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Asparagine Biosynthesis in Soybean Nodules[1]

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ABSTRACT

Asparagine biosynthesis in soybean (*Glycine max* [L.] Merr.) nodules has been difficult to demonstrate due to the poor conversion of suspected immediate precursors to asparagine and the instability of the key enzyme asparagine synthetase. The present study was designed to explore the effects of two ammonium assimilation inhibitors on the metabolism of $^{14}CO_2$ to [^{14}C]asparagine and to demonstrate the existence in nodules of the enzyme asparagine synthetase. When detached nodules were incubated in $^{14}CO_2$, radioactivity in asparagine (as a percentage of amino acid cpm) increased 10-fold over 4 hours. Vacuum infiltration of 10 mM methionine sulfoximine or 10 mM azaserine prior to $^{14}CO_2$ incubations decreased both the rate of dark fixation and the radioactivity in the amino acid fraction. These inhibitors also decreased the recovery of label in aspartate and asparagine. These results, plus the sequence of labeling of metabolites from $^{14}CO_2$, are consistent with a glutamine-dependent synthesis of asparagine from aspartate with oxalacetate as a precursor to aspartate.

An enzyme catalyzing the ATP- and glutamine-dependent amidation of aspartic acid to form asparagine was isolated from soybean nodules. High levels of sulfhydryl protectants were required and the inclusion of glycerol and substrates in the extraction buffer helped to stabilize the enzyme. Enzyme activity in taproot nodules increased between 38 and 41 days after planting and peaked soon after flower initiation (45 days). The activity then declined to basal levels by 70 days. On a total enzyme activity basis, there was 170-fold more asparagine synthetase activity in the infected zone of the nodule than in the cortex, and 205-fold more activity in the cytosol than the bacteroid fraction. The enzyme has a broad pH maximum around pH 8.25, and the apparent K_m values for the substrates aspartate, MgATP, and glutamine are 1.24 mM, 0.076 mM, and 0.16 mM, respectively. Ammonium ion can replace glutamine as the nitrogen donor, but the K_m value of the enzyme for ammonium ion is 40-fold higher than that for glutamine.

Asparagine is the amino acid found in the highest concentration in the bleeding sap of soybean nodules (31) and the xylem sap of nodulated soybean plants grown with or without combined *N* (6, 19). Ohyama and Kumazawa (21) found 3-fold more asparagine than ureides in soybean nodule cytosol even though the ureides are the principal form in which *N* is transported from soybean nodules (19). The results of numerous *in vitro* enzymic analyses (17, 18, 26, 29) have demonstrated glutamine-linked asparagine synthesis in higher plant tissue, including legume nodules (1, 3, 12, 24, 27). In addition, tracer studies utilizing $^{15}NH_4^+$ and [amide-^{15}N]glutamine have confirmed glutamine-dependent asparagine formation in legume nodules *in vivo* (10).

The pathway providing carbon skeletons for asparagine formation in legume nodules is thought to be dependent upon dark CO_2 fixation *via* PEP carboxylase[3] (4, 5, 15, 16). The oxalacetate formed is transaminated with glutamate to form aspartate. This aspartate is then converted to asparagine *via* AS (27). Laing *et al.* (15) have demonstrated that amino acid formation from specifically labeled glucose in lupin nodules, at least with short incubation times, is *via* PEP carboxylase rather than *via* pyruvate kinase. High correlations between *in vitro* PEP carboxylase activity ($r = 0.93$) or *in vivo* CO_2 fixation rates ($r = 0.88$) and nodule acetylene reduction activity throughout nodule development have been reported (4). Coker and Schubert (5) have shown that the ratio of CO_2 fixed to N_2 reduced approximates unity which agrees well with the theoretical ratio on incorporation of two NH_4^+ and one CO_2 to form asparagine. Finally, several workers have reported the metabolism of dark-fixed $^{14}CO_2$ to asparagine in legume nodules (5, 12, 16).

