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Communication

Estimation of Ammonium Concentration in the Cytosol of Soybean Nodules[1]

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ABSTRACT

Analysis of ammonium concentration in the cytosol of soybean (*Glycine max* [L.] Merr.) root nodules gave high levels of error variation. When the separation of cytosol and bacteroids was deliberately delayed following nodule maceration, a large increase in ammonium concentration was found in the cytosol. When a series of samples was subjected to delay intervals of 0 to 60 minutes, extrapolation of the regression line to time zero indicated that the ammonium concentration in cytosol at the time of nodule maceration was essentially nil. The source of ammonium buildup following maceration was not found, but hydrolysis of free amino acids or ureides was ruled out. An extremely low concentration of ammonium in the cytosol is consistent with a model for movement of ammonia (or ammonium) from bacteroids to host cytoplasm by diffusion.

In conjunction with studies on the effect of nitrate on nodule composition, the concentration of ammonium in nodules was determined (20). As effects of nitrate on ammonium were pursued further, it became evident that there was substantial error variation associated with our analysis of ammonium. The use of 10 mM HCl in place of water to extract nodules gave slightly lower estimates, but measured concentrations still seemed high (3–4 μmol/g fresh weight) and variable.

We ultimately noted that estimates of ammonium concentration were higher when extraction time was increased. This led to a systematic evaluation of the effect of time delay on ammonium concentration. We have shown previously that when the processing of extracts is deliberately delayed following the maceration of nodules, the regression of concentration on time can be extrapolated to provide an estimate of metabolite concentrations at the time of maceration of the tissue (19). This approach was used here with the objective of providing an accurate estimate of the ammonium concentration in bacteroids and cytosol.

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MATERIALS AND METHODS

Soybean seeds (*Glycine max* (L.) Merr.) cv Beeson were planted in autoclaved silica sand and inoculated with *Bradyrhizobium japonicum* USDA 110. Plants were irrigated with a nutrient solution lacking combined N and were grown in a greenhouse with supplemental light during the period from October through March (20). Nodules were used from plants 30 to 45 d after planting.

After washing sand from roots, nodulated roots were held in ice water until nodules were picked. Detached nodules were kept on moist paper towels on ice until all nodules were picked (generally 16–20 g fresh weight). All subsequent operations were carried out at 2°C. Nodules were mixed and weighed to give 0.95 to 1.00 g samples.

Nodules were placed in chilled mortars and macerated with 5.0 mL of extraction solution. The slurry was transferred to a 50 mL centrifuge tube and two 2.5 mL rinses of the mortar and pestle were added to the first extract. For samples subjected to time delay, samples were allowed to stand at 2°C for intervals up to 60 min.

Samples were centrifuged in a Beckman J2-21 centrifuge using an angle head rotor. The rotor was accelerated to 15,000 rpm (27,200*g*) and held for 1 min followed by deceleration with maximum brake. The supernatant ('cytosol') was mixed with 200 μL of 0.5 N HCl to give a final HCl concentration of about 10 mM. The pellet ('bacteroids') was suspended in 10 mL of 10 mM HCl.

A difference from the previously reported procedure (19) is that nodule extracts were not filtered prior to centrifugation. Thus, the bacteroid samples contained cellular debris in addition to bacteroids. It was assumed that the debris would not make a significant contribution to ammonium concentration, and, by appearance, debris comprised an insignificant portion of the bacteroid sample. Bacteroids were sonicated to rupture the cell walls and were recentrifuged at 27,200*g*. The supernatant was diluted with 10 mL water and made basic using 200 μL of 10 N NaOH just prior to the analysis of ammonia.

Precipitate formed upon acidification of the cytosol was removed by centrifugation. The clear supernatant was diluted with 39.5 mL water and made basic by adding 500 μL of 10 N NaOH prior to the analysis of ammonia.

Ammonia concentration was measured using an ammonia electrode (Orion Research, Inc., Boston, MA). Standard curves were determined once or twice per day and were always

linear in the range from 1.0 to 0.05 mM ammonia. Response of the electrode to ammonia concentrations below 50 μM was not linear and ammonia concentrations were estimated with a hand-drawn curve. Standards gave reproducible millivolt readings down to about 10 μM ammonia as long as sufficient time was allowed for equilibration.

Amino acid composition of extracts was determined by HPLC analysis of the phenylisothiocyanate derivatives (2, 19). Ureides, ureidoglycolate, and glyoxylate were determined by the method of Vogels and Van Der Drift (22).

RESULTS

In experiment 1, nodule samples were allowed to stand for 0 to 60 min at 2°C following maceration in water. The minimum time between maceration of nodules and acidification of cytosol and bacteroids was 7 min (Fig. 1). The combined data points for cytosol samples resulted in a highly significant regression which, when extrapolated to time zero, indicated that ammonium concentration in the cytosol was close to zero.

