Pathways of Nitrogen Metabolism in Nodules of Alfalfa (Medicago sativa L.)

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ABSTRACT
Exposure of intact alfalfa nodules to \( ^{15} \text{N} \) showed that in bacteroids the greatest flow of \( ^{15} \text{N} \) 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 \( ^{15} \text{N} \) was incorporated into Asn than into other compounds. Detached nodules were also used to study the metabolic pathway of N assimilation after exposure to \( ^{15} \text{N} \) or vacuum infiltration with \( \left( {^{15} \text{N}} \right) \text{SO}_4 \). In the presence or absence of different inhibitors of nitrogen assimilation: methionine sulfoximine (MSO), azaserine (AZA), or amino-oxycacetate (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. Azs, which inhibits the formation of Gln from Glu 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 \( ^{15} \text{N} \) 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 \( ^{15} \text{N} \)-labeled amide group of glutamine. Data from \( ^{15} \text{NH}_4^+ \) 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 enzymatic 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 amminated 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 \( ^{15} \text{N} \) tracer technique reported the predominant 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 \( ^{15} \text{N} \) tracer techniques.

The objective of this work was to examine the pattern of \( ^{15} \text{N} \) incorporation into various nitrogenous compounds in alfalfa nodules exposed to \( ^{15} \text{N} \) or supplied with \( ^{15} \text{NH}_4^+ \) and (\( ^{15} \text{N}-\text{amide} \)) 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 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \))/dark cycle at 25/20°C and 70% RH. Nodules from 6-week old plants (early bloom stage) were exposed to \( ^{15} \text{N}_2 \), \( ^{15} \text{NH}_4^+ \) or (\( ^{15} \text{N}-\text{amide} \)) glutamine.

Reagents. Labeled (\( ^{15} \text{NH}_4 \))SO\(_4 \), (\( ^{15} \text{N}-\text{amide} \)) glutamine and \( ^{15} \text{N} \) gas were purchased from MSD isotopes, Montreal at 99 atom % excess. Other chemicals were obtained from Sigma.

\( ^{15} \text{N} \) 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 placed in one hole, another hole held a serum stopper for \( ^{15} \text{N} \) gas injection and the remaining hole was connected to a vacuum system. When \( ^{15} \text{N}_2 \) gas was used, an excess (200 ml for 30 min exposure) mixture of \( ^{15} \text{N}_2 \cdot \text{O}_2 \cdot \text{Ar} (20:20:60) \) 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. \( ^{15} \text{N}_2 \) gas or a solution of either 2 mm (\( ^{15} \text{NH}_4 \))SO\(_4 \) or 2 mm (\( ^{15} \text{N}-\text{amide} \)) glutamine was then injected into each vial. Twenty min after introduction of \( ^{15} \text{N} \), the nodules were washed several times with distilled H\(_2\)O 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 \( ^{15} \text{N} \) 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 \( ^{15} \text{N}_2 \), attached nodules were sampled and macerated at 4°C in 0.2 M sorbitol solution. The

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2. Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; MSO, methionine sulfoximine; AZA, azaserine; AOA, amino-oxycacetate; GDH, glutamate dehydrogenase; AS, asparagine synthetase.
macerate was filtered through double layers of nylon net (25 μm). The filtrate was centrifuged at 6,000 g 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 NH₃, Amino Acids and Amidases. NH₃ was separated by distillation of the extract, 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 NH₃ 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 NH₃ 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. ¹⁵N 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 Amidases in Alfalfa Nodules. Table I shows the composition of free nitrogenous compounds in both cytosol and bacteroid fractions of alfalfa nodules. NH₃ was the predominant compound found in the bacteroids; however, in the plant nodule cytosol Asn was present in the highest concentration followed by NH₃, Glu, α-aminobutyric acid, Ala, Asp, and Ser. Ureides were not detected in either fraction.

Time Course of ¹⁵N Incorporation from ¹⁵N₂ into Various Nitrogenous Substances in Nodules. During the supply of ¹⁵N₂ to intact alfalfa nodules, label was found to increase steadily in various nitrogenous compounds in both bacteroid and cytosol fractions. NH₃ showed a high ¹⁵N abundance in bacteroids, while in the cytosol, ¹⁵N atom % excess was high in Asp, Glu, Gln, NH₃, and Asn (data not shown). This is consistent with the early finding (15) that NH₃ is the first product of N₂ fixation and that the assimilation of NH₃ takes place in the cytosol.

