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Role of Amides, Amino Acids, and Ureides in the Nutrition of Developing Soybean Seeds

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ABSTRACT

The various nitrogenous solutes important to embryo development in symbiotic soybean plants were determined during the midpodfilling stage. Glutamine was the principal form of nitrogen, contributing 55% of the embryo nitrogen requirement. Asparagine was the second most important, contributing 20%. The ureides allantoin and allantoic acid directly contributed only insignificantly to the total nitrogen requirement of the embryo. These conclusions were based upon analyses of tissue extracts, translocation studies of radiolabeled solutes, analysis of *in vivo* seed coat exudate collected from the freespace of attached, surgically altered seeds, and the *in vitro* culture of isolated immature soybean embryos.

The source of nitrogen for storage protein synthesis in developing seeds of symbiotic soybean plants has remained ambiguous. In these plants, the ureides allantoin and allantoic acid are the principal transported forms of assimilated nitrogen, comprising greater than 80% of the nitrogen in the xylem sap (8–10). That allantoin and allantoic acid are the principal nitrogenous source for embryo growth, however, has not been demonstrated. The growth of isolated, immature soybean embryos *in vitro* is very slow in liquid culture media supplemented with ureides, but rapid when supplemented with the amides asparagine and glutamine (J. Thompson, Cornell University, personal communication). Furthermore, the activity of one of the principal enzymes predicted from animal and microbial studies to catabolize ureides, allantoicase, has not been detected at physiological levels in developing soybean embryos. In the N-fixing legume *Lupinus albus*, asparagine is the principal nitrogen source for storage protein synthesis in the developing embryo (2). This amide also constitutes the major nitrogen source assimilated by the nodules and exported in the xylem stream to the reproductive shoot (7). Collectively, these observations question the direct involvement of allantoin and allantoic acid in soybean seed development, and suggest that they may be converted to other nitrogenous solutes prior to or during import by the fruit. This study addresses the question of the principal N sources supplying the embryo for growth and storage protein synthesis. With a number of techniques, we demonstrate that the amides glutamine and asparagine are of prime importance to seed nutrition.

MATERIALS AND METHODS

Plant Material. Nodulated plants of soybean (*Glycine max* L. Merr. cv Clark) were grown in vermiculite in controlled environment chambers with a 12-h diurnal regime (30/25°C, day/night). The light intensity during the photoperiod was about 700 μE m^{-2} s^{-1} and the RH was maintained at about 75%. The soybeans were inoculated with *Rhizobium* strain SR 166, and were fertilized twice weekly with quarter-strength Hoagland solution lacking N. Reproductive soybean plants of midpodfilling stage 54 to 75 days after sowing were used exclusively.

Chemicals and Radiolabel. Unless otherwise stated, all chemicals and enzymes used were purchased from Sigma. Radiolabel was purchased from two sources, L-[2-^{14}C]uric acid, L-[U-^{14}C] glutamine, and L-[U-^{14}C]asparagine from Amersham and L-[U-^{14}C]aspartate and L-[U-^{14}C]glutamate from New England Nuclear.

Preparation of [^{14}C]Allantoin and [^{14}C]Allantoic Acid. L-[2-^{14}C] Uric acid (50 μCi/μmol) was dissolved in 1 ml Tris-HCl buffer (100 mM, pH 8.8) and incubated (2 h, 30°C) with 0.03 unit of urate oxidase (urate:oxygenase oxidoreductase, EC 1.7.3.3), isolated from *Candida utilis*) in a total volume of 1.1 ml. Because the enzyme preparation contained a small contaminant of allantoicase, care was taken to monitor the progress of the reaction. This was done with the HPLC technique described below. The [^{14}C]uric acid was converted to [^{14}C]allantoin with about a 90% yield. Purification was as follows.

Separation and Identification of ^{14}C-Labeled Allantoin, Allantoic Acid, Uric Acid, and Glyoxylate. Allantoin was separated from the acidic products of uric acid oxidation using a small column (5 × 50 mm) of formate ion exchange resin (Bio-Rad). Allantoin and other components of the neutral fraction were eluted from the column with distilled water and the acidic fraction was then eluted with 1 N HCOOH. The acidic and neutral fractions were evaporated to dryness and resuspended in a known volume of distilled H_2O. The various acidic components were separated and quantitated with a Du Pont HPLC system equipped with a Bio-Rad organic acid column (300 × 7.8 mm Aminex HPX-87H) and 0.01 N H_2SO_4 solvent with refractive index detection (Fig. 1). Neutral components were assayed by HPLC as previously described (16). In both cases, identification of component fractions was by co-elution with authentic standards.

