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Asparagine Biosynthesis in Alfalfa (*Medicago sativa* L.) Root Nodules[1]

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ABSTRACT

Rapid direct conversion of exogenously supplied [^{14}C]aspartate to [^{14}C] asparagine and to tricarboxylic cycle acids was observed in alfalfa (*Medicago sativa* L.) nodules. Aspartate aminotransferase activity readily converted carbon from exogenously applied [^{14}C]aspartate into the tricarboxylic acid cycle with subsequent conversion to the organic acids malate, succinate, and fumarate. Aminooxyacetate, an inhibitor of aminotransferase activity, reduced the flow of carbon from [^{14}C]aspartate into tricarboxylic cycle acids and decreased $^{14}CO_2$ evolution by 99%. Concurrently, maximum conversion of aspartate to asparagine was observed in aminooxyacetate treated nodules (30 nanomoles asparagine per gram fresh weight per hour. Metabolism of [^{14}C]aspartate and distribution of nodule-fixed $^{14}CO_2$ suggest that two pools of aspartate occur in alfalfa nodules: (a) one involved in asparagine biosynthesis, and (b) another supplying a malate/aspartate shuttle. Conversion of [^{14}C]aspartate to [^{14}C]asparagine was not inhibited by methionine sulfoximine, a glutamine synthetase inhibitor, or azaserine, a glutmate synthetase, inhibitor. The data did not indicate that asparagine biosynthesis in alfalfa nodules has an absolute requirement for glutamine. Radioactivity in the xylem sap, derived from nodule $^{14}CO_2$ fixation, was markedly decreased by treating nodulated roots with aminooxyacetate, methionine sulfoximine, and azaserine. Inhibitors decreased the [^{14}C]aspartate and [^{14}]asparagine content of xylem sap by greater than 80% and reduced the total amino nitrogen content of xylem sap (including nonradiolabeled amino acids) by 50 to 80%. Asparagine biosynthesis in alfalfa nodules and transport in xylem sap are dependent upon continued aminotransferase activity and an uninterrupted assimilation of ammonia via the glutamine synthetase/glutamate synthase pathway. Continued assimilation of ammonia apparently appears crucial to continued root nodule CO_2 fixation in alfalfa.

Asparagine biosynthesis and transport have pivotal roles in assimilation and utilization of symbiotically fixed N_2 (2, 3, 6). Asparagine is the dominant amino acids in nodules and xylem sap of lupine (*Lupinus subcarnosus* L.) (2), alfalfa (*Medicago sativa* L.) (16), and pea (*Pisum sativum* L.) (28). Although ureides are the major N transport product in soybeans (Glycine max L. Merr.), substantial quantities of asparagine were detected in soybean nodules and in the xylem sap of nodulated soybeans (26). Glutamine-dependent AS[2] (EC 6.3.5.4) in nodules was demonstrated with *in vivo* studies utilizing metabolic inhibitors, with *in vitro* enzyme analysis (3, 9, 22), and has been purified from soybean (10). Recent data indicate that both NH_3- and glutamine-dependent asparagine synthesis occurs in alfalfa nodules (27). However, the regulation of carbon skeletons into asparagine biosynthesis remains unclear (15, 17, 24, 25).

In alfalfa, birdsfoot trefoil (*Lotus corniculatus* L.) and soybean, radioactive asparagine was readily formed when excised nodules were exposed to $^{14}CO_2$ (9, 16). Radiolabeled asparagine and aspartate were transported in the xylem sap of nodulated root systems of alfalfa and birdsfoot trefoil exposed to $^{14}CO_2$ (16, 29). In ineffectively nodulated plants, or when nodules were removed from plants, little radiolabeled asparagine and aspartate were detected in xylem sap. These results suggest that oxaloacetate formed from dark CO_2 fixation by nodule PEPC [EC 4.1.1.31] is transaminated by AAT [EC 2.6.1.1] to aspartate. Aspartate is then converted to asparagine by AS. However, *in vivo* conversion of exogenous [^{14}C]aspartate to asparagine has been difficult to demonstrate (9, 25).

