film for 2 or 4 weeks. Subsequently, the film was developed for 8 min in developer (Kodak GBX), followed by 1 min in a water wash, then 5 min in Kodak fixer (473 mL solution A and 55 mL solution B).

Statistical Treatment

Each comparison between the distribution of [^{14}C]inulin and a ^{14}C-labeled (two plants each) nitrogenous solute was conducted on 3 separate days, giving a total of six plants per treatment. Treatment of the data by two-way analysis of variance indicated no day or interaction effect and therefore the data were pooled to determine the mean ± SE. Significant differences between the solutes at the 5% level were found using the Student's t test.

RESULTS

HPLC Analysis of Xylem Sap

The xylem sap was composed predominantly of the ureides, ALA and ALN, on a concentration and a percentage N basis (Table I). The amides, Asn and Gln, were the next most abundant, followed by a variety of amino acids, of which Arg and Asp were the most prominent.

Whole Shoot Distribution of ^{14}C from Xylem-Borne Ureido and Amino Compounds

The distribution of total ^{14}C in the shoot after a 0.5-h feed/2.0-h chase period was dependent on the N form supplied in the transpiration stream. The retention of ^{14}C by the stem and petioles followed the sequence Arg > Asp > Gln > ALN > Asn > ALA (Table II).

The relative distribution of ^{14}C recovered in the leaves from each nitrogenous compound was compared with the distribution of [^{14}C]inulin, a xylem marker (4, 20). The distribution of ^{14}C from ALA and ALN was similar (Fig. 1). In both cases, the ^{14}C-recovery was higher than for [^{14}C]inulin in the second and third trifoliolates, and the bud, but lower in the first trifoliolate. The ^{14}C from ALN was also lower in the unifoliolate than [^{14}C]inulin. The distribution of ^{14}C from Asp and Asn was similar to [^{14}C]inulin for the unifoliolate, and the first and second trifoliolates, however, more ^{14}C was found in the third trifoliolate and bud. The percentage of ^{14}C from Arg was similar to [^{14}C]inulin for the unifoliolate and second trifoliolate, but it was lower in the first trifoliolate and higher in the third trifoliolate and bud. With Gln, the only significant deviations from the [^{14}C]inulin distribution occurred in the third trifoliolate and the bud.

Table I. *Composition of Ureido and Amino Compounds in Xylem Sap from V_4 Soybean Plants*

Compound	Composition	
	μmol/mL	*% total N*
Ureides		
ALA	7.34	72.5
ALN	1.99	19.7
Amides		
Asn	0.59	2.91
Gln	0.36	1.78
Amino acids		
Arg	0.11	1.09
Asp	0.34	0.84
Abu[a]	0.12	0.30
Lys	0.06	0.30
Ser	0.06	0.15
Glu	0.05	0.12
Ala	0.04	0.10
Val	0.03	0.07
Leu	0.02	0.05
Ile	0.02	0.05
Phe	0.02	0.05
Thr/Cit	0.01	0.02
Met/Trp	0.01	0.02
Tyr	0.01	0.02

[a] γ-Aminobutyric acid.

Table II. *Percent Distribution of Total ^{14}C (soluble and protein) in Vegetative Soybean Shoots Fed via the Transpiration Stream for 0.5 h with Various ^{14}C-Labeled Compounds followed by a 2-h Chase with Unlabeled Xylem Sap*

Data are the mean of three experiments ± SE (*n* = six plants). The plants had a mean fresh weight in mg (±SE) of 839 ± 22 for the stem, 265 ± 8 for the petioles and petiolules, and 1380 ± 36 for the leaves.

Plant Part	Compound					
	ALA	ALN	Asn	Asp	Arg	Gln
Stem	24.3 ± 1.9	39.2 ± 2.2	37.0 ± 0.5	50.7 ± 3.0	74.6 ± 2.4	56.4 ± 7.9
Petioles	8.7 ± 0.6	8.6 ± 0.7	3.3 ± 0.2	14.9 ± 0.8	4.9 ± 0.5	2.2 ± 0.2
Leaves	67.3 ± 2.3	52.2 ± 2.0	59.8 ± 0.6	35.6 ± 2.6	20.5 ± 2.1	41.4 ± 7.8

A small proportion of the ^{14}C from ALA and ALN was incorporated into the protein fraction in the unifoliolates, and the first and second trifoliolates (Fig. 2). In contrast, up to 30 and 15% was incorporated in the third trifoliolate and bud, respectively. Similar results were found with Asp, Asn, and Gln. In the case of Arg, incorporation of ^{14}C into protein was similar in the lower leaves to the other compounds, however, in the third trifoliolate and bud up to 60% of the ^{14}C was incorporated. Note that in all cases except one (Arg), the largest percentage incorporation of ^{14}C into protein occurred in the third trifoliolate, then the bud. With Arg, similar amounts of ^{14}C were incorporated into protein in the bud and the third trifoliolate.