Xylem Transport of ^{14}C-Solutes in Explants. Soybean plants were submerged in water and stem sections were cut from fifth, sixth, and seventh nodes to provide one-node explants, each having a trifoliolate leaf and one or two subtending fruit. The basal stem end was immersed in a solution designed to mimic xylem sap (1 mM asparagine, 0.5 mM glutamine, 1 mM allantoin, 1 mM allantoic acid, 1 mM malate, pH 5.5). The explants were allowed to transpire in the light (1300 μE m^{-2} s^{-1}) for at least 1 h before replacing the artificial xylem sap with a pulse (1 μCi/explant) of ^{14}C-labeled nitrogenous solutes. The 15-min pulse was administered through the cut stem and then chased with artificial xylem sap for specified lengths of time in the light (300

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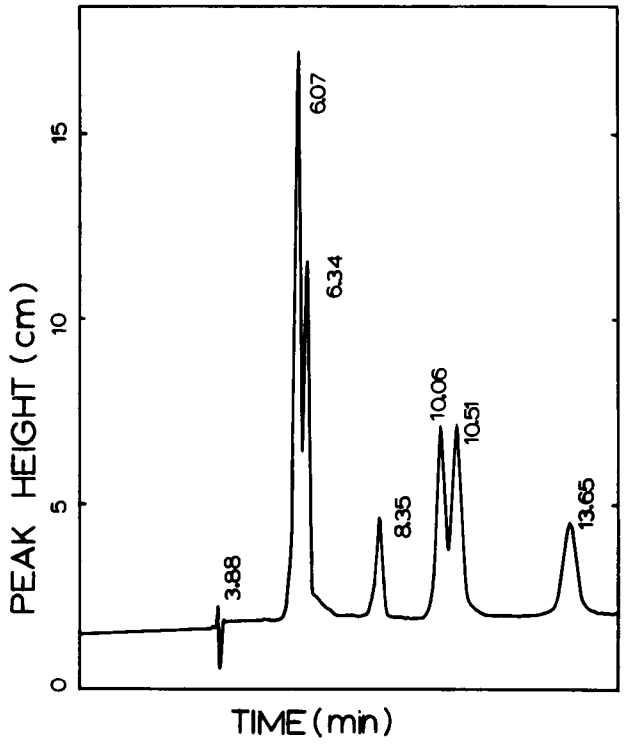

FIG. 1. A typical chromatograph from HPLC separation of the products of uric acid catabolism and malate. Represented in 10-nmol fractions are glyosylate (6.07 min), malate (6.34 min), ureidoglycollate (8.35 min), allantoin (10.06 min), uric acid (10.52 min), and allantoic acid (13.65 min).

$\mu E\ m^{-2}\ s^{-1}$).

Determination of Dry Matter, Total Carbon, and Nitrogen Accumulation. Reproductive plants were sequentially harvested from d 54 to 75 and dissected into nodulated roots, stems and petioles, leaflets, pod walls, seed coats, and embryos. The plant material was dried at 80°C, weighed, and finely ground in a mill. A subsample (2 mg) was analyzed for C and N content by elemental analysis (Perkin-Elmer).

Collection of Xylem Sap. Root exudate was collected from the cut stem base of nodulated soybean plants. The first 10 min of exudation was discarded and the next 60 min was sampled at periodic intervals and stored at −20°C until analysis.

Collection of Seed Coat Exudate. Seed coat exudate was collected from surgically exposed seed coats as previously described (17).

Studies of Embryo Solute Uptake. Embryos were isolated and assayed for uptake of nitrogenous solutes as previously described for sucrose uptake (16).

***In Vitro* Embryo Culture.** Embryos were isolated under sterile conditions, weighed, and cultured using the technique of Thompson *et al.* (15). The sucrose medium was supplemented with 62 mM N in the form of either glutamine, asparagine, or allantoin. After 8 d of incubation at 25°C, the increase in fresh weight, dry weight, and protein content of the embryos was determined.

