Plant Physiol. (1986) 80, 1002–1005
0032-0889/86/80/1002/04/$01.00/0

Pathways of Nitrogen Metabolism in Nodules of Alfalfa (*Medicago sativa* L.)[1]

Received for publication October 16, 1985 and in revised form January 2, 1986

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ABSTRACT

Exposure of intact alfalfa nodules to $^{15}N_2$ showed that in bacteroids the greatest flow of ^{15}N was to NH_3. Label was also detected in glutamic acid, aspartic acid, and asparagine (Glu, Asp and Asn), but at far lower levels. In the host plant cytosols, more ^{15}N was incorporated into Asn than into other compounds. Detached nodules were also used to study the metabolic pathway of N assimilation after exposure to $^{15}N_2$ or vacuum infiltration with $(^{15}NH_4)_2SO_4$ in the presence or absence of different inhibitors of nitrogen assimilation: methionine sulfoximine (MSO), azaserine (AZA), or amino-oxyacetate (AOA). Treatment with MSO, an inhibitor of glutamine synthetase (GS), inhibited the flow of the label to glutamine (Gln)-amide, resulting in subsequently decreased label in Asn-amide. Aza, which inhibits the formation of Glu from Gln by glutamate synthase (GOGAT), enhanced the labeling of the amide groups of both Gln and Asn, while that of Asn-amino decreased. When AOA was used to block the transamination reaction very little label was found in Asp and Asn-amino. The results are consistent with the role of GS/GOGAT in the cytosol for the assimilation of NH_3 produced by N_2 fixation in the bacteroids of alfalfa nodules. Asn, a major nitrogen transport compound in alfalfa, is mainly synthesized by a Gln-dependent amidation of Asp, according to feeding experiments using the ^{15}N-labeled amide group of glutamine. Data from $^{15}NH_4^+$ feeding support some direct amidation of Asp to form Asn.

In legume nodules, the first product of N_2 fixation in bacteroids is NH_3. The NH_3 is then exported to the host plant cytosol where it is further metabolized to amino acids and amides (1, 9–11, 14).

In vitro enzymic studies indicate that GS/GOGAT[2] rather than GDH are involved in the assimilation of NH_3 to produce Glu and Gln. The resulting Glu and Gln are utilized to give a variety of amino acids including Asp, and this Asp is amidated by the Gln-dependent AS to form Asn (18, 20, 21). While information is available from *in vitro* studies which may be insufficient to explain the authentic metabolic pathway, little *in vivo* work has been conducted. Ohyama and Kumazawa (14, 15, 16), Matsumoto *et al.* (12), and Fujihara and Yamaguchi (3, 4) using the ^{15}N tracer technique reported the predominent role of GS/GOGAT in the assimilation of N in soybean, a ureide-producing legume. However, in amide-producing legumes such as pea, lupin, and alfalfa, the exact pathway of N assimilation has not been established with ^{15}N tracer techniques.

The objective of this work was to examine the pattern of ^{15}N incorporation into various nitrogenous compounds in alfalfa nodules exposed to $^{15}N_2$ or supplied with $^{15}NH_4^+$ and (^{15}N-amide) Gln solution in the presence or absence of different inhibitors of nitrogen metabolism.

MATERIALS AND METHODS

Plant Culture. Alfalfa seeds (*Medicago sativa* L. cv Saranac) were germinated in vermiculite which had been inoculated with a suspension of *Rhizobium meliloti*, strain 102F70 (Nitragin Co.). Two-week old seedlings were then transferred to jars (500 ml) containing aerated N-free Hoagland nutrient solution, which was renewed twice a week. The lower portion of the roots was partially immersed in nutrient solution and the upper part (5 cm) was exposed to air. The plants were grown in a growth room with 16/8 h light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$)/dark cycle at 25/20°C and 70% RH. Nodules from 6-week old plants (early bloom stage) were exposed to $^{15}N_2$, $^{15}NH_4^+$ or (^{15}N-amide) glutamine.

Reagents. Labeled $(^{15}NH_4)_2SO_4$, (^{15}N-amide) gluatamine and $^{15}N_2$ gas were purchased from MSD isotopes, Montreal at 99 atom % excess. Other chemicals were obtained from Sigma.