Distribution of ^{14}C among Leaf Soluble Products

Analysis of soluble products from the trifoliolates revealed that with Asn and Arg, considerable ^{14}C was still recovered as the fed compound (Table III). Most of the ^{14}C from Asp and Gln was found outside the fed compound. With ALA and ALN there was substantial labeling of amino compounds, but the methodology used here did not allow the determination of ^{14}C remaining in the fed compound.

Whole Plant Autoradiography

Autoradiograms (Fig. 3, A and B) indicated that [^{14}C]inulin was recovered mainly at the leaf margins, whereas [^{14}C]AIB, a synthetic amino acid used previously (4), was recovered largely in the stem and throughout the trifoliolates and the bud. Autoradiograms produced from [^{14}C]ALA and [^{14}C]ALN exhibited similar patterns of ^{14}C-distribution, with intense labeling of the stem, petioles, and second and third trifoliolates (Fig. 3, C and D). In the first trifoliolate, the ^{14}C was located primarily in the veinal regions, whereas the veinal and the mesophyll regions were both intensely labeled in the other trifoliolates. The ^{14}C from Asp and Asn was located throughout the entire shoot (Fig. 3, E and F). In the first trifoliolate, the ^{14}C from Asp was located mainly in the veins, with a small amount in the mesophyll, whereas most of the ^{14}C from Asn was located in the mesophyll. The mesophyll of the second and third trifoliolates was intensely labeled with ^{14}C

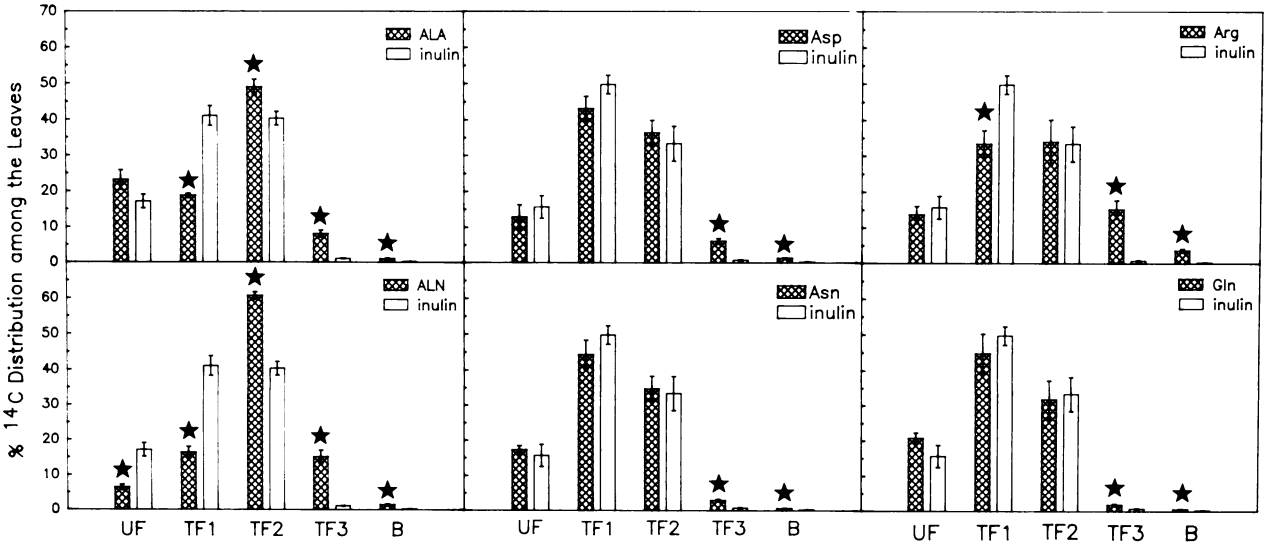

Figure 1. Distribution of total ^{14}C (soluble and protein) among the foliage of soybean shoots fed via the transpiration stream with ^{14}C-labeled nitrogenous compounds for 0.5 h followed by a 2-h chase period in unlabeled xylem sap. The unfoliolates; the first, second, and third trifoliolates; and the bud are depicted as UF, TFI, TF2, TF3, and B, respectively. Bars, mean of three experiments ± SE (*n* = six plants). ★, Significance between the nitrogenous compound and inulin at the 95% level of confidence.