Extraction of Plant Material for Analysis of Soluble Fraction. Plant material was dissected, weighed, and then pulverized in liquid N_2 with a mortar and pestle. The powdered tissue was allowed to thaw and then repeatedly extracted at room temperature with 80% ethanol. The lipids were removed by washing with petroleum ether, chilling to −20°C, and pouring off the ether, leaving behind the frozen aqueous extract.

Amino Acid and Ureide Analysis. Amino acids in the seed coat exudate, xylem sap, and tissue ethanol extracts were separated and quantitated with a Beckman amino acid analyzer. Ureide content in the plant extract was determined by the phenylhydrazine glyoxylate technique (4) or by the HPLC techniques described above.

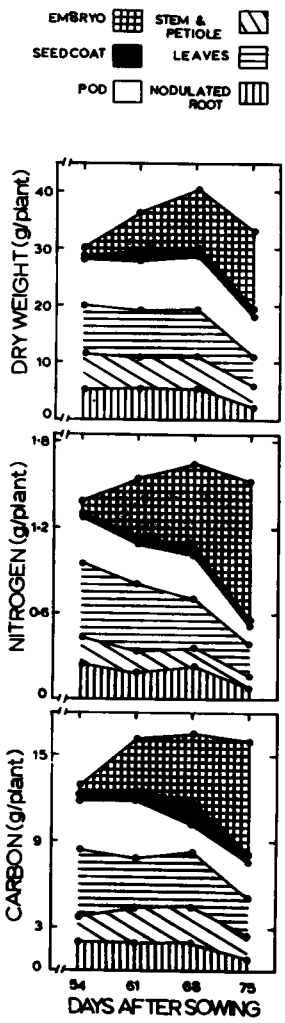

FIG. 2. Cumulative increases and decreases in dry weight (top), nitrogen (middle), and carbon (bottom) in different plant parts during the study period.

Specific radioactivities of ^{14}C-ureides were determined by HPLC and scintillation spectroscopy.

Analysis of Total ^{14}C in Plant Material. In some experiments, radiolabeled plant material was combusted with an Intertechnique Oxymat (Fairfield, NJ), and the $^{14}CO_2$ was collected and analyzed as previous described (16).

RESULTS

Accumulation of Dry Matter, Carbon, and Nitrogen. Total dry matter of the plants increased from 30 to 40 g during a 2-week study period, 54–68 d. The major contribution to this dry weight increase was embryo growth (Fig. 2), because the pods, leaflets, and stems and petioles all lost C and N during this period.

The C acquired by the growing soybean embryos during the study period was 4.2 g, exceeding the total plant C increment of 3.6 g. Similarly, the total plant N increased approximately 0.2 g and the embryo nitrogen increased 0.6 g. This is consistent with the observation by Warenbourg *et al.* (19) that at least 60% of the N used by developing embryos for growth and storage product formation is derived from remobilization. This is unlike cowpea, for Herridge and Pate (5) have shown the majority of

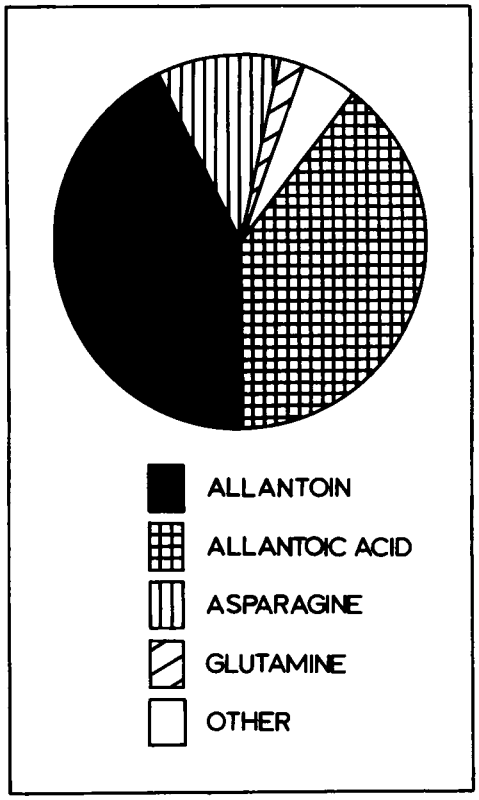

FIG. 3. Analysis of xylem sap collected from the severed stumps of 62-d-old soybean plants.

the seed's N is derived from current N_2 fixation rather than remobilization.