^{15}N Administration. For experiments with attached nodules, jars containing the nodulated-root system were filled with N-free Hoagland solution and sealed with three-hole rubber stoppers. Four plants were held in one hole, another hole held a serum stopper for N_2 gas injection and the remaining hole was connected to a vacuum system. When $^{15}N_2$ gas was used, an excess (200 ml for 30 min exposure) mixture of N_2:O_2:Ar (20:20:60) was injected into the jars to displace the solution to 5 cm below the stopper. For experiments with detached nodules, nodules were removed from the root systems, placed in 20 ml vials and vacuum infiltrated with different inhibitors or water (as control) for 15 min. $^{15}N_2$ gas or a solution of either 2 mM $(^{15}NH_4)_2SO_4$ or 2 mM (^{15}N-amide) glutamine was then injected into each vial. Twenty min after introduction of ^{15}N, the nodules were washed several times with distilled H_2O on filter paper held in a Büchner funnel connected to an aspirator and then rapidly frozen in liquid N_2. No attempt was made to measure the ^{15}N atom % excess and the concentration of the gases in the mixture after nodule exposures on the assumption that no major change took place.

Extraction of Soluble Nitrogenous Compounds. Soluble compounds were extracted from nodules by crushing the nodules in 80% cold ethanol and allowing to stand overnight. The supernatant obtained by centrifugation was concentrated under reduced atmosphere, below 40°C, using a rotary evaporator.

After various times of exposure to $^{15}N_2$, attached nodules were sampled and macerated at 4°C in 0.2 M sorbitol solution. The

[1] Paper contribution No. 804 from Ottawa Research Station, and No. 1580 from Chemistry and Biology Research Institute, Agriculture Canada.

[2] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; MSO, methionine sulfoximine; AZA, azaserine; AOA, amino-oxyacetate; GDH, glutamate dehydrogenase; AS, asparagine synthetase.

macerate was filtered through double layers of nylon net (25μm). The filtrate was centrifuged at 6,000g for 15 min and separated into cytosol (supernatant) and bacteroid (pelleted) fractions. The bacteroid fractions were extracted with 80% ethanol as described above.

Separation of NH_3, Amino Acids and Amides. NH_3 was separated by distillation of the extracts, at 40°C (pH 10), into 0.2 N HCl. The sample solution (adjusted to pH 6.5) was then passed through Dowex 1, acetate form. Glu and Asp were eluted separately with 0.1 and 0.5 N acetic acid, respectively. Glutaminase enzyme (from Sigma, grade V) was added to the sample solution and incubated at 30°C (pH 5) for 2 h. The resulting NH_3 from the Gln-amide group was obtained by distillation as above. The amide group of Asn was separated from the sample solution by using asparaginase enzyme (from Sigma, grade V) incubated at 30°C (pH 8) for 2 h; the resulting NH_3 was also obtained by distillation. Finally, the amino group of both Gln and Asn was separated from the sample solution in the form of Glu and Asp by using Dowex 1 as described above. Each fraction was concentrated under an IR lamp and then introduced into 4 mm o.d. pyrex tubes. Discharge tubes were prepared according to the direct Dumas combustion method, in evacuated tubes. ^{15}N was analyzed by emission spectrophotometry (Ta *et al.* [23]).

The amounts of each amino acid and amide were determined using an amino acid analyzer (Beckman model 121-M, with lithium citrate buffer on physiological mode).

RESULTS AND DISCUSSION

Under the growing conditions of this experiment, the roots of alfalfa were well nodulated. When the plants reached 10% bloom stage, the nodules were very active and pink in color. At this stage about 0.2 g of fresh nodules were obtained from each plant.

Amounts of Amino Acids and Amides in Alfalfa Nodules. Table I shows the composition of free nitrogenous compounds in both cytosol and bacteroid fractions of alfalfa nodules. NH_3 was the predominant compound found in the bacteroids; however, in the plant nodule cytosol Asn was present in the highest concentration followed by NH_3, Glu, α-aminobutyric acid, Ala, Asp, and Ser. Ureides were not detected in either fraction.

Table I. *Amounts of Various Nitrogenous Compounds in Bacteroid and Cytosol Fractions of Alfalfa Nodules*

t, Trace amounts (less than 10 nmol/g fresh wt). Each value is the mean of 5 observations ± SE.