from both Asp and Asn. With Arg, most of the ^{14}C was present in the stem, third trifoliolate, and the leaf veinal regions (Fig. 3G). With Gln, the ^{14}C was distributed throughout most of the plant, with the exception of the unifoliolates (Fig. 3H). The first trifoliolate was not intensely labeled, and most of the ^{14}C was recovered in the mesophyll. The second and third trifoliolates were labeled in both the veins and mesophyll.

DISCUSSION

Xylem Sap Composition

ALA and ALN were the predominant compounds found in the xylem sap of soybean, with Asn, Gln, Asp, and Arg next in order of abundance, followed by a variety of amino acids (Table I). In other studies (6, 8, 9, 17, 18), the xylem sap had ureide concentrations ranging from 82 to 91% of the organic N, values entirely consistent with this study. The ratio of ALN-N to ALA-N (22:78) observed in this study was similar to that found elsewhere (8, 17). However, one study (6) differed dramatically with an ALN:ALA ratio of 1:99. Although an explanation is not readily apparent, that result could be due in part to collection of xylem sap at 4-d and 2-night sampling times, since the proportion of ALN to ALA decreases drastically after 12:30 PM (18). The concentration and order of abundance of the amino acids in this study were similar to those reported previously (6, 9, 17). Therefore, in the absence of combined N in the nutrient solution, the predominant nitrogenous solutes compounds exported in the xylem stream of nodulated soybean plants are ALA, ALN, Asn, Gln, Arg, and Asp.

Xylem-to-Phloem Transfer of Xylem-Borne [^{14}C] Nitrogenous Solutes

The adoption of a technique, first described for transpiring tomato shoots (20), allowed the identification of xylem-to-

Table III. *Distribution of Soluble ^{14}C in Trifoliolates of Soybean Shoots Fed via the Transpiration Stream with ^{14}C-Labeled Nitrogenous Compounds for 0.5 h followed by a 2-h Chase Period in Unlabeled Xylem Sap*

Each value represents the mean of two replicates.

Fed ^{14}C Compound	Trifoliolate	^{14}C Recovered in Trifoliolate		
		As nonamino compounds	As fed compound	As other amino compounds
		%		
ALN	1[a]	-----54[b]-----		46
	2	-----62-----		38
	3	-----57-----		43
ALA	1	-----33-----		67
	2	-----58-----		42
	3	-----25-----		75
Asn	1	33	37	30
	2	38	27	35
	3	24	23	53
Asp	1	78	2	20
	2	71	3	26
	3	53	7	40
Arg	1	55	18	27
	2	56	13	31
	3	30	12	58
Gln	1	68	3	29
	2	66	3	31
	3	18	2	80

[a] One to three indicates the oldest to the youngest trifoliolates. [b] As fed compound and in nonamino acid compounds; the methodology used did not allow the determination of ^{14}C remaining in ALN or ALA only.