Similarly, Macnicol (15) showed that [^{14}]aspartate was not incorporated into asparagine after 5 h of labeling in developing pea cotyledons. Low conversion of [^{14}C]aspartate to asparagine has also been noted in soybean seedlings (24).

Rapid metabolism of aspartate to tricarboxylic cycle acids may contribute to the apparently low conversion of [^{14}C]aspartate to [^{14}C]asparagine (14, 15, 17, 24). Since aspartate enter the tricarboxylic acid cycle as oxaloacetate via AAT, treatments that inhibit AAT activity may increase conversion of aspartate to asparagine. Joy *et al.* (11) demonstrated that addition of AOA, an aminotransferase inhibitor, decreased the flow of [^{14}C]aspartate radiolabel to organic acids and enhanced ^{14}C-labeling of asparagine in pea leaves.

The inhibitors MSO and AZA have also been useful in evaluating the pathway for ammonia assimilation into asparagine (9, 23). MSO is a GS inhibitor (8) and AZA is a glutamine analog that inhibits GOGAT (18). Conversion of aspartate to asparagine was inhibited in soybean leaves infiltrated with either MSO or AZA during 60 and 90 min labeling periods (23). Recent studies with soybean nodules showed that infiltration with MSO and AZA decreased nodule CO_2 fixation and decreased the recovery of label derived from $^{14}CO_2$ in aspartate and asparagine (9).

Although indirect evidence suggests that the pathway for asparagine biosynthesis in nodules involves aspartate as a substrate, little direct conversion of exogenous aspartate to asparagine has been demonstrated. The objectives of this research were to: (a) determine if rapid direct conversion of [^{14}C]aspartate to [^{14}C] asparagine occurs in alfalfa nodules; (b) assess if AOA blocks aspartate carbon flow into tricarboxylic cycle acids and its effects on asparagine biosynthesis; (c) assess the effects of MSO and AZA on asparagine biosynthesis; and (d) determine if the move-

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[2] Abbreviations: AS, asparagine synthetase; AAT, aspartate aminotransferase; AOA, aminooxyacetate; AZA, azaserine; GOGAT, glutamate synthase; GS, glutamine synthetase; MSO, methionine sulfoximine; PEPC, phosphoenolpyruvate carboxylase.

ment of carbon from nodule CO_2 fixation into aspartate and asparagine is altered by AOA, MSO, and AZA.

MATERIALS AND METHODS

Plant Material and Culture. Alfalfa (*Medicago sativa* L., cv Saranac) seedlings were grown in glasshouse sandbenches under supplemental light and nutrient conditions as previously described (30). Sand was inoculated at the time of seeding with *Rhizobium meliloti* strain 102F51 (Nitragin Co., Milwaukee, WI[3]). Hydroponically grown plants were initiated and maintained as described by Maxwell *et al.* (16). All plant material was used at bud to midbloom stage.

^{14}C-Labeling Studies. *In vivo* [^{14}C]aspartate labeling was performed on nodules excised from plants grown in sandbenches. Before exposure to [^{14}C]aspartate, nodules (100 mg fresh weight) were vacuum infiltrated for three (1 min) periods in either water (control) or 5 mM inhibitor (AOA, MSO, or AZA). AOA inhibits AAT activity (11), while MSO and AZA block ammonia assimilation (8, 18). Infiltrated nodules were sliced in half and placed cut face down on filter paper, premoistened with water or inhibitor, at the bottom of a 7.5 ml plexiglass reaction vessel. The sealed vessel was equipped with stainless steel inlet and outlet ports to facilitate air circulation with a circulatory pump (model MB-41, Metal Bellows, Sharon, MS). Labeling was initiated by adding 2 μCi of [U-^{14}C]-L-aspartic acid (200 mCi mmol^{-1}, ICN, Irvine, CA) directly to the nodules on the moistened filter paper. $^{14}CO_2$ evolved during the incubation period was collected by gently flushing the chamber for 45 s at 10 min intervals. Nodules remained moist throughout the experiment. The $^{14}CO_2$ evolved was trapped by bubbling the flushed air through Carbo-Sorb II (Packard Instrument Company, Inc.) and then radioactivity was determined by liquid scintillation spectroscopy. After incubations of 20 and 60 min at 23°C, nodules were quickly placed on a Büchner funnel and rinsed three times with distilled H_2O to remove excess [^{14}C]aspartate. The reaction was terminated by homogenizing the nodules in hot 50% (v/v) ethanol, followed by extraction in a 45°C water bath for 20 min and centrifugation at 18,100*g* for 15 min. Radioactivity in an aliquot of the supernatant was determined by liquid scintillation spectroscopy.