A second experiment was conducted to confirm the unusual result obtained in the first experiment and to provide samples for analysis of other constituents in the cytosol and bacteroids. Experiment 2 was similar to experiment 1 except that the shortest delay was 6 min, more samples were taken with short delay intervals, and the maximum delay was only 30 min. A similar regression resulted with a highly significant slope and a predicted zero-time ammonium concentration close to zero. The numerous samples with short time delay were intended to show any curvature which might exist at short time intervals, but curvature was not evident, and the overall data indicated that ammonium concentration in the cytosol was essentially nil (Fig. 1).

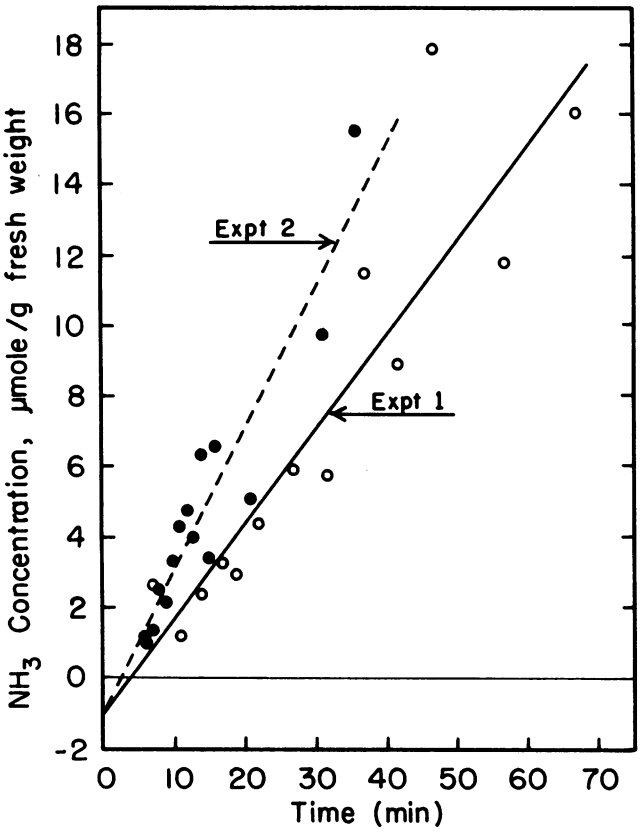

Figure 1. Effect of a time delay after maceration of soybean nodules on the concentration of ammonia in the cytosol fraction following centrifugation. The linear regression equations for experiments 1 and 2 were $[NH_3] = -1.0 + 0.27 \cdot$ time and $[NH_3] = -1.0 + 0.40 \cdot$ time, respectively. The correlation coefficients for the data sets in experiments 1 and 2 were 0.91 and 0.94, respectively; both correlation coefficients are highly significant. Ammonia concentration is expressed on a g fresh weight of nodule basis.

Ammonium concentration in bacteroids was measured in all samples in both experiments, but results were not plotted because concentration was statistically invariant as a function of time. Mean ± SE for the ammonium concentration in bacteroids in experiment 1 was 1.20 ± 0.08 μmol/g fresh weight nodules and in experiment 2 was 2.00 ± 0.08 μmol/g fresh weight nodules.

Results shown in Figure 1 indicated formation of 15 to 20 μmol ammonium/h in nodule cytosol from 1 g of nodules and an obvious question is the source of this ammonium. It seemed unlikely that free amino acids could be the source because only minor variations in amino acid composition of cytosol had been found in earlier time-delay experiments (19). Nevertheless, a portion of each cytosol sample in experiment 2 was used for analysis of amino acids. The concentration of three major amino acids was different from results reported previously (19); namely, the mean concentration of aspartate, glutamate, and asparagine in 15 cytosol samples from experiment 2 was 0.95, 0.69, and 4.5 μmol/g fresh weight, respectively. However, there was no major change in concentration of any amino acid as a function of time delay after nodule maceration. For example, asparagine, which would be a potential source of ammonium because of its high concentration and susceptibility to deamidation (17), had a standard error across the 15 samples which was equal to only 7% of the mean concentration.

The method used for amino acid analysis also provides an estimate of ammonium concentration (2). There was more scatter in the HPLC results for ammonium concentration than in the data obtained with the ammonia electrode. Even so, the general trend was clearly for increasing ammonium concentration in cytosol samples with increasing time delay after nodule maceration (data not shown). Thus, the results for amino acid analysis confirmed the accumulation of ammonium in cytosol by a second analytical method.

Amino acid concentration in bacteroids are low relative to those in cytosol (19). Results for the analysis of amino acids in bacteroid extracts from experiment 2 confirmed this point and gave no indication that gradual loss of some amino acid from bacteroids could make a significant contribution to the accumulation of ammonium in cytosol samples (data not shown). However, when bacteroids were removed from the cytosol prior to a 30 min time delay, the increase in ammonium concentration was reduced by about 80%. Thus, bacteroids were involved somehow in the time-dependent increase in ammonium shown in Figure 1.