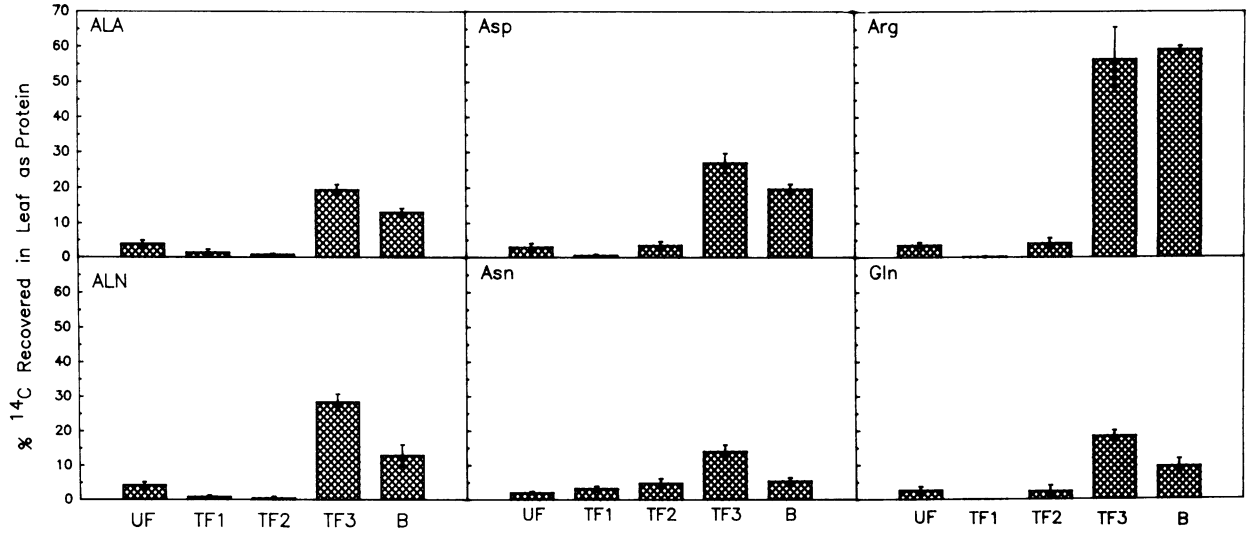

Figure 2. Partitioning of ^{14}C into the protein fraction of each leaf of soybean shoots fed via the transpiration stream with ^{14}C-labeled nitrogenous compounds for 0.5 h followed by a 2-h chase in unlabeled xylem sap. The unifoliolates; the first, second, and third trifoliolates; and the bud are depicted as UF, TFI, TF2, TF3, and B, respectively. The data are the mean of three experiments ± SE (n = six plants).

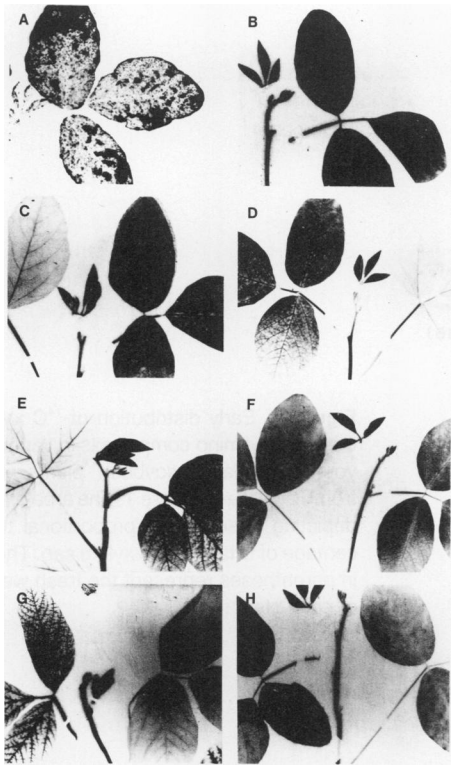

Figure 3. Autoradiograms of the upper regions of V_4 soybean shoots fed via the transpiration stream with ^{14}C-labeled nitrogenous compounds for 0.5 h followed by a 2-h chase in unlabeled xylem sap. A, Inulin; B, AIB; C, ALA; D, ALN; E, Asp; F, Asn; G, Arg; H, Gln.

phloem transfer in cut transpiring soybean shoots at the V_4 growth stage (4). Two h after the completion of a 30-min feed period with a ^{14}C-labeled marker compound for either xylem (inulin) or xylem and phloem movement (AIB), AIB was more readily retained in the stem, and a significantly greater proportion of the foliar AIB compared with inulin was recovered in the developing trifoliolate. Evidence from girdling studies and the absence of AIB metabolism suggested that direct xylem-to-phloem transfer, occurring in the lower stem and in the veins of the second trifoliolate, supplied AIB to this indicator trifoliolate. Another process that might have been involved is xylem-to-xylem transfer (7, 13). However, girdling the petiole leading to the indicator trifoliolate reduced the percentage [^{14}C]AIB in that leaf to the same level as [^{14}C]inulin (4). Because [^{14}C]AIB, unlike [^{14}C]inulin, can undergo transfer to xylem as well as to phloem, this evidence indicates that xylem-to-xylem transfer was of little importance in those young vegetative soybean plants.