Procedures for the *in vivo* $^{14}CO_2$ labeling study were modified from Maxwell *et al.* (16). Excised alfalfa nodules (100 mg fresh weight) were vacuum infiltrated three times with water (control) or 5 mM inhibitor (AOA, MSO, or AZA) and were then placed in the bottom of a 13-ml reaction flask (Kontes) on filter paper premoistened with distilled H_2O or inhibitor. The reaction flask was sealed at the side arm and the top with rubber serum stoppers. The top serum stopper held a plastic well suspended above the nodules, inside the flask. The well contained 8 μCi of aqueous NaH$^{14}CO_3$ (50 mCi mmol^{-1}, ICN). Labeling was initiated by injecting 4 M lactic acid through the serum stopper into the center well, releasing $^{14}CO_2$. After incubation for 20 min at 23°C, the reaction was terminated by injection of 1.5 ml of hot 50% (v/v) ethanol onto the nodules. Flasks were opened to release any unreacted $^{14}CO_2$. Nodules were extracted as described for the [^{14}C]aspartate labeling studies.

Xylem sap was radiolabeled and collected from plants as described by Maxwell *et al.* (16). The only modification was the inclusion of 1 mM inhibitor (AOA, MSO, or AZA) treatments added to the nutrient solution wetting the root systems over the 2-h labeling period.

Analysis of Products of ^{14}C-Labeling Studies. Xylem sap exudates and nodule extracts were separated into neutral, basic (amino acid), and acidic (organic acid) fractions by Dowex ion exchange chromatography (16). ^{14}C-Labeled amino and organic acid fractions were separated into individual compounds by TLC (19). TLC plates were autoradiographed to detect radiolabeled amino and organic acids, which were then eluted from the plates. The associated radioactivity was quantified by liquid scintillation spectroscopy (16).

RESULTS

[^{14}]Aspartate Metabolism. Nodule-associated radioactivity derived from exogenously added [^{14}C]aspartate was relatively similar for all treatments except AOA and AZA at 20 min (Table I). Aspartate was completely metabolized to CO_2 as evidenced by the evolution of $^{14}CO_2$. Comparable amounts of $^{14}CO_2$ were evolved from control, MSO, and AZA-treated nodules at both 20 and 60 min. In contrast, $^{14}CO_2$ evolution by AOA-treated nodules was reduced by 99 and 91% after 20 and 60 min, respectively. Radioactivity in AZA- and MSO-treated and control nodules was distributed approximately 70% in the acid fraction, 28% in the basic fraction, and 2% or less in the neutral fraction. However, in AOA-treated nodules after 20 min, only 9% of the label was in the acid fraction, with 90% in the basic fraction. After 60 min, 45% of the label in AOA-treated nodules was in the acid fraction and over 50% was in the basic fraction.

Further analysis of the basic fraction showed that for all treatments [^{14}C]aspartate was primarily metabolized to asparagine, glutamate, alanine, glutamine, and two unknown compounds (Table II). Radioactivity in the basic fraction of control, MSO, and AZA-treated nodules was comparable after 20 and 60 min. Nodules treated with AOA had about double the radioactivity in the basic fraction than other treatments at both 20 and 60 min. The majority of the label in the basic fraction of AOA-treated nodules was in asparagine. Within 20 min [^{14}C]asparagine was readily formed from [^{14}C]aspartate in all treatments. Maximum [^{14}C]asparagine formation occurred in AOA-treated nodules and corresponded to a 15-fold increase over that found in 20 min control nodules. Furthermore, in AOA-treated nodules initial (20 min) radioactivity in glutamate, glutamine, alanine, and unknown 2 was reduced compared to control nodules, while radioactivity in unknown 1 was higher. MSO initially stimulated asparagine formation accompanied by decreased label in glutamate, glutamine, and unknown 1. After 60 min, MSO-treated nodules had slightly reduced label in asparagine and glutamine as compared to 60 min control nodules. AZA-treated nodules (20 min) had more label in glutamine and unknown 1 than did control nodules. After 60 min distribution of label in amino acids was similar in AZA-treated control nodules.