Compounds	Bacteroid	Cytosol
	nmole·g nodule fresh weight^{-1}	
Asp	27 ± 2	753 ± 69
Thr	t	417 ± 51
Ser	15 ± 2	686 ± 35
Asn	648 ± 50	43,690 ± 1,383
Gln	62 ± 6	329 ± 33
Pro	t	1,554 ± 126
Glu	48 ± 3	4,194 ± 549
Gly	t	355 ± 41
Ala	t	2,248 ± 518
Val	t	367 ± 25
Cys	t	594 ± 50
Met	t	72 ± 7
Ile	t	246 ± 22
Leu	t	689 ± 56
Tyr	t	68 ± 9
Phenylalanine	t	148 ± 16
γ-Aminobutyric acid	51 ± 5	2,702 ± 425
Orn	t	71 ± 10
NH_3	1,533 ± 72	4,275 ± 216
Lys	t	231 ± 23
His	t	201 ± 7
Arg	t	227 ± 19

Time Course of ^{15}N Incorporation from $^{15}N_2$ into Various Nitrogenous Substances in Nodules. During the supply of $^{15}N_2$ to intact alfalfa nodules, label was found to increase steadily in various nitrogenous compounds in both bacteroid and cytosol fractions. NH_3 showed a high ^{15}N abundance in bacteroids, while in the cytosol, ^{15}N atom % excess was high in Asp, Glu, Gln, NH_3, and Asn (data not shown). This is consistent with the early finding (15) that NH_3 is the first product of N_2 fixation and that the assimilation of NH_3 takes place in the cytosol.

Within 5 min of labeling with $^{15}N_2$, Asn was the most predominantly labeled compound in the cytosol (Fig. 1). This pattern became even more evident after 30 min. ^{15}N appeared at one-tenth or less of that rate in NH_3, Glu, Asp, and Gln in the cytosol, suggesting that Asn can be synthesized very rapidly from newly fixed N_2 in the cytosol of nodules. The actual ^{15}N content of Gln was quite low due to its small pool size, and possibly rapid turnover. In the bacteroid fraction, NH_3 was the most rapidly labeled substance and it accumulated far more than labeled Asn and Glu. These results agree with enzymic studies (5–8, 13, 18–20, 22) showing that high levels of GS and GOGAT consistently operate in the cytosol fraction of various legumes, although they also have been detected in the bacteroid fraction at much lower levels (2). The accumulation rates of $^{15}NH_3$ in the bacteroids were apparently far lower than those of ^{15}N in various N compounds in the cytosols (Fig. 1), suggesting NH_3 produced from N_2 in the bacteroids is exported rapidly into the plant cytosols for further metabolism.

When nodulated roots were transferred to $^{14}N_2$ after 30 min in $^{15}N_2$ (Fig. 1), the levels of ^{15}N in NH_3, Glu, Asp, and Gln decreased immediately, while the rate of decrease in Asn was relatively slow. These results indicate that NH_3 is very rapidly metabolized and that Asn is the final compound which was synthesized and transported to the shoot.

Effect of Various Inhibitors on the Assimilation of N Compounds in Nodules. The amounts of major amino acids in detached nodules were very similar to those in intact nodules and the amounts were relatively unchanged in nodules treated with inhibitors other than MSO (data not shown). This indicated no major perturbations in the amino acid pool sizes of detached nodules during experimental periods. However, treatment with MSO caused a decrease in the level of Gln and increased that of NH_3.

$^{15}N_2$ and $^{15}NH_4^+$ Feeding. The flow of ^{15}N from $^{15}N_2$ or $^{15}NH_4^+$