In this study, which used similar experimental methodology and plants, all the naturally occurring ^{14}C-labeled nitrogenous compounds supplied to the cut transpiring soybean shoot also underwent xylem-to-phloem transfer. The phloem supply of ALN, ALA, Asn, Asp, Gln, and Arg, respectively, provided about 20, 10, 3, 5, 2, and 20 times the ^{14}C delivered to the indicator trifoliolate by the xylem stream (Fig. 1). As with AIB (4), in the cases where the magnitude of transfer was great (ALN, ALA, Arg), the proportion of ^{14}C in the first trifoliolate declined (Figs. 1 and 3), suggesting that that leaf was largely bypassed by direct stem transfer at the departing leaf trace. Where the xylem-to-phloem supply to the indicator trifoliolate was low (Asn, Asp, Gln), the distribution of ^{14}C in the remaining foliage was not significantly different from inulin (Figs. 1 and 3). Little support was found for the proposal that the anionic solutes such as ALA and Asp predominantly supply mature leaves, whereas their respective neutral forms, ALN and Asn, predominantly supply developing organs after direct xylem-to-phloem transfer (1, 13, 14).

The recovery of ^{14}C in the third trifoliolate as the fed compound (*e.g.* Arg) supports the existence of some direct xylem-to-phloem transfer of that compound, but it did not exclude indirect transfer. Similarly, when the fed compound was not recovered intact, the involvement of direct transfer was not entirely excluded. The pool size of each ^{14}C-compound reflects the extent to which various organs are dependent on *in situ* biosynthesis *versus* an external source of specific amino acids and their metabolites for protein synthesis (Fig. 2) (2). Here, as suggested earlier (17), the major xylem-borne nitrogenous solutes, ALA and ALA, were readily metabolized (Table III), and the extent of direct xylem-to-phloem transfer remained uncertain. In cowpea, a species that also exports principally ureides from root nodules, investigations using aphids (3) and cryopunctured fruits (16) have demonstrated that although xylem-borne [^{14}C]ALN is largely metabolized in leaves, a significant amount of the ^{14}C in phloem is present as ureides. The phloem sap of soybean plants is not readily collected from vegetative parts (6), but analysis of seedcoat exudate from fruiting plants (17) could provide valuable information about the xylem-to-phloem transfer process.

Distribution of Xylem-Borne [^{14}C]Nitrogenous Solutes

Previous research has shown that the concentration of xylem-borne amino acids is an important factor in the uptake and distribution pattern in tomato (22) and cottonwood (23) stems. Therefore, we supplied ureido and amino compounds at high specific activity against the background of the solutes that are normally delivered from nodulated roots to shoots in the xylem stream. It is generally accepted that root bleeding sap from the stem base provides a reasonably accurate picture of the relative composition of nitrogenous compounds present in intact transpiring plants (12). Calculations by Pate *et al.* (15) indicate that in lupin bleeding sap, N concentrations were four to 29 times higher than in xylem from intact plants. Thus, to approximate the actual N concentrations in intact soybean plants, the root bleeding sap was diluted fivefold before addition of a ^{14}C-labeled compound.

It was possible to estimate, from the relative concentration of xylem-borne solutes (Table I), the early partitioning of the major ureido and amino compounds in a developing intact nodulated soybean plant (Fig. 4). Total available solute was distributed in the following manner: 30% each to the stem and second trifoliolate; 13% each to the unifoliolates and first trifoliolate; 8% to the petioles; 5% to the third trifoliolate; and less than 1% to the bud. No close relation was found between leaf ^{14}C distribution and transpiration rates per unit leaf fresh weight, as determined by inulin distribution (Fig. 1). A diversity of distribution patterns was found between