The primary organic acids labeled when excised nodules were exposed to [^{14}C]aspartate were malate, succinate, and fumarate (Table III). Within 20 min of exposure to AOA, incorporation of label into malate, succinate, and fumarate was reduced 90, 85, and 72%, respectively. Exposure to AOA not only reduced the amount of [^{14}C]aspartate metabolized into tricarboxylic acids but also altered the distribution of label in organic acids. After 20 min of exposure to AOA, acid fraction radioactivity was distributed approximately 60, 11, and 26% in malate, succinate, and fumarate, respectively. By contrast, in all other 20 min treatments radioactivity was distributed approximately 75, 10, and 15% in malate, succinate, and fumarate, respectively. After 60 min, radioactivity increased 100% in succinate, while radioactivity in fumarate decreased approximately 70% for AOA- and AZA-treated nodules and control nodules. Radioactivity associated with fumarate decreased by 70% in MSO-treated nodules after 60 min, while radioactivity associated with succinate remained constant for 20 and 60 min MSO treatments.

$^{14}CO_2$-Labeling Experiments. Short term $^{14}CO_2$ fixation was

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Table I. *Total Nodule-Associated Radioactivity Derived from Metabolism of [^{14}C]Aspartate, Distribution of Radioactivity in the Neutral, Acid, and Basic Fractions, and $^{14}CO_2$ Evolved from Excised Alfalfa Nodules*

The nodules (100 mg) were vacuum infiltrated with 5 mM AOA, MSO, AZA, or water (control) and incubated with 2 μCi [^{14}C]aspartate for 20 or 60 min. Each value is the mean ± SE of at least three replicates.

Treatment Time	Nodule Associated Radioactivity	$^{14}CO_2$ Evolved	Neutral	Acid	Basic
min	*dpm × 10^{-3}*		*% of nodule ^{14}C*		
Control					
20	62 ± 4	54 ± 19	2.0 ± 0.2	70.7 ± 2.7	27.3 ± 2.7
60	71 ± 16	226 ± 42	1.4 ± 0.2	60.7 ± 2.1	38.3 ± 2.2
AOA					
20	44 ± 10	1 ± 0	0.4 ± 0.1	9.1 ± 3.0	90.5 ± 2.9
60	88 ± 7	20 ± 1	0.6 ± 0.1	45.7 ± 6.4	53.7 ± 6.5
MSO					
20	63 ± 13	28 ± 5	1.8 ± 0.5	69.9 ± 3.3	28.3 ± 3.2
60	85 ± 16	251 ± 62	1.8 ± 0.5	69.3 ± 3.4	29.1 ± 2.9
AZA					
20	80 ± 4	42 ± 1	1.5 ± 0.2	70.6 ± 2.9	28.0 ± 3.0
60	85 ± 6	239 ± 26	2.3 ± 0.8	78.6 ± 3.8	19.1 ± 4.4

Table II. *Metabolism of [^{14}C]Aspartate into Asparagine, Glutamate, Glutamine, Alanine, Unknown 1 and Unknown 2 by Excised Alfalfa Nodules*

The nodules (100 mg) were vacuum infiltrated with 5 mM, AOA, MSO, AZA, or water (control) and incubated with 2 μCi [^{14}C]aspartate for 20 or 60 min. Each value is the mean ± SE of at least three replicates.