Xylem Sap Composition. Analysis of xylem sap exudate collected from severed stems confirmed earlier reports (8, 9, 14) that the principal nitrogenous compounds exported from the nodulated root system to the shoot were the ureides allantoin and allantoic acid (Fig. 3). The ureides comprised 82% of the N transported, and asparagine and glutamine provided a further 10 and 2%, respectively. The remaining 6% N was made up of various other amino acids.

Distribution of ^{14}C-Solutes within the Explant System. When ^{14}C-labeled nitrogenous solutes were fed into the transpiration stream of soybean explants, the pattern of ^{14}C distribution depended upon the form of N solute presented. When [^{14}C]asparagine was fed into the transpiration stream, 70 to 80% of the label remained in the stem after a 5-h period (Fig. 4A). The leaflets and petioles contained only 12% of the total label. The fruit contained approximately 14% of the label, half of which was in the embryo. A similar distribution pattern was observed when [^{14}C]glutamine was fed into the transpiration stream (Fig. 4B). Again the stem was the primary sink after 5 h. However, label entered the fruit at a somewhat slower rate than when asparagine was fed, and only 4% was found in the embryo after a 5-h period, compared to 7% when [^{14}C]asparagine was fed to the transpiration stream.

When [^{14}C]allantoin or [^{14}C]allantoic acid was fed to the transpiration stream of explants, a larger proportion of the label entered the leaves and less entered the fruit than when amides were fed (Fig. 4, C and D). The distribution of label from allantoin (Fig. 4C) after 5 h was largely associated with the stem (approximately 60% of the total). The leaves contained 18%, the petiole 15%, and the remaining 12% was in the fruit. Unlike asparagine (Fig. 4A) and glutamine (Fig. 4B), when [^{14}C]allantoin was fed very little of the label (0.1%) was accumulated by the embryo and, instead, the label imported by the fruit was accumulated by the pod. Similarly, when [^{14}C]allantoic acid was fed, the distribution of ^{14}C label was almost equally divided between the leaflets, stem, and petiole (31, 33, and 37% of the total label, respectively) after a 5-h chase. Only 9% of the total label was found in the fruit and, like allantoin, very little was found in the embryo (approximately 1%).

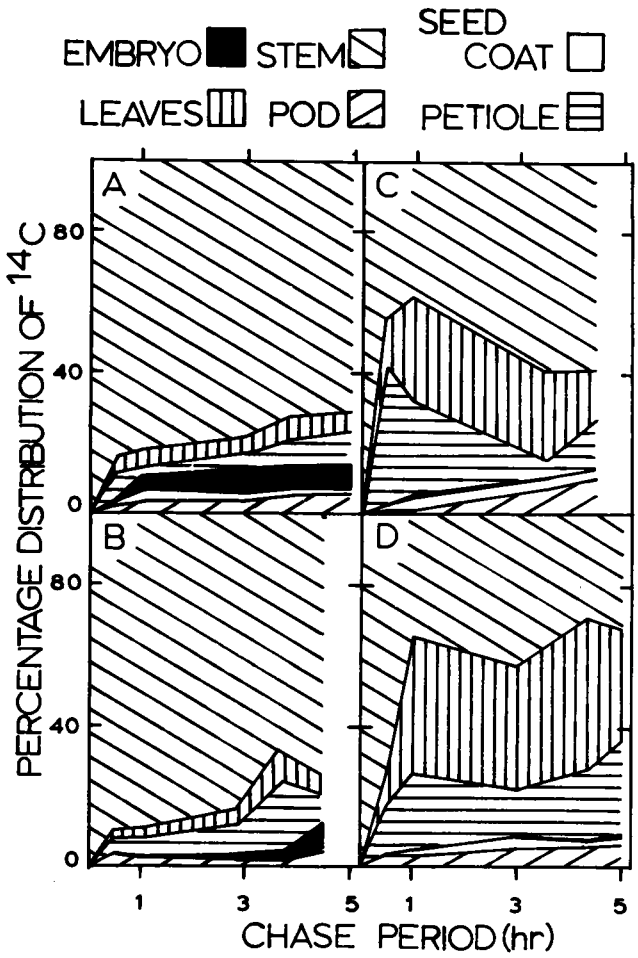

FIG. 4. Distribution with time of various nitrogenous solutes with varying concentrations. [^{14}C]Asparagine (A), [^{14}C]glutamine (B), [^{14}C] allantoin (C), and [^{14}C]allantoic acid (D).