More immediate carbon precursors of asparagine have been difficult to demonstrate. Attempts by Streeter (30) to demonstrate asparagine formation in soybean nodules from various labeled precursors, including aspartate, have failed, probably due to asparaginase activity in nodules (10, 30). In addition, many plant tissues rapidly metabolize added aspartate to tricarboxylic acid cycle intermediates rather than to asparagine (18, 29, 30). Stewart (28), however, was able to show a significant glutamine-dependent conversion of [^{14}C]aspartate to [^{14}C]asparagine in soybean leaves, which was inhibited by MSX and AZA.

Asparagine formation in the ureide-producing soybean nodule is likely to be similar to the pathway proposed by Scott *et al.* (27) for the amide-producing lupin. The pathway is directly dependent upon the enzymes AAT and AS. Nitrogen donors are supplied via the activities of GS and GOGAT. The presence of all of these enzymes has been confirmed in lupin nodules (1, 18) and more recently in soybean nodules (2, 24, 25). The work reported here was designed to elucidate the pathway of asparagine formation in soybean nodules. Three approaches were used, including (a) a time course study following the metabolism of dark-fixed $^{14}CO_2$ to [^{14}C]asparagine, (b) an investigation of the effect of the inhibitors MSX and AZA on this metabolic pathway, and (c) demonstration of the enzyme AS in soybean nodules. A preliminary report of this work was previously published (12).

MATERIALS AND METHODS

Plant Material. 'Beeson' soybean (*Glycine max* [L.] Merr.) seeds were sown in pots containing rinsed silica sand. Seeds were

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[3] Abbreviations: PEP carboxylase, phosphoenolpyruvate carboxylase; AS, asparagine synthetase; MSX, methionine sulfoximine; AZA, azaserine; GOGAT, glutamate synthase; GS, glutamine synthetase; AAT, aspartate aminotransferase.

inoculated at the time of planting with a commercial peat inoculant (Agricultural Laboratories, Inc., Columbus, OH) containing *Rhizobium japonicum* strains 110, 8-0, 8-T, and 138. Plants were irrigated with a nutrient solution containing no nitrogen (30).

Plants for the experiment to determine the effect of nodule age on asparagine synthetase activity were grown outdoors during the summer months without alteration of the natural photoperiod. Plants for all other experiments were grown either outdoors during the summer or in a greenhouse between November and May with supplemental light provided by metal halide lamps. These lamps provided approximately 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (400–700 nm) and a photoperiod of 12 h.

$^{14}CO_2$ Dark Fixation. All *in vivo* feedings were performed using micro-diffusion chambers (Bel-Art Products, Pequannock, NJ) with three concentric chambers (rings) and an outer diameter of 6 cm. The outer chamber contained sufficient 0.01 N HCl to seal the inner two chambers when the lid was in place. The innermost chamber contained $H^{14}CO_3^-$ when gaseous $^{14}CO_2$ was to be employed. The $^{14}CO_2$ was released by adding a solution of 50% (v/v) lactic acid just prior to replacing the lid. The middle chamber contained whole or sliced nodules and two droplets of water to maintain the humidity in the closed chamber.

A preliminary experiment was designed to determine which of four methods of introducing $^{14}CO_2$ would result in the largest incorporation of label in asparagine. The four treatments were (a) whole, detached nodules placed with the attachment site in droplets of $H^{14}CO_3^-$, (b) once-sliced nodules placed sliced face down on droplets containing $H^{14}CO_3^-$, (c) detached nodules vacuum infiltrated with $H^{14}CO_3^-$, and (d) detached nodules exposed to gaseous $^{14}CO_2$. Each of the four treatments was performed in duplicate with five freshly picked taproot nodules weighing about 40 mg each per treatment. Approximately 20 μCi ^{14}C was used per treatment. Incubations were allowed to proceed for 3 h. At the end of the incubation, the microdiffusion chambers were opened and the nodules extracted.

Experiment 2 was designed to explore the effects of two ammonium assimilation inhibitors on the metabolism of $^{14}CO_2$ to [^{14}C]asparagine. Groups of detached taproot nodules (400 mg fresh weight) were weighed and vacuum infiltrated with a 10-mM solution of either AZA or MSX. Azaserine is a glutamine analog which inhibits all glutamine amide transfer reactions (28) including GOGAT(28) and AS (17). Methionine sulfoximine is an analog of the γ-glutamyl phosphate enzyme complex of GS and is known to be a potent inhibitor of that enzyme (28). An additional group of nodules was vacuum infiltrated with water as a control. Nodules were placed in the middle chamber of a microdiffusion chamber as detailed above. After incubating the nodules with the inhibitors for 30 min, the $^{14}CO_2$ in the center chamber was released with 50% (v/v) lactic acid. Lids were immediately replaced and the nodules were incubated with $^{14}CO_2$ for 30, 60, 120, or 240 min.