Treatment Time	Basic Fraction	Asparagine	Glutamate	Glutamine	Alanine	Unknown 1	Unknown 2
min	*dpm × 10^{-3}*			*% of ^{14}C in basic fraction*			
Control							
20	17 ± 2	15.1 ± 1.6	22.8 ± 0.6	5.3 ± 0.4	13.1 ± 2.0	13.2 ± 1.8	30.0 ± 2.2
60	27 ± 2	15.3 ± 2.2	28.7 ± 3.6	5.3 ± 0.4	19.5 ± 5.9	8.1 ± 1.0	22.9 ± 1.7
AOA							
20	40 ± 1	58.6 ± 3.9	3.8 ± 0.8	3.4 ± 0.5	2.8 ± 0.4	24.1 ± 2.5	6.1 ± 0.3
60	47 ± 6	54.5 ± 2.9	13.4 ± 1.6	2.9 ± 0.2	7.3 ± 1.9	12.0 ± 1.7	9.9 ± 0.5
MSO							
20	18 ± 2	25.7 ± 3.6	16.4 ± 1.1	3.2 ± 1.3	10.5 ± 1.3	9.3 ± 0.5	34.2 ± 3.6
60	25 ± 3	11.5 ± 2.8	28.5 ± 4.6	2.7 ± 0.4	23.0 ± 2.3	8.7 ± 1.3	25.7 ± 3.9
AZA							
20	22 ± 2	16.7 ± 0.9	13.6 ± 1.6	11.6 ± 0.8	10.4 ± 0.7	23.7 ± 3.4	24.1 ± 1.6
60	16 ± 4	21.2 ± 4.0	23.9 ± 1.7	12.2 ± 1.5	14.5 ± 2.5	8.9 ± 1.7	19.7 ± 0.7

Table III. *Acid Fraction Radioactivity and Distribution of Radioactivity in Malate, Succinate, and Fumarate from Excised Alfalfa Nodules*

The nodules (100 mg) incubated with 5 mM AOA, MSO, AZA, or water (control) and 2 μCi [^{14}C]aspartate for 20 or 60 min. Each value is the mean ± SE of at least three replicates.

Treatment Time	Acid Fraction Radioactivity	Malate	Succinate	Fumarate
min	*dpm × 10^{-3}*		*% of ^{14}C in acid fraction*	
Control				
20	44 ± 2	78.3 ± 2.5	9.0 ± 1.2	12.4 ± 1.6
60	43 ± 2	76.2 ± 1.7	20.4 ± 1.5	3.3 ± 0.2
AOA				
20	4 ± 1	59.5 ± 2.9	11.2 ± 2.5	26.0 ± 0.2
60	40 ± 6	74.0 ± 6.6	22.7 ± 6.2	3.4 ± 0.9
MSO				
20	44 ± 2	66.5 ± 3.6	14.1 ± 1.2	18.7 ± 2.9
60	59 ± 3	80.6 ± 1.9	13.2 ± 1.4	6.3 ± 1.1
AZA				
20	56 ± 2	82.0 ± 1.3	7.3 ± 0.8	10.5 ± 1.1
60	66 ± 3	76.2 ± 3.7	20.6 ± 3.9	3.2 ± 0.2

Table IV. *Total Radioactivity, Specific Activity of Xylem Sap and Distribution of Radioactivity in the Neutral, Acid, and Basic Fractions of Attached Nodules and Xylem sap in alfalfa root systems*

The roots were incubated for 2 h with 80 μCi $^{14}CO_2$ and 1 mM AOA, MSO, AZA, or no inhibitor (control). Each value is the mean ± SE of at least three replicates.

Treatment	Total Radioactivity	Specific Activity	Neutral	Acid	Basic
	dpm × 10^{-3}	*dpm*/μ*l*		% of ^{14}C	
Control					
Xylem sap	72 ± 13	215 ± 41	0.2 ± 0.1	32.6 ± 4.1	67.5 ± 4.0
Attached nod	87 ± 26		2.4 ± 0.3	25.1 ± 4.5	72.5 ± 4.4
AOA					
Xylem sap	19 ± 6	72 ± 20	0.3 ± 0.1	44.0 ± 6.1	55.8 ± 6.1
Attached nod	57 ± 27		1.3 ± 0.1	33.9 ± 10.5	64.8 ± 10.6
MSO					
Xylem sap	11 ± 5	49 ± 16	0.3 ± 0.3	50.9 ± 5.4	48.9 ± 5.5
Attached nod	10 ± 6		1.1 ± 1.0	41.4 ± 7.6	57.6 ± 6.8
AZA					
Xylem sap	20 ± 13	44 ± 20	0.1 ± 0.2	52.5 ± 5.3	47.5 ± 5.3
Attached nod	18 ± 10		1.7 ± 0.9	51.0 ± 6.8	47.3 ± 6.1