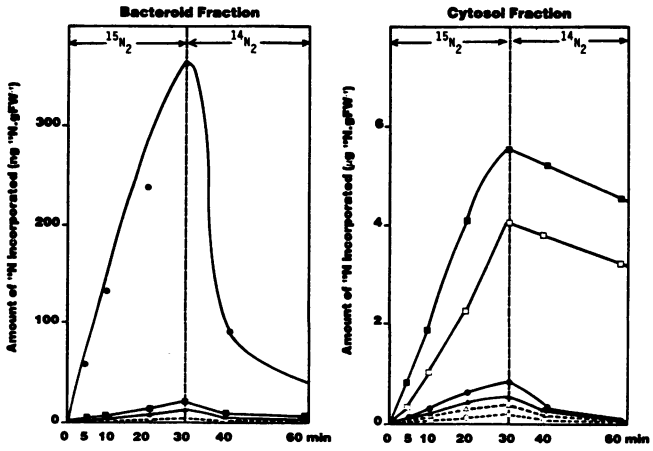

FIG. 1. Time course of ^{15}N flow from $^{15}N_2$ into various nitrogenous compounds in bacteroid and cytosol of alfalfa nodules attached to roots. (●—●), NH_3; (■—■), Asn-amide; (□—□), Asn-amino; (▲—▲), Asp; (Δ---Δ), Glu; (○---○), Gln-amide.

into various amino acids and amides of alfalfa nodules treated with MSO, AZA, or AOA is presented in Figures 2 and 3, respectively. $^{15}N_2$ was supplied equally to detached and attached nodules, but the total $^{15}N_2$ fixed was lower in detached nodules due to the known rapid decrease in nitrogenase activity after detachment from root system.

When nodules were treated with MSO (2 mM), an inhibitor of GS, the labeling of NH_3 dramatically increased and that of Gln decreased in nodules supplied with either $^{15}N_2$ or $^{15}NH_3$ (Figs. 2 and 3). This again indicates the important role of GS in NH_3 assimilation. On the other hand, label of the amide group of Asn was also depressed as the flow of ^{15}N into the same group of Gln

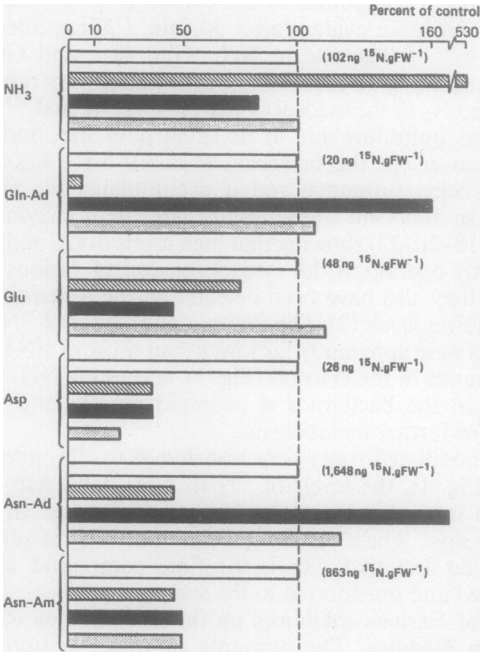

FIG. 2. Flow of ^{15}N from $^{15}N_2$ into various nitrogenous compounds in detached alfalfa nodules in the presence or absence of different inhibitors of N assimilation. (▭), Control; (▨), +MSO (2 mM); (▬), +AZA (2 mM); (▩), +AOA (4 mM). Ad and am denote amide and amino group, respectively. Numbers in parenthesis represent total actual amounts of ^{15}N content.

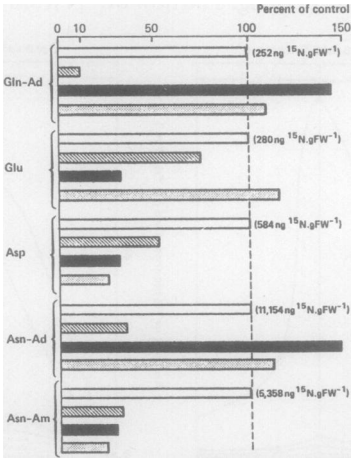

FIG. 3. Flow of ^{15}N from $^{15}NH_4^+$ into various nitrogenous compounds in detached alfalfa nodules infiltrated with $(^{15}NH_4)_2SO_4$ solution (2 mM). (▭), Control; (▨), +MSO (2 mM); (▬), +AZA (2 mM); (▩), +AOA (4 mM). Ad and am denote amide and amino group, respectively. Numbers in parenthesis represent total actual amounts of ^{15}N content.