Extraction and Analysis of Radioactive Metabolites. Nodule incubations were stopped by grinding each sample in 3 ml of extraction medium (29 ml 75% (v/v) ethanol + 1 ml 0.5 N HCl) in a ground glass tissue grinder containing a small amount of acid-washed sand. The extraction medium contained HCl in order to inactivate bacteroid asparaginase activity (30). The homogenate and several rinses were combined and centrifuged at 40,000*g* for 10 min and the supernatant collected. The pellet was extracted twice and the supernatants combined and dried under a stream of air at room temperature. The samples were dissolved in 0.5 ml water and fractionated by Dowex column chromatography in a cold room (4°C). Samples were passed first through Dowex-50 (H^+, 5 × 50 mm) and then immediately through Dowex-1 (formate, 5 × 50 mm) ion exchange resins. After several 1-ml water rinses of the column pairs, effluent of the Dowex-1 column was taken as the neutral fraction. The Dowex-50 column was eluted with 50% (v/v) NH_4OH, followed by water, to provide the basic fraction containing amino acids. The Dowex-1 column was eluted with 2 N HCl to provide the acidic fraction containing organic acids. After adding a few drops of chloroform, each fraction was dried under a stream of air at room temperature and redissolved in 1 (acidic fraction) or 0.5 (basic and neutral fractions) ml of water.

Amino acids were separated by two-dimensional TLC and detected using 1,2-naphthoquinone-4-sulfonic acid as previously described (29). Spots to be analyzed were scraped into liquid scintillation vials, eluted by shaking with 2 ml of water for 1 h, and counted after mixing with 8 ml of scintillation cocktail (24 g PPO + 1 g POPOP + 2 L toluene + 2 L Triton X-100).

Organic acids were separated by descending paper chromatography on Schleicher and Schuell 589 White Ribbon paper using the 1-butanol:acetic acid:water (24:6:10) solvent and detected using a solution of 0.02% acridine in 95% ethanol as previously described (29). Spots to be analyzed were cut out of the papers and eluted and counted as described above.

Extraction and Assay of Asparagine Synthetase. Freshly picked or previously frozen (−70°C) nodules were ground using a chilled mortar and pestle in buffer (2 ml/g fresh weight of nodules) containing 100 mM Na phosphate (pH = 8.5), 25% (v/v) glycerol, 0.1 mM EDTA, 10 mM $MgCl_2$, 2 mM aspartic acid, 50 mM mercaptoethanol, 0.5 mM DTE, and 0.1 mM ATP. These and all subsequent operations were performed at 4°C. Acid-washed, insoluble PVP (Polyclar AT, GAF Co., NY) was added to the macerate at a rate of 0.25 g/g fresh weight and carefully mixed without grinding. After 15 min, the macerate was centrifuged (30,100*g*, 20 min) and the supernatant collected. Solid $(NH_4)_2SO_4$ (Sigma, Grade 1) was slowly added to 42% saturation, the pH being kept at pH 8.5 with the addition of NH_4OH. An additional 0.5 ml mercaptoethanol/L of extract was added. After stirring for 30 min, the precipitate was collected by centrifugation (30,000*g*, 15 min) and gently dissolved in a minimal amount of extraction buffer. This enzyme fraction was desalted by passing through a Sephadex G-25 (Pharmacia) column which was equilibrated and eluted with extraction buffer.

The standard reaction mixture is given in a footnote to Table III. Enzyme activity was determined by measuring the conversion of [4-^{14}C]aspartate to [4-^{14}C]asparagine and radioactive aspartate and asparagine were separated with Dowex-1 (formate) columns as previously described (29). A complete reaction mixture minus glutamine was used as the control. Asparagine formation was not linear with respect to protein content or time when crude, gel-filtered preparations were assayed. The use of the 0% to 42% $(NH_4)_2SO_4$ fraction yielded an enzyme preparation that gave approximately linear asparagine formation for 60 min when less than 1.5 mg protein was assayed. In all assays, less than 5% of the available aspartate was converted to asparagine.