Substantial ureide concentrations in soybean nodule extracts have been reported (18) and ureide hydrolysis could

account for at least a portion of the ammonium accumulation in cytosol (Fig. 1). However, analysis of samples from experiment 2 showed no decline in allantoin + allantoic acid or in ureidoglycolate concentrations and no increase in glyoxylate related to time delay following maceration. Mean ± SE values for 15 cytosol samples were, for total allantoin + allantoate, 15.6 ± 0.5 μmol/g fresh weight; for ureidoglycolate, 0.44 ± 0.01 μmol/g fresh weight; and for glyoxylate, 0.72 ± 0.02 μmol/g fresh weight. Ureide concentrations in bacteroid fractions were too low to be of any consequence.

The above results indicated that ammonium accumulating in the cytosol may be liberated from proteins. Aminopeptidases are proteases which might liberate ammonium from protein-bound amino acid amides (16), and nodule aminopeptidases can be inhibited by low concentrations of Cu^{2+} or Zn^{2+} ions (G Sarath, personal communication). To test for the presence of ammonium-liberating aminopeptidase activity in cytosol, nodules were extracted with either 5 mM $ZnCl_2$ or 5 mM $CuCl_2$. Two concentrations of HCl were also compared and samples from approximately 10 and 40 min after nodule maceration were analyzed. Extraction with 5 mM $CuCl_2$ was not different from extraction with water (Table I). Extraction with $ZnCl_2$ decreased the rate of ammonium accumulation but this effect may have been due to the lowering of pH (Table I).

The 50 mM HCl treatment resulted in very low ammonia concentrations and almost no change in concentration with a delay of 30 min after nodule maceration (Table I). Thus, it appears that true cytosolic ammonium concentration can be estimated by extraction of nodules with 50 mM HCl. However, relative error variation associated with these estimates was high because the ammonia concentration in these samples was below the range in which the ammonia electrode gives accurate results with the sample size used. In all, six samples of cytosol from nodules ground in 50 mM HCl were analyzed. The mean ammonia concentration in the solutions used for ammonia analysis was 4.8 μM; values ranged from 1.5 to 9.6 and the SE was 1.2 μM. The mean ± SE concentration of ammonia in cytosol for the three rapidly processed samples was 0.16 ± 0.04 μmol/g fresh weight. Because of the error level, the extrapolated result shown in Figure 1 is probably more accurate, but regardless of the result one chooses, the concentration of ammonium in nodule cytosol appears to be close to zero.

Table I. *Effect of Extraction Medium on the Rate of Increase in Ammonium Concentration in the Cytosol following Maceration of Soybean Nodules*

Data represent average results for two separate experiments. In each experiment a large sample of nodules was divided into 10 subsamples, each of 0.95 g fresh weight. Bacteroids and cytosol were separated by centrifugation immediately or after a delay of 30 min and the change in ammonium concentration is based on these two time points.

Extraction Medium	Increase in $[NH_4^+]$ in 30 min	pH of Crude Extract[a]
	μmol	
Water	6.18	6.2
5 mM $CuCl_2$	6.12	6.0
5 mM $ZnCl_2$	3.33	4.6
10 mM HCl	2.34	3.9
50 mM HCl	0.18	2.2

[a] pH was determined using the 10 mL – mixture of bacteroids + cytosol prior to centrifugation. Temperature of the mixture at the time of pH measurement was <5°C.

DISCUSSION

There are relatively few published estimates of ammonium concentration in legume nodule cytosol. Klucas (7) reported concentrations ranging from 7 to 20 μmol/g fresh weight in cytosol and 0.8 to 1.7 μmol/g fresh weight in bacteroids from soybean nodules. Ta *et al.* (21) have reported values of 4.3 μmol/g fresh weight for cytosol and 1.5 μmol/g fresh weight for bacteroids from alfalfa (*Medicago sativa* L.) nodules. These results are in reasonable agreement with our results for bacteroids, but previously published estimates of ammonium concentration in cytosol must be viewed with caution because nodules were extracted with buffer (7) or 80% ethanol (21). Soybean nodule extracts have a pH of 6.0 to 6.5 (8, 14; Table I), and some increase in ammonium might occur even in the presence of 80% ethanol because some nodule enzymes are stable under these conditions (17).

The source of the ammonium which accumulated in cytosol samples was not identified, but amino acid hydrolysis and ureide hydrolysis were ruled out. Because of the high rate of ammonium generation (Fig. 1), direct cleavage of ammonium from some protein is suggested. However, such a deamination activity has not been described in reports of nodule proteases (9, 13, 14). Because of the large quantity of ammonium generated, cleavage of ammonium from leghemoglobin, a major component of nodule cytosol, is suggested. In fact, catalytic activity for hydrolysis of hemoglobin (13) or leghemoglobin (14) has been reported in nodule cytosol, but assays were for amino acid formation.

The fact that ammonium accumulation in cytosol is largely eliminated by removal of bacteroids indicates either that bacteroids cause the deamination/deamidation of exposed amino/amide groups in a cytosolic protein or that bacteroids have exposed proteins which are susceptible to deamination/deamidation reactions catalyzed by cytosolic enzymes. The latter explanation is favored. Pladys *et al.* (15) have reported that proteases in *Phaseolus vulgaris* nodules release amino acids when mixed with purified bacteroids. They suggested that the peptidoglycan layer of the bacteroid cell