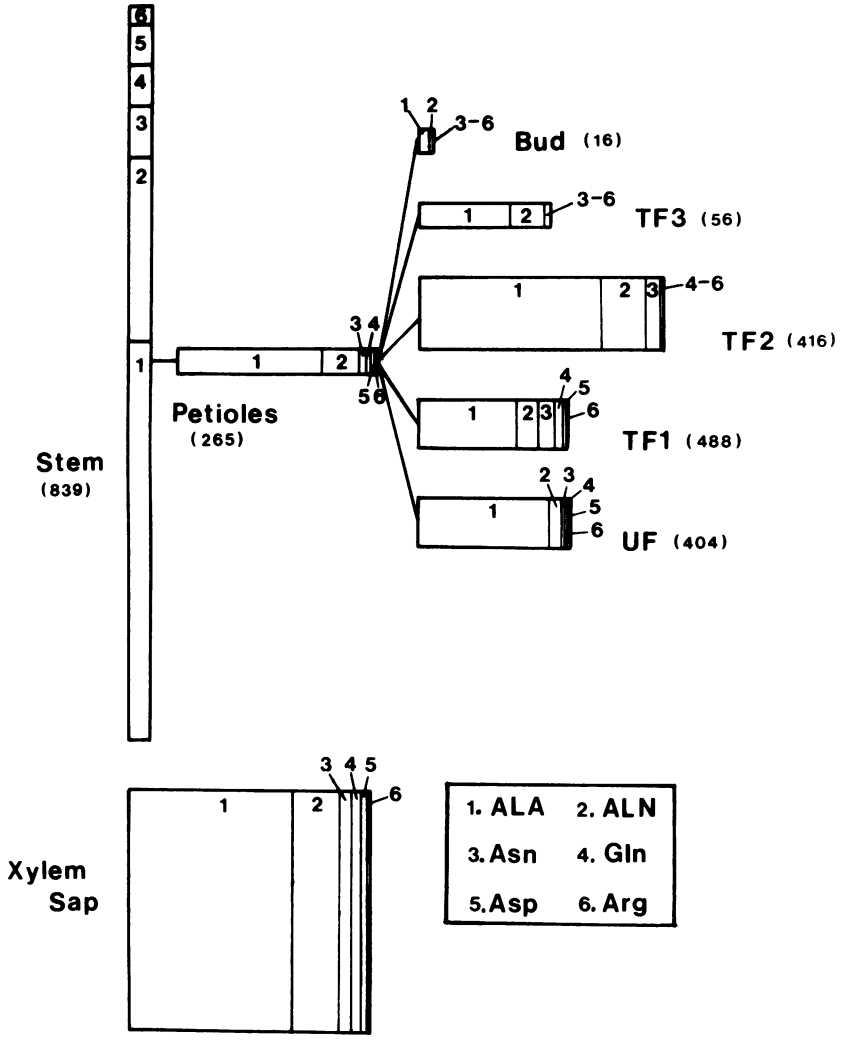

Figure 4. Early distribution of ^{14}C xylem-borne ureido and amino compounds in various parts of young nodulated soybean plants. Calculated from Table II and Figure 1. The area of rectangles depicting N solutes is proportional to the percentage of total N in the xylem sap. The numbers in parentheses represent the fresh weight in mg of each plant part.

solutes. Although the ureides were the predominant sources of carbon and nitrogen in each plant part, compared with their relative proportions in xylem sap, ^{14}C from ALN was enriched in the stem and the third trifoliolate, and ^{14}C from ALA in the unifoliolates and first trifoliolate. Similarly, enrichment of ^{14}C from Arg was found in the stem and third trifoliolate, and enrichment of ^{14}C from Asp with respect to Asn was evident in the stem and third trifoliolate, and with respect to Gln in the third trifoliolate.

Ionic interactions of the xylem solutes with the cell walls could probably account for the lower stem retention of ALA than ALN, and for the high stem retention of Arg, but not for the retention of the anion/neutral pair, Asp/Asn (10, 21). Some of the discrepancy in distribution of nitrogenous compounds between this study and those of previous workers (5, 10, 19) may be attributed to differences in the physiological age of the plant used. However, the information about stem retention (Table II) and xylem-to-phloem supply to an indicator trifoliolate (Fig. 1) could be interpreted as evidence for differentiation between the ureido and amino compounds based on their prominence in xylem sap. Of the ureides, ALN had a lower concentration and exhibited a greater retention by the stem and a greater degree of xylem-to-phloem transfer. Of the amino compounds, Arg, Asp, Gln, and Asn, from the lowest to the highest concentration, showed the same order of decreasing stem retention and with the exception of Gln, the same order of decreasing xylem-to-phloem transfer. It is noteworthy that the Gln concentration in xylem sap was overestimated by the methods used here (Table I). Thus, retention by the stem reflected the ease that a particular nitrogenous compound underwent xylem-to-phloem transfer, and it is possible that the capacity and selectivity of absorption by parenchyma cells lining the xylem pathway, particularly at departing leaf traces (10), were the overriding factors in determining the distribution of ureido and amino compounds.

The processes involved in the distribution of nitrogenous compounds in soybean, a species that transports predominantly ureides, are not inconsistent with those proposed for lupin (10, 13, 14), pea (19), and cottonwood (5, 23), species that transport amides. In each case a portion of the solutes moving upward would be available for phloem transport to and growth of immature organs with the remainder going to more mature transpiring leaves, which possess the metabolic machinery and capacity for the transformation of all incoming N. During development, the changing demands for and con-

tributions of nitrogenous solutes by the leaf of a higher plant are accommodated by the increasing contribution of xylem *versus* phloem.

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