The identity of the radioactive product when assaying crude preparations was shown to be asparagine by two chromatographic separation techniques. Effluent from the Dowex-1-formate column was immediately made to 50% (v/v) ethanol and boiled. After concentrating with a flash evaporator, the product was separated using the two-dimensional TLC method described above or a descending paper chromatography method using phenol:H_2O (3:1) as previously described (29). Recovery of radioactivity in the asparagine spot was greater than 90% with either method. If the Dowex-1-formate effluent was not brought to 50% (v/v) ethanol and boiled all of the label was recovered in aspartate. This result is most likely due to the very active and stable asparaginase reported to be present in soybean nodules (30). Asparagine was present in Dowex-1-formate effluent from complete reaction mixtures but was absent in effluent from controls.

Localization of Asparagine Synthetase Activity. Nodules from plants inoculated with suboptimal numbers of rhizobia were considerably larger than typical nodules. These large nodules were halved or quartered by hand and the red, infected zone tissue was scooped out with a spatula and placed in a chilled mortar. The remaining cortex was carefully scraped and rinsed with extraction buffer to remove any additional infected cells and was also placed in a chilled mortar. The nodule fractions were extracted as described above and assayed for asparagine synthetase activity.

Bacteroids and cytosol were obtained using the method of Reibach *et al.* (23) with minor modifications. All solutions contained 25 mM mercaptoethanol. Ten g of nodules were picked and gently crushed at 4°C in 13 ml of extraction buffer (described above) with thiols freshly added. Two g of Polyclar AT/g fresh weight nodules were added and the mixture allowed to set for 15 min. The slurry was squeezed through four layers of cheesecloth. One and one-half ml of the filtered extract was carefully layered on top of each of four Percoll gradients. The tubes were centrifuged and the bacteroid and cytosol fractions collected (23). The bacteroid and whole nodule fractions were sonicated for 3 × 1 min using a Sonicator Cell Disrupter (Model W 185 F, Ultrasonics, Inc. Plainview, NY) at a maximal setting. Each sample (whole nodules, bacteroid, and cytosol) was taken to 42% saturation with solid $(NH_4)_2SO_4$ and processed as described above.

RESULTS AND DISCUSSION

***In Vivo* Experiments.** Results of a preliminary experiment corroborate reports (6, 12, 16) that the dark fixation of $^{14}CO_2$ leads to a high level of radioactivity in asparagine in legume nodules. Approximately 30% of the total radioactivity in nodules was recovered in the basic fraction from each treatment after the 4-h incubation period (data not shown). The percentages of the amino acid cpm recovered in glutamine, glycine, serine, and alanine were also similar. The treatments differed, however, in the amount of label recovered in asparagine and, to a lesser degree, in recoveries in aspartate and glutamate. Whole nodules exposed to $^{14}CO_2$ gas contained 11% of the amino acid cpm in asparagine as compared to less than 2% with other treatments. This treatment also resulted in a greater recovery of label in aspartate and less label in glutamate when compared with the other treatments. None of the various methods tested to introduce $H^{14}CO_3^-$ resulted in significant levels of radioactivity recovered in asparagine. These results may be attributed to the localization of AS in the same nodule tissue as PEP carboxylase (discussed later).

Previous work in this laboratory (J. G. Streeter, unpublished data) has demonstrated that the patterns of amino acid labeling are similar if attached or detached nodules are exposed to $^{14}CO_2$ for 5 h. Because the whole nodules exposed to $^{14}CO_2$ in the present experiment resulted in labeling patterns similar to these previous experiments using attached or detached nodules, this system was chosen to study the time course of asparagine biosynthesis and the effects of specific inhibitors on this pathway.