Uptake of Nitrogenous Solutes by Isolated Embryos. These data suggested that either the maternal fruit tissue was metabolizing the incoming ureides and preventing them from reaching the embryo or that the embryos could not accumulate the ureide when presented to them by the seed coats. We examined this latter alternative by determining the capacity of immature, isolated embryos to accumulate various ^{14}C-labeled solutes. *In vitro* uptake (30°C, pH 6.0) varied with the compound and its concentration; embryos accumulated asparagine much more rapidly than glutamine, allantoin, aspartate, or glutamate (Fig. 5). The uptake of allantoin was equivalent to that of glutamine at low concentrations (<5 mM), but increased only slowly at higher concentrations. The uptake of allantoic acid was very slow at all concentrations tested (Fig. 5). The amino acids aspartate and glutamate were also accumulated at a slow rate when compared to the accumulation by the embryo of the amides asparagine and glutamine (Fig. 5).

***In Vitro* Embryo Growth on Different Nitrogen Sources.** When isolated, immature cotyledons were cultured *in vitro*, growth and storage protein accumulation varied, depending on the N source (Table I). Embryos grew at the fastest rate when supplied glutamine. Asparagine supported growth at a somewhat slower rate,

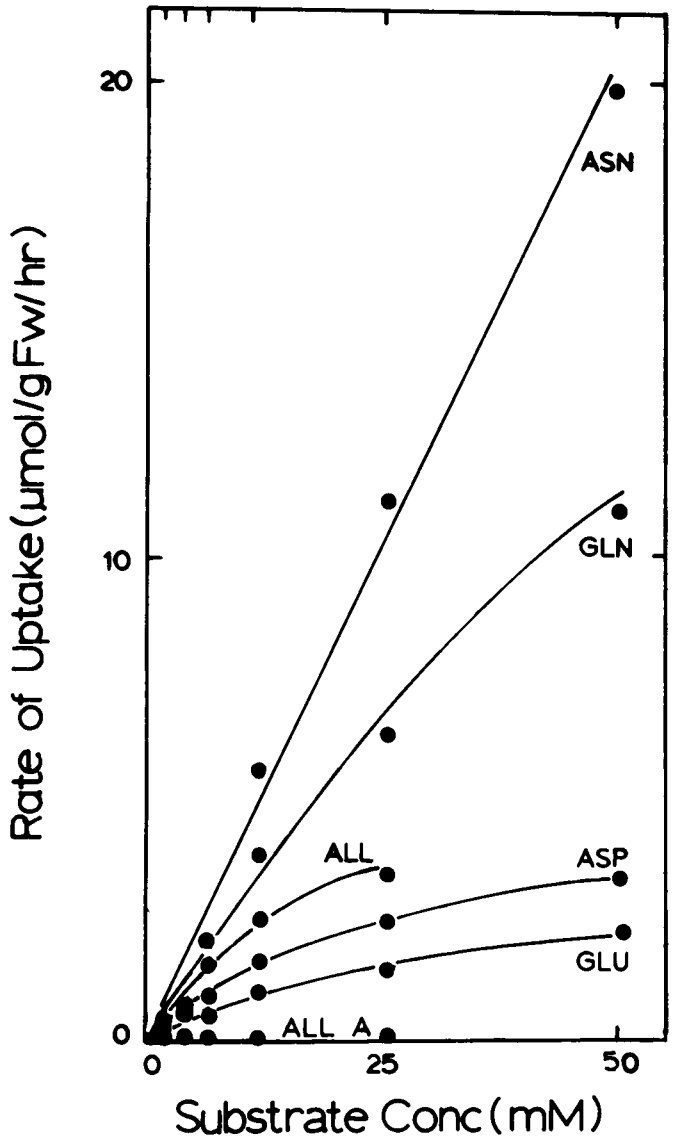

FIG. 5. *In vitro* uptake of nitrogenous solutes by isolated immature soybean embryos at pH 6.0 and 30°C. Asn (asparagine), Gln (glutamine), All (allantoin), Asp (aspartate), Glu (glutamate), and All A (allantoic acid).