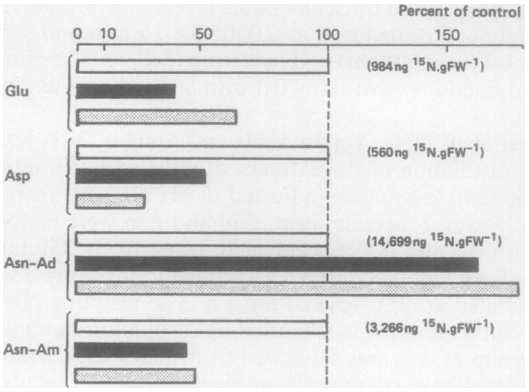

FIG. 4. Flow of ^{15}N from ^{15}N-amide of glutamine into various nitrogenous compounds in detached alfalfa nodules infiltrated with ^{15}N-amide of glutamine solution (2 mM). (▭), Control; (▬), +AZA (2 mM); (▢), +AOA (4 mM). Ad and am denote amide and amino group, respectively. Numbers in parenthesis represent total actual amounts of ^{15}N content.

declined. An interesting point should be noted here that in the presence of MSO, the flow of ^{15}N from both $^{15}N_2$ and $^{15}NH_4^+$ into the amide group of Gln was inhibited by 90%, but that into the same group of Asn was retarded by only 60%. This suggests that NH_3 directly contributed to the formation of Asn in alfalfa root nodules, although Gln-amide is the main N donor for amidation of Asp.

AZA (2 mM), an inhibitor of GOGAT, decreased the flow of ^{15}N from either $^{15}N_2$ or $(^{15}NH_4)_2SO_4$ into Glu and Asp and also depressed the label of the amino group of Asn (Figs. 2 and 3). This supports the conclusion of Groat and Vance (5, 6) that GS/GOGAT are the main enzymes involved in the assimilation of NH_3 in alfalfa nodules. Data from Figures 2 and 3 also showed a greater effect of MSO on the incorporation of ^{15}N from $^{15}N_2$ or $^{15}NH_4$ into the amide group of Gln (90% inhibition) than into Glu (40% inhibition) suggesting the presence of GDH in alfalfa nodules (5).

When AOA (4 mM) was used to stop the transamination reaction there was no change in the ^{15}N abundance of Glu, Gln-amide, and Asn-amide while that in Asp and Asn-amino was decreased. In other words, the labeling pattern of the amino group of Asn, followed a trend similar to that of Asp.

(^{15}N-Amide)Gln Feeding. When alfalfa nodules were supplied with (^{15}N-amide)Gln (Fig. 4), the label was rapidly incorporated into the same group of Asn. This is consistent with the role of asparagine synthetase in the formation of Asn by Gln-dependent amidation of Asp. Fujihara and Yamaguchi (4) also reported high efficiency of use of the Gln-amide as N donor for Asn synthesis when soybean nodules were fed with (^{15}N-amide)Gln. In alfalfa nodules, the label from (^{15}N-amide)Gln was also detected in the amino group of Asn, Glu, and Asp. Again, treatment with either AZA or AOA resulted in a decrease of the label in all these amino acids. This supports the conclusion of an active operation of GOGAT and transaminase in the N assimilation.

In summary, *in vivo* studies using labeled $^{15}N_2$ and its intermediate metabolites in combination with different inhibitors of N metabolism have shown that NH_3 is the first product of N_2 fixation in the bacteroids of alfalfa nodules. This compound is then rapidly exported to the plant cytosols, where it is assimilated mainly via the GS/GOGAT cycle to form Gln and Glu. Besides these two key enzymes for the primary assimilation of NH_3, GDH appears to operate in alfalfa nodules. Transamination of Glu to a range of amino acids, including Asp, provides further metabolism of Glu. Synthesis of Asn, the major N transport compound in alfalfa plants occurs by amidation of Asp, where both Gln-amide and NH_3 act as N donors. Considering the proportions of ^{15}N incorporated into Gln-amide and Asn-amide

from $^{15}N_2$ and $^{15}NH_4^+$ in the presence and absence of MSO, about 35% of the Asn may be synthesized by NH_3-dependent amidation of Asp.

Acknowledgments—We thank Dr. K. W. Joy for helpful discussion, Drs. R. W. Miller, C. Preston, and Mr. D. Tutte for their cooperation in the use of equipment for ^{15}N and amino acid analyses.