In experiment 2, detached nodules were vacuum infiltrated with AZA or MSX, or with water as a control, before incubating with $^{14}CO_2$ gas for 0.5, 1, 2, and 4 h. Both inhibitors decreased the rate of incorporation of label into the basic, acidic, and neutral fractions (Table I). In general, the amount of total recovered radioactivity increased from 0.5 to 4 h with all treatments, demonstrating CO_2 fixation throughout the incubation periods. The ratios of total radioactivity at 4 h to total radioactivity at 0.5 h for the control, AZA, and MSX treatments were 1.6:1, 7.6:1, and 5.7:1, respectively. The data for the water control indicate an equilibrium with external $^{14}CO_2$ may have been reached after 1 h. In all treatments, less than 10% of the supplied ^{14}C was recovered in nodules. Because of the difference in the

TABLE I. *Radioactivity in Basic, Acidic, and Neutral Fractions Isolated from Detached Soybean Nodules which had been Vacuum Infiltrated with AZA, MSX, or Water (Control) and Incubated in $^{14}CO_2$ Gas for 0.5, 1, 2, and 4 Hours*

Plants were grown in a greenhouse during the spring and were harvested 60 d after planting. Less than 10% of the supplied ^{14}C was recovered from nodules. The results shown are the means of two replicates. The average deviations from the mean were 11%, 7.4%, and 4.3% when considering the data as water control, azaserine, and methionine sulfoximine, respectively, or 5.3%, 5.4%, and 12% when considering the data as basic, acidic, and neutral fractions, respectively. Experiment 2.

Fraction	Water Control (h)				Azaserine (h)				Methionine Sulfoximine (h)			
	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4
	cpm × 10^{-3}/400 mg fresh wt and (% of total cpm)											
Basic	228.0 (38)	481.0 (48)	504.0 (56)	466.0 (49)	27.7 (22)	133.0 (21)	131.0 (27)	297.0 (36)[a]	29.0 (24)	72.0 (26)	176.0 (34)	338.0 (48)
Acidic	368.0 (61)	500.0 (50)	345.0 (39)	421.0 (44)	96.3 (77)	481.0 (77)	342.0 (72)	512.0 (62)[a]	93.4 (76)	201.0 (73)	346.0 (66)	353.0 (51)
Neutral	7.23 (1.2)	20.0 (2.0)	45.2 (5.1)	63.6 (6.7)	0.566 (0.45)	7.73 (1.2)	6.12 (1.3)	23.9 (2.9)[a]	0.382 (0.31)	1.00 (0.37)	1.40 (0.27)	8.55 (1.2)

[a] Single observation.

Table II. *Radioactivity in Glutamate, Glutamine, Aspartate, Asparagine, Glycine, Serine, and Alanine Isolated from Detached Soybean Nodules which had been Vacuum Infiltrated with AZA, MSX, or Water (Control) and Incubated in $^{14}CO_2$ Gas for 0.5, 1, 2, and 4 Hours*

Plants were grown as described in Table I. The results shown are the means of two replicates. The recovery of label after TLC was fairly constant at different treatment times, but varied depending upon the incubation condition. Between 75% and 83% of the label in the basic fraction of the water control was recovered in the six amino acids shown. Recoveries of the basic fraction cpm in MSX and AZA treated nodules in these same amino acids were 75% and 65%, respectively. Additional amino acids separated by the TLC method include γ-aminobutyrate, Ile, Leu, Met, Phe, Pro, Thr, and Val. Negligible radioactivity was detected in these amino acids as well as in compounds eluted from two unknown spots and the origin. The average deviation from the mean was about 14% for the values shown. Experiment 2.

Amino Acid	Water Control (h)				Azaserine (h)				Methionine Sulfoximine (h)			
	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4
	% of amino acid fraction cpm											
Glutamate	30.5	37.0[a]	18.8	44.5	32.6	35.6	28.0	23.3[a]	41.8	45.2	49.2	54.7
Glutamine	3.04	3.1	21.8	6.23	17.3	23.2	24.3	46.2[a]	0.027	1.06	1.08	0.607
Aspartate	49.1	42.5[a]	28.8	11.8	8.03	7.36	2.74	1.70[a]	11.1	8.46	5.03	3.22
Asparagine	2.35	3.75	14.0	20.3	1.20	4.91	1.47	2.25[a]	0.735	0.287	0.186	0.173
Glycine	3.56	6.18	3.69	2.54	14.6	6.35	7.28	5.15[a]	16.7	10.4	7.61	5.83
Serine	9.24	5.50	11.9	13.8	21.5	18.1	32.6	15.8[a]	25.5	29.6	31.5	31.3
Alanine	2.29	2.18	1.29	2.17	4.87	4.61	3.65	2.07[a]	4.18	4.98	5.41	4.21

[a] Single observation

rates of $^{14}CO_2$ incorporation, the remaining data will be presented on a percentage basis so that comparisons are more easily made.