Table V. *Total Amino Nitrogen Distribution of Radioactivity in Amino Acids of the Basic Fraction of Xylem Sap from Alfalfa Nodules Attached to a Root System*

The root system was incubated for 2 h with 80 μCi $^{14}CO_2$ and 1 mM AOA, MSO, AZA, or no inhibitor (control). Each value is the mean ± of at least three replicates.

Treatment	Total Amino Nitrogen	Aspartate	Asparagine	Glutamate	Glutamine	Alanine
	μg			*dpm* × 10^{-3}		
Control						
Xylem sap	294 ± 17	36.0 ± 1.0 (74)[a]	10.4 ± 1.4 (21)	0.6 ± 0.1 (1)	1.5 ± 0.1 (3)	0.6 ± 0.1 (1)
AOA						
Xylem sap	152 ± 34	4.9 ± 0.7 (44)	4.7 ± 0.9 (42)	0.4 ± 0.1 (4)	0.7 ± 0.4 (6)	0.4 ± 0.2 (4)
MSO						
Xylem sap	165 ± 36	2.6 ± 0.4 (46)	1.4 ± 0.3 (25)	0.7 ± 0.4 (13)	0.4 ± 0.1 (7)	0.5 ± 0.2 (9)
AZA						
Xylem sap	74 ± 20	5.6 ± 0.9 (59)	2.7 ± 0.8 (28)	0.2 ± 0.1 (2)	0.7 ± 0.2 (7)	0.3 ± 0.1 (3)

[a] Number in parentheses indicates percent total basic fraction label in specific compound.

not affected by AOA, MSO, or AZA. Similar to previous studies (16, 29) excised nodule CO_2 fixation rates of all treatments averaged 5.2 ± 1.0 nmol·min^{-1} g^{-1} fresh weight. The distribution of nodule-fixed ^{14}C was similar to that reported previously (16) with the acid, basic, and neutral fractions containing 79, 20, and less than 1%, respectively.

By contrast, long-term exposure of intact nodulated roots to AOA, MSO, or AZA inhibited nodule CO_2 fixation (Table IV, see attached nod). Nodule-associated radioactivity in AOA-, MSO-, and AZA-treated root systems was reduced 35, 89, and 80%, respectively. The distribution of nodule-associated radioactivity in neutral, acid, and basic fractions averaged over treatments was 2, 38, and 60%, respectively (data not shown).

Xylem sap collected during the 120 min $^{14}CO_2$ exposure of treated, intact, nodulated root systems also reflected the inhibition of nodule CO_2 fixation (Table IV). Radioactivity in xylem sap of AOA-, MSO-, and AZA-treated plants was reduced 72, 85, and 72%, respectively. The distribution of radioactivity in xylem sap of controls was 33% in the acid fraction and 67% in the basic formation. However, radioactivity in the xylem sap of AOA-, MSO-, and AZA-treated plants was approximately 50% in both acid and basic fractions.

Further analysis of the xylem sap showed that greater than 80% of the ^{14}C in the acid fraction was in malate. Malate in control xylem sap contained 17,600 ± 200 dpm, while malate in AOA, MSO, and AZA contained 6,200 ± 600, 4,900 ± 300, and 6,600 ± 100 dpm, respectively.

Radioactivity in the basic fraction of xylem sap from control plants was found primarily in aspartate and asparagine, with lesser amounts in glutamate, glutamine, and alanine (Table V). Radioactivity associated with xylem sap aspartate and asparagine was strikingly reduced in AOA, MSO, and AZA-treated plants. MSO appeared to have a greater effect than AOA or AZA on radioactivity associated with aspartate and asparagine (Table V). Consistent with known *in vivo* effects, radioactivity associated with xylem sap glutamine was reduced 72% by MSO, while AZA reduced radioactivity associated with glutamate by 65%.