Within 5 min of labeling with ¹⁵N₂, Asn was the most predominantly labeled compound in the cytosol (Fig. 1). This pattern became even more evident after 30 min. ¹⁵N appeared at one-tenth or less of that rate in NH₃, Glu, Asp, and Gln in the cytosol, suggesting that Asn can be synthesized very rapidly from newly fixed N₂ in the cytosol of nodules. The actual ¹⁵N content of Gln was quite low due to its small pool size, and possibly rapid turnover. In the bacteroid fraction, NH₃ 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 ¹⁵NH₃ in the bacteroids were apparently far lower than those of ¹⁵N in various N compounds in the cytosols (Fig. 1), suggesting NH₃ produced from N₂ in the bacteroids is exported rapidly into the plant cytosols for further metabolism.

When nodulated roots were transferred to ¹⁴N₂ after 30 min in ¹⁵N₂ (Fig. 1), the levels of ¹⁵N in NH₃, Glu, Asp, and Gln decreased immediately, while the rate of decrease in Asn was relatively slow. These results indicate that NH₃ 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 NH₃.

¹⁵N₂ and ¹⁵NH₄⁺ Feeding. The flow of ¹⁵N from ¹⁵N₂ or ¹⁵NH₄⁺ into various nitrogenous compounds in bacteroid and cytosol of alfalfa nodules attached to roots.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Bacteroid</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmole·g nodule fresh weight⁻¹</td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>27 ± 2</td>
<td>753 ± 69</td>
</tr>
<tr>
<td>Thr</td>
<td>1 ± 0</td>
<td>417 ± 51</td>
</tr>
<tr>
<td>Ser</td>
<td>15 ± 2</td>
<td>686 ± 35</td>
</tr>
<tr>
<td>Asn</td>
<td>648 ± 50</td>
<td>43,690 ± 1,383</td>
</tr>
<tr>
<td>Gln</td>
<td>62 ± 6</td>
<td>329 ± 33</td>
</tr>
<tr>
<td>Pro</td>
<td>1,554 ± 126</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>48 ± 3</td>
<td>4,194 ± 549</td>
</tr>
<tr>
<td>Gly</td>
<td>355 ± 41</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2,248 ± 518</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>367 ± 25</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>594 ± 50</td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>72 ± 7</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>246 ± 22</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>689 ± 56</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>68 ± 9</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>148 ± 16</td>
<td></td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>51 ± 5</td>
<td>2,702 ± 425</td>
</tr>
<tr>
<td>Orn</td>
<td>1 ± 0</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>NH₃</td>
<td>1,533 ± 72</td>
<td>4,275 ± 216</td>
</tr>
<tr>
<td>Lys</td>
<td>231 ± 23</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>201 ± 7</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>227 ± 19</td>
<td></td>
</tr>
</tbody>
</table>
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 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$_3$ 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$_3$)$_2$SO$_4$ 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$_3$ 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.

**(15N-Amide)Gln Feeding.** When alfalfa nodules were supplied with (15N-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 (15N-amide)Gln. In alfalfa nodules, the label from (15N-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.

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**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). (c), Control; (m), +MSO (2 mM); (M), +AZA (2 mM); (E), +AOA (4 mM). Ad and am denote amide and amino group, respectively. Numbers in parenthesis represent total actual amounts of $^{15}$N content.

**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. (c), Control; (m), +MSO (2 mM); (M), +AZA (2 mM); (E), +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$_3$ into various nitrogenous compounds in detached alfalfa nodules infiltrated with ($^{15}$NH$_3$)$_2$SO$_4$ solution (2 mM). (c), Control; (m), +MSO (2 mM); (M), +AZA (2 mM); (E), +AOA (4 mM). Ad and am denote amide and amino group, respectively. Numbers in parenthesis represent total actual amounts of $^{15}$N content.
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.

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**LITERATURE CITED**

11. Kennedy IR 1966 Primary products of symbiotic nitrogen fixation. II. Pulse-labeling of Serratella nodules with $^{15}$N$_2$. Biochim Biophys Acta 130: 295–303