Results in Table I demonstrate that the apparent metabolism of labeled compounds was from the acidic fraction to the basic and neutral fractions. The amount of recovered radioactivity in the acidic fraction decreased from 61% to 39% after 2 h in the water control, while label increased from 38% to 56% and from 1.2% to 5.1% in the basic and neutral fractions, respectively. A similar trend is apparent in nodules incubated with either inhibitor, although there was less decline in the label in the acidic fraction. The neutral fraction contained the least radioactivity at all times and the recovery of label in neutral compounds was particularly sensitive to the inhibitors.

The recovery of radioactivity in individual amino acids, expressed as the percentage of amino acid cpm, indicated that at early times, aspartate contained the most radioactivity in the water control (Table II). This agrees well with reports by Lawrie and Wheeler (16) and Minchin and Pate (20), and is attributed to the metabolic proximity of this metabolite to oxalacetate. Furthermore, these results are consistent with PEP carboxylase functioning as the principal agent of CO_2 fixation (9). During the 4-h incubation, label in aspartate decreased 4-fold while label in glutamate and asparagine increased 1.5-fold and 8.5-fold, respectively. This result is consistent with a precursor product relationship and has previously been reported in broad beans (16). At times shorter than 20 min, little label has been recovered in asparagine in soybean nodules (6, 7). The apparent movement of label is from organic acids to amino acids (especially aspartate and glutamate) and from aspartate to asparagine (Tables I and II). Label in glutamine and alanine was usually low in the water control and did not change in a consistent manner. Label in glycine plus serine was higher and may have increased slightly during the incubation.

When AZA was vacuum infiltrated prior to incubation with $^{14}CO_2$, a very different labeling pattern was obtained (Table II). Glutamate and serine contained the most radioactivity at earliest times. Label in the glutamine pool increased dramatically, from 17.3% to 46.2%, during the 4-h incubation, probably at the expense of the glutamate pool. This trapping of label in the glutamine pool demonstrates that the inhibitor functioned in a similar manner as previously reported (22, 28). Aspartate never contained more than 8% of the label even at the earliest time. Label in asparagine and alanine was always low and showed no consistent pattern. The recovery of label in glycine plus serine was higher than in the water control but showed no consistent pattern.

When MSX was included as an inhibitor, virtually no label was found in glutamine (Table II). This is consistent with both the previously reported inhibition of glutamine synthetase by MSX (28) and the effect of this inhibitor *in vivo* (22, 28). The recovery of radioactivity in aspartate and asparagine was much lower than that found in the water control. At all times, over 80% of the amino acid radioactivity was recovered in the combined glutamate, serine, and glycine pools. Label in glutamate and serine increased during the incubation period, while label in glycine decreased.

Both inhibitors greatly reduced the radioactivity recovered in both aspartate and asparagine and demonstrate the need for glutamine and for glutamate as nitrogen donors. Either inhibitor should interfere with the GS/GOGAT pathway for ammonia assimilation. This is reflected in the decreased recovery of label in amino acids when the inhibitors were present (Table I). The

Table III. *Substrate Requirements for Asparagine Synthetase from Soybean Root Nodules*

Plants were grown indoors and were harvested 60 d after planting. Values shown are the means of two replicates.

Reaction Mixture	Radioactivity in Dowex-1-formate Resin Effluent	Cpm from Complete Reaction Mixture
	cpm	*%*
Complete[a]	9312	100
Minus aspartate	772	8.3
Minus glutamine	614	6.6
Minus $MgCl_2$	403	4.3
Minus ATP	364	3.9
Minus protein	383	4.1

[a] The complete reaction mixture consisted of 100 mM Tris, pH 7.75, 10 mM $MgCl_2$, 10 mM glutamine, 0.8 mM aspartate (10^6 cpm), 5 mM ATP, 1 mM DTE, and 2.34 mg of crude, gel-filtered protein preparation in a total volume of 1.0 ml. Reaction mixtures were incubated at 30°C for 60 min.