The total quantity of amino nitrogen, both labeled and unlabeled, transported in xylem sap during the 120 min collection and also sharply reduced by inhibitors (Table V). Xylem sap of control plants contained 294 μg of amino nitrogen. Compared to the control, the total quantity of amino nitrogen in xylem sap of AOA-, MSO-, and AZA-treated plants was reduced by 50, 44, and 75%, respectively.

DISCUSSION

Asparagine is biosynthesized directly from aspartate in alfalfa nodules. Aspartate involvement in asparagine biosynthesis has been suggested previously in studies of pea shoots (11, 15), rice seedlings (12), and soybean leaves (7, 23). However, direct coversion of [^{14}C]aspartate to [^{14}C]asparagine has been difficult to

show in legume nodules (9, 25). This is surprising in view of the presence of AS activity in lupine (22), soybean (9, 10), and alfalfa nodules (CP Vance, unpublished data). Previous studies documented conversion of aspartate primarily into tricarboxylic cycle organic acids (11, 14, 24). This may have contributed to difficulties reported in efforts to demonstrate [^{14}C]asparagine biosynthesis from [^{14}]aspartate (17, 25). We also observed rapid movement of [^{14}C]aspartate carbon into tricarboxylic cycle acids, indicating high rates of AAT activity. Short-term exposure (20 min) to AOA dramatically reduced the flow of carbon in nodules from aspartate into tricarboxylic cycle acids and subsequent respiration. This confirms and further documents the suggestion of Joy *et al.* (11) that AAT plays a pivotal role in aspartate metabolism.

Significant biosynthesis of [^{14}C]asparagine from [^{14}C]aspartate was previously found only in pea cotyledons (11, 15) and in soybean leaves (7, 23). We calculate that Duke *et al.* (7) and Stewart (23) observed production of [^{14}C]asparagine from [^{14}C]aspartate in soybean leaves (1 h incubation) at the rate of 30 and 21 nmol g^{-1} fresh weight h^{-1}, respectively. Joy *et al.* (11) reported in pea shoots (after 5 h) biosynthesis of 20 nmol [^{14}C] asparagine g^{-1} fresh weight h^{-1}, which increased upon the addition of AOA to 66 nmol [^{14}C]asparagine g^{-1} fresh weight h^{-1}. Untreated alfalfa nodules biosynthesize [^{14}C]asparagine from [^{14}C]aspartate at the rate of 2.1 nmol asparagine g^{-1} fresh weight. However, upon exposure of nodules to AOA, [^{14}C]asparagine biosynthesis increased 15-fold (30 nmol asparagine g^{-1} fresh weight h^{-1}). Thirty nmol asparagine g^{-1} fresh weight h^{-1} could supply 3.0 nmol asparagine h^{-1} plant $^{-1}$ to the xylem, about 0.1 to 4.0% of the observed asparagine concentration in alfalfa xylem sap (6, 16). This indicates that excised nodules do not provide optimal conditions for asparagine biosynthesis.

The rapid labeling of asparagine from aspartate is consistent with a direct role for aspartate in asparagine biosynthesis. Our data do not, however, resolve whether asparagine biosynthesis in alfalfa nodules is glutamine dependent. MSO and AZA produced the expected effects on glutamine and glutamate labeling patterns, indicating that they were effective in inhibiting GS and GOGAT, respectively. Yet these inhibitors appeared to have little effect on overall accumulation of label in asparagine. While AZA inhibited glutamine-dependent AS in soybean (9), we saw little effect of this inhibitor on asparagine formation after 60 min. Previous studies of soybean nodules (9) and soybean leaves (23) infiltrated with MSO and AZA implicated glutamine-dependent AS in asparagine biosynthesis. In addition, a glutamine-dependent AS was purified from soybean (9, 10) and lupine nodules (22). While we used 5 mM MSO and AZA, the lack of effect on conversion of aspartate to asparagine could have resulted from a high concentration of free glutamate and glutamine in alfalfa nodules (16, 27) preventing the complete binding of inhibitors to GS and GOGAT. Our data corroborate recent evidence by Trung-Chan *et al.* (27) regarding assimilation of $^{15}NH_4^+$ and $^{15}N_2$ by alfalfa nodules which indicated that both glutamine- and NH_3-dependent amidation of aspartate occurred.