Table I. *In Vitro Soybean Embryo Culture Using Different Nitrogen Sources*

Growth for 8 d on sucrose media supplemented with 62 mM glutamine, asparagine, or allantoin; allantoic acid was not used due to its instability in long term experiments at pH 6.0. Other components of the medium were as published by Thompson *et al.* (15). FW, fresh weight; DW, dry weight.

Nitrogen Source	Initial FW	Final FW	Increase in FW	Final DW	Final Protein
	mg	*mg*	*%*	*mg*	*mg*
Glutamine	344.6	709.3	105.8	173	16.8
Asparagine	362.2	552.0	52.5	138	11.9
Allantoin	355.2	436.2	22.8	125	11.9

but growth on allantoin was very much retarded. These results are similar to those of Thompson (personal communication), indicating that ureides are a poor N source for soybean embryo growth *in vitro*.

Ureide and Amino Acid Pools. When harvested at midpodfilling (54 d of age), the highest concentrations of ureides were found in the seed coat (37 μmol/g fresh weight), pod walls (28 μmol/g fresh weight), and stem (20 μmol/g fresh weight). Expressed as mg ureide-N/plant part (Table II), the pod walls contained the predominant pool of ureides (62 mg). Ureides generally constituted between 60 and 80% of the total soluble N in a given tissue except in the embryo where they comprised only 14%. Asparagine was the predominant nitrogenous solute in the embryo (58% of the soluble N). Other plant parts had between 8 and 18% of their soluble N in the form of asparagine. Glutamine was relatively high in seed coats (18% of the soluble N).

Seed Coat Exudate. Using a recently developed *in vivo* technique (17), solutes were collected from soybean seed coats and the components analyzed (Table III). Glutamine was found to be the major nitrogenous solute released by the seed coat, comprising 52% of the N present. A further 19% of the N was asparagine. Arginine and histidine accounted for 5 and 4% of the seeds' nutrition, respectively. Ammonia and the other 14 amino acids constituted 24% of the total N. The ureides allantoin and allantoic acid were an insignificant N component of this phloem sap. The major C component of the seed coat exudate (Table III) was sucrose, constituting 90% of the total C exported to the embryo, while the C skeletons of the N solutes constituted the remaining 10%.

By addition of the C and N associated with the N compounds and the sugars, it was possible to calculate the C:N ratio of the seed coat exudate. As shown in Table III, this was computed to be 31 mg C/mg N, similar to the C:N ratios of fruit phloem sap published previously (11, 12).

DISCUSSION

As the ureides are the major nitrogenous solute exported in the symbiotic transpiration stream of soybean (Fig. 3), it is often suggested that they may play a direct major role in developing embryo nutrition. However, several lines of experimental evidence suggest that ureides do not directly play an important role in the nutrition of the developing embryo: (a) isolated soybean embryos preferentially accumulated amides rather than ureides or amino acids when presented *in vitro* in equimolar concentrations (Fig. 4); (b) exogenous amides, but generally not ureides, are transported in the transpiration stream of cut soybean stems to embryos of attached fruit (Fig. 5); (c) isolated embryos cultured *in vitro* grow poorly on sucrose supplemented with allantoin (Table I); (d) analysis of the solutes delivered by the intact seed coat showed the principal nitrogenous solutes to be not ureides but glutamine (52%), asparagine (19%), ammonia (7%, perhaps resulting from the breakdown of ureides in maternal tissues), and various amino acids (29%). The ureides allantoin and allantoic acid comprised only trace amounts of the nitrogenous solutes delivered to the embryo *in vivo* (Table III).

The indirect role of ureides in embryo nutrition can also be demonstrated by constructing a C/N budget, if two assumptions are made. The first is that the nitrogenous compounds transported during N remobilization from the shoot to the seed are amino acid residues from protein degradation rather than *de novo* ureide resynthesis in the shoot. This is supported by the low levels of ureides found in nonsymbiotic plants (3, 8, 9). The second assumption is that N requirements will be satisfied by depletion of soluble N pools prior to remobilization of N from 'structural' sources. With these assumptions, a budget was constructed for embryo nutrition during the period of 54 to 68 d after sowing.