Table IV. *Asparagine Synthetase Activity in the Infected Tissue and Cortex of Soybean Root Nodules*

Plants were grown in a greenhouse and were harvested 60 d after planting. There were fewer, larger taproot nodules due to a lower dose of inoculant. These nodules could be separated into infected tissue and cortex by hand. The values shown are the means of two enzyme assays on the same material. The experiment was repeated with similar results.

Locale	Asparagine Synthetase Activity	
	Specific activity	Total activity
	nmol/min·mg protein	*nmol/min·g fresh wt*
Infected tissue	2.17	48.6
Cortex	0.04	0.28

Table V. *Asparagine Synthetase Activity in the Plant Fraction and the Bacteroids of Soybean Root Nodules*

Plants were grown in a greenhouse and were harvested 45 d after planting. The enzyme preparation required 2 h to complete which probably explains the relatively low enzyme activities. Only the bacteroid and whole nodule fractions were sonicated. The values shown are the means of two enzyme assays on the same material. Adding bacteroid extract to cytosol extract did not affect the activity measured using cytosol extract alone.

Locale	Asparagine Synthetase Activity	
	Specific activity	Total activity
	nmol/h·mg protein	*nmol/h·g fresh wt*
Cytosol	12.7	15.0
Bacteroids	0.27	0.073
Whole nodules	3.67	5.73

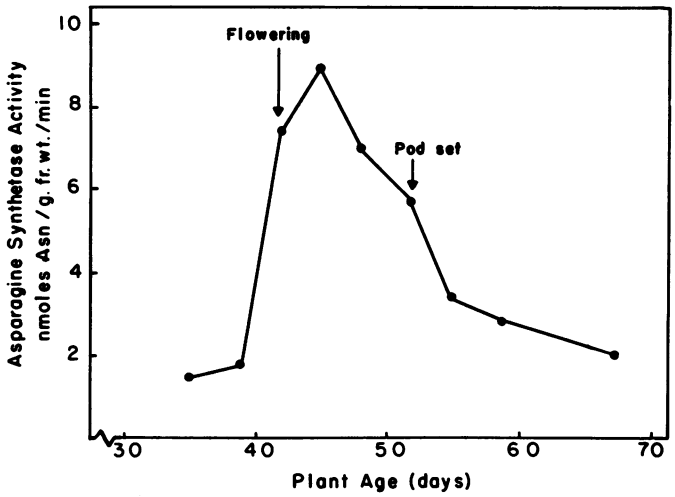

FIG. 1. Effect of plant age on asparagine synthetase activity. Plants were grown outdoors from May to August without alteration of the natural photoperiod. Every 3 or 4 d, from 35 to 70 d after planting, a pot was randomly chosen and harvested. Only nodules from the upper 4 cm of the tap root were used. Although this experiment was not repeated, routine assays for enzyme activity consistently indicated that the highest activities occurred between flowering and pod set.

decreased label in asparagine was probably due to direct inhibition of asparagine synthetase (AZA) or lack of glutamine for enzyme activity (MSX). The decreased label in aspartate was most likely a result of a diminished extramitochondrial glutamate pool for transamination in the proplastid. The AAT isozyme activity which increases concurrently with other key enzymes of nitrogen assimilation is localized in proplastids, as is AS (2).

The increasing amount of label recovered in glutamate when MSX was present (Table II) was unexpected, since little glutamine should be present for GS activity. Givan (11) has suggested, however, that GDH, which is not inhibited by either AZA or MSX, may assimilate ammonia when the GS/GOGAT pathway is nonfunctional. Additional evidence for this concept may be provided by the increased labeling of glycine and serine when the inhibitors are present. The glutamate formed via GDH would be mitochondrial and could be used in glycine and serine synthesis (14). A similar pathway has been proposed by Cookson *et al.* (8) to provide C_2 units for ureide synthesis in *Phaseolus vulgaris*. Cookson *et al.* (8) suggested that CO_2 fixation plays an anapleurotic role to replace carbon lost from the Kreb's cycle via isocitrate lyase rather than aspartate aminotransferase. The glyoxylate so formed may be transaminated with mitochondrial glutamate.