LITERATURE CITED

1. BERGENSEN FJ 1965 Ammonia—an early stable product of nitrogen fixation by soybean root nodules. Aust J Biol Sci 18: 1–9
2. DUNN SD, RK KLUCAS 1976 Studies on possible routes of ammonia assimilation in soybean root nodule bacteroids. Can J Microbiol 19: 1493–1499
3. FUJIHARA S, M YAMAGUCHI 1981 Assimilation of $^{15}NH_3$ by root nodules detached from soybean plants. Plant Cell Physiol 22: 797–806
4. FUJIHARA S, M YAMAGUCHI 1980 Asparagine formation in soybean nodules. Plant Physiol 66: 139–141
5. GROAT RG, CP VANCE 1981 Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Plant Physiol 67: 1198–1203
6. GROAT RG, CP VANCE 1982 Root and nodule enzymes of ammonia assimilation in two plant-conditioned symbiotically ineffective genotypes of alfalfa (*Medicago sativa* L.). Plant Physiol 69: 614–618
7. HENSON CA, M COLLINS, SH DUKE 1982 Subcellular localization of enzymes of carbon and nitrogen metabolism in nodules of *Medicago sativa*. Plant Cell Physiol 23: 227–235
8. HUBER TA, J STREETER 1984 Asparagine biosynthesis in soybean nodules. Plant Physiol 74: 605–610
9. KENNEDY IR 1966 The probable site of nitrogen fixation in root nodules of *Ornithopus sativus*. Biochim Biophys Acta 130: 517–519
10. KENNEDY IR 1966 Primary products of symbiotic nitrogen fixation. I. Short term exposures of *Serradella* nodules to $^{15}N_2$. Biochim Biophys Acta 130: 285–294
11. KENNEDY IR 1966 Primary products of symbiotic nitrogen fixation. II. Pulse-labelling of *Serradella* nodules with $^{15}N_2$. Biochim Biophys Acta 130: 295–303
12. MATSUMOTO T, M YATAZAWA, Y YAMAMOTO 1977 Incorporation of ^{15}N into allantoin in nodulated soybean plants supplied with $^{15}N_2$. Plant Cell Physiol 18: 459–462
13. MCCORMACK DK, KJ FARDEN, MJ BOLAND 1982 Purification and properties of glutamine synthetase from the plant cytosol fraction of lupin nodules. Arch Biochem Biophys 218: 561–571
14. OHYAMA T, K KUMAZAWA 1978 Incorporation of ^{15}N into various nitrogenous compounds in intact soybean nodules after exposure to $^{15}N_2$ gas. Soil Sci Plant Nutr 24: 525–533
15. OHYAMA T, K KUMAZAWA 1980 Nitrogen assimilation in soybean nodules. I. The role of GS/GOGAT system in the assimilation of ammonia produced by N_2 fixation. Soil Sci Plant Nutr 26: 109–115
16. OHYAMA T, K KUMAZAWA 1980 Nitrogen assimilation in soybean nodules. II. $^{15}N_2$ assimilation in bacteroid and cytosol fractions of soybean nodules. Soil Sci Plant Nutr 26: 205–213
17. RAWSTHORNE S, ER MINCHIN, RJ SUMMERFIELD, C COOKSON, J COOMBS 1980 Carbon and nitrogen metabolism in legume root nodules. Phytochemistry 19: 341–355
18. REYNOLDS HSP, DG BLEVINS, MJ BOLAND, KR SCHUBERT, DD RANDALL 1982 Enzymes of ammonia assimilation in legume nodules: a comparison between ureide- and amide-transporting plants. Physiol Plant 55: 255–260
19. ROGNES SE 1975 Glutamine-dependent asparagine synthetase from *Lupinus luteus*. Phytochemistry 14: 1975–1982
20. SCOTT DB, KJF FARDEN 1976 Ammonia assimilation in lupin nodules. Nature 263: 703–704
21. SHELP BJ, CA ATKINS 1984 Subcellular location of enzymes of ammonia assimilation and asparagine synthesis in root nodules of *Lupinus albus* L. Plant Sci Lett 36: 225–230
22. STREETER JG 1973 *In vivo* and *in vitro* studies on asparagine biosynthesis in soybean seedlings. Arch Biochem Biophys 157: 613–624
23. TA TC, KW JOY, RJ IRELAND 1984 Amino acids metabolism in pea leaves. Utilization of both amino and amide group of [^{15}N]asparagine. Plant Physiol 74: 822–826