As shown in Table IV, ureides resulting from recent N assimilation (represented by ^{14}C-ureides) are relatively unavailable to the developing soybean embryo. Atkins *et al.* (1) have shown a similar pattern for the symbiotic legume cowpea, supporting the

Table II. *Distribution and Concentration of Soluble Fraction Nitrogenous Compounds in Soybean Plants during Midpodfilling Stage of Reproductive Growth*

Data are from the first day of the study period (day 54) and ureides are allantoin and allantoic acid. FW, fresh weight.

	Ureides		Asparagine		Glutamine		Other	
	μmol/g FW	*mg N/plant*	*μmol/g FW*	*mg N/plant*	*μmol/g FW*	*mg N/plant*	*μmol/g FW*	*mg N/plant*
Embryo	4.9	2.5	21.7	5.5	1.4	0.4	9.0	1.1
Seed coat	37.3	5.8	16.9	1.3	20.9	1.6	10.3	0.4
Pod	27.5	62.4	7.0	7.9	3.0	3.8	2.0	1.1
Stem and petiole	20.2	29.9	7.9	5.8	2.5	1.9	2.2	0.8
Leaflets	6.4	14.2	1.6	1.8	2.0	2.1	9.0	4.9
Total		114.8		22.3		9.8		8.3

Table III. *Components of Seed Coat Exudate*

C:N ratio = 30.8 mg C/mg N.

Component	Mol %	Carbon	Nitrogen
		%	%
Sugars			
Sucrose	78.7	90.7	
Hexoses	Trace		
Totals	78.7	90.7	
Amino acids			
Glutamine	10.4	5.0	52.6
Asparagine	3.7	1.4	18.8
Ammonia	1.3	0	6.9
Serine	0.8	0.4	2.2
Histidine	0.6	0.3	4.4
Proline	0.5	0.3	1.4
Threonine	0.5	0.2	1.3
Arginine	0.5	0.3	4.9
Valine	0.5	0.2	1.2
Alanine	0.4	0.1	1.1
Aspartate	0.4	0.2	1.1
Leucine	0.4	0.2	0.9
Glutamate	0.3	0.1	0.7
Isoleucine	0.3	0.1	0.7
Glycine	0.2	0.04	0.6
Phenylalanine	0.2	0.2	0.5
Tyrosine	0.2	0.2	0.5
Methionine	Trace		
Totals	21.2	9.24	100.0
Ureides			
Allantoin and allantoic acid	Trace		

Table IV. *Relative Metabolism of Xylem-Borne ^{14}C-Ureides in Various Tissues from 54–68 Days after Sowing*

Plant Part	Imported[a] Ureide-N	Ureide Metabolism	Ureide-N Remaining after 5 h
	mg	*%*	*mg*
Stem and petiole	142	69	44
Leaves	48	93	3
Pods	17	48	9
Seed coat	3	100	0
Embryo	1	100	0
Total	211		56

[a] Calculated knowing (a) the amount of recently assimilated N during the 14-d period, (b) percentage ureide in the xylem sap, and (c) percentage distribution of ^{14}C-labeled ureides after a 5-h pulse chase for 62 d-old plants.

hypothesis that recently assimilated ureides make a minor contribution to the nitrogen nutrition of the embryo. Only 1 mg of ureide-N can be calculated to enter the embryo and of the remaining recently assimilated ureide, the majority (14%) was extensively metabolized (Table IV). Assuming no further metabolism than what occurred during the first 5 h after translocation to the shoot, 56 mg of ureide-N assimilated in the study period would be available for possible translocation (Table IV). By adding the total free pools of ureide N (115 mg N) laid down before the study period (Table II) to the recently assimilated ureide (Table IV), the direct maximum possible ureide-N contribution to embryo nutrition was calculated to be 171 mg of N. Since the embryos increased their N content by 600 mg N during the study period (Fig. 2), the ureide compounds could have directly contributed a maximum of 29% of this N requirement (using the above assumptions).

In conclusion, these observations indicate that glutamine and, to a lesser extent, asparagine provide most of the N nutrition for seed development in symbiotic soybean plants. This selectively occurs in spite of very high concentrations of ureides in the xylem sap (Fig. 3) and in the maternal fruit tissues and phloem sap (6) responsible for the nutrition of the seed. The apparent inability of the embryo to utilize ureides appears to be due to a combination of (a) rapid metabolism of ureides in maternal tissues, (b) low rates of uptake by the embryo, and (c) their inability to efficiently utilize any incoming ureides for growth and storage protein formation.

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