The recovery of radioactivity in the organic acids malate, fumarate, succinate, and citrate was also determined for each incubation time and treatment (data not shown). These four organic acids accounted for 95% to 100% of the acidic fraction cpm. At all times, under all treatments, malate contained the most (>78%) radioactivity (16, 20). Fumarate and citrate always contained less than 1% and 5% of the acidic fraction cpm, respectively. Succinate was intermediate. The most significant trend was the conversion of label from the malate pool to the succinate pool. The percentage of acidic fraction radioactivity in succinate rose from 3% to 17% in the water control while the label in malate fell from 94% to 78% during the 4-h incubation. When inhibitors were present, radioactivity in succinate rose only to 13% to 15% and label in malate fell to 82% to 85%. The effect of the inhibitors on this conversion was, therefore, minimal.

Due to the low amounts of label recovered in the neutral fraction, no attempt was made to determine the nature of individual compounds.

***In Vitro* Experiments.** The glutamine-dependent AS from soybean nodules required magnesium ion and ATP in addition to glutamine and aspartate (Table III) as has been previously reported (12, 26, 29). Glutamine could be partially replaced by NH_4^+, although the K_m for the latter was 40-fold greater than for glutamine.

Extraction of the enzyme required the presence of high sulfhydryl protection and glycerol. In addition, the use of Polyclar AT and the inclusion of substrates (especially ATP and aspartate) helped stabilize the enzyme (18). Large losses (>40%) of activity were realized when the enzyme was precipitated with ammonium sulfate. This slight purification (4-fold) was needed, however, to provide an enzyme preparation exhibiting linear activity with increasing assay time and added protein.

Results in Table IV show that both on a specific activity basis and a total activity basis there is considerably more (50-fold and 170-fold, respectively) AS activity in nodule-infected tissue than in the cortex. In separate experiments, only trace levels of enzyme activity could be detected in uninoculated roots of plants grown without combined *N*. When nodules were separated into bacteroid and host cytosol fractions the activity was clearly localized in the host cytosol (Table V). This was true both on a specific activity basis (47-fold difference) and a total activity basis (205-fold difference). This localization of AS activity in the host cytoplasm of the infected zone is consistent with the recent localization of AS in the proplastid (2).

AS activity increased starting at 38 d after planting and peaked soon after flowering (Fig. 1). The activity steadily declined to near basal levels by 68 d after planting. The highest specific activity of the crude gel-filtered enzyme measured in soybean nodules in the present study was about 1 nmol/min·mg protein which agrees well with the value determined by Chan and Klucas

(3).

The AS from soybean nodules has recently been purified 240-fold to a specific activity of 226 nmol asparagine formed/min·mg protein in our laboratory (unpublished data). This enzyme preparation was used to determine the pH optimum of the enzyme and the K_m values for each substrate. The purified enzyme has a broad pH optimum around pH 8.25 with 80% of this activity at pH 7.25 and pH 9.25. The K_m values for the substrates aspartate, glutamine, and Mg ATP are 1.24 mM, 0.16 mM, and 0.076 mM, respectively. These values are similar to those reported for the enzyme extracted from various plant tissues (18). The relatively low K_m values for glutamine and ATP suggest that the enzyme can successfully compete with other enzymes for these substrates.

Maximum specific enzyme activity in crude extracts was about 1 nmol/min·mg protein. Assuming 10 mg protein/g fresh weight of nodules and 1.5 g fresh weight of nodules per plant, a biosynthetic rate of 15 nmol asparagine formed per min is obtained. Data for inoculated, 53-d-old soybean plants with N_2 as the only nitrogen source provide an estimated asparagine translocation rate of 84 nmol asparagine per min per plant (19). Thus, the *in vitro* rate of asparagine synthesis is inadequate to account for observed translocation rates. Two well documented reasons for this are the extreme instability of the enzyme (18) and the presence of an active asparaginase activity (30). Alternatively, an asparagine synthetase inhibitor similar to the one recently discovered in pea leaves (13) may be present in soybean root nodules. It is quite likely, therefore, that the *in vitro* values are an underestimation of the AS activity present.

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