The dramatic effect of AOA on aspartate metabolism to either organic acids or asparagine supports a key role for AAT in nodule metabolism. At least two cytosolic and one bacteroid isoform of AAT exist in alfalfa nodules (CP Vance, unpublished data). Multiple cytosolic and bacteroid isoforms of AAT have been reported in soybean (21) and lupine (20) nodules. Taken inclusively, these data suggest that there may be two pools of aspartate controlled by distinct isozymes. One isozyme may be involved in aspartate biosynthesis supporting nodule N assimilation while another isozyme may provide the link facilitating movement of carbon between organic acid and amino acid pools. This latter role is an essential element of a malate/aspartate shuttle (1). Our data support previous studies suggesting that such a shuttle may transfer reducing equivalents from the host plant cytosol to the bacteroid (1, 21). Further support for two pools of aspartate came from our analysis of radiolabeled amino acids in xylem sap derived from nodule CO_2 fixation. Data from this study and from Maxwell *et al.* (16) indicated that xylem sap aspartate contains the major portion of label derived from nodule $^{14}CO_2$ fixation. Yet, asparagine is the predominant amino acid transported from alfalfa nodules (16). This suggests that one pool of labled aspartate derived from $^{14}CO_2$ fixation is rapidly loaded into xylem sap, while another pool of aspartate with less label may be used for asparagine biosynthesis.

Alfalfa nodule CO_2 fixation rates, partitioning of fixed $^{14}CO_2$, and labeling patterns of organic and amino acids via nodule CO_2 fixation were comparable to those reported previously in alfalfa, soybean, and lupine (4, 5, 16, 29). Organic acids are the initial products of nodule CO_2 fixation with subsequent metabolism to amino acids and transport in the xylem sap as aspartate and asparagine (Tables IV and V). The data support the contributions of PEPC, AAT, and AS to aspartate and asparagine biosynthesis. In short-term assays (20 min) the lack of inhibition of nodule CO_2 fixation by MSO, AZA, and AOA provided further support for a buffering capacity by large pools of free amino acids in nodules. However, in long-term assays (120 min, Table V) inhibitor treatment reduced the total radioactivity in attached nodules exposed to $^{14}CO_2$, suggesting inhibition of nodule CO_2 fixation. In long-term assays (60–240 min) of soybean nodules, Huber and Streeter (9) reported that MSO and AZA inhibited nodule CO_2 fixation and nodule aspartate and asparagine biosynthesis. The inhibition of CO_2 fixation by MSO and AZA is probably an indirect effect resulting from reduction in ammonia assimilation. Previous studies of lupine (13) and alfalfa nodules (M Anderson, personal communication) have shown that treatments which reduce N_2 fixation and the supply of ammonia result in a concomitant decrease in nodule CO_2 fixation.

Inhibition of nodule, AAT, GOGAT, and GS by AOA, AZA, and MSO, respectively, not only inhibited nodule CO_2 fixation, but also reduced xylem sap amino nitrogen transport 50 to 70% and incorporation of label from $^{14}CO_2$ into xylem sap by 70 to 85%. The reduction in xylem sap radioactivity and amino nitrogen was directly related to reduced labeled asparagine and aspartate in xylem sap. Since 95% of the amino acids in the xylem sap of effectively nodulated alfalfa plants are derived from actively fixing nodules (16), the effect of inhibitors on xylem sap characteristics was mediated through inhibitor effects on nodules. These data demonstrate the striking interdependence of nodule ammonia assimilation, CO_2 fixation, asparagine biosynthesis, and transport of fixed N_2.

In summary, these data demonstrate rapid biosynthesis of asparagine from aspartate in alfalfa nodules. Nodule CO_2 fixation also served as a precursor for aspartate for asparagine production and transport in alfalfa xylem sap. CO_2 fixation and amino acid biosynthesis in alfalfa nodules were sensitive to inhibition of aminotransferase activity or interruption of ammonia assimilation. The key roles of nodule AAT and AS are apparent, and the need for further investigations of these possibly regulatory enzymes is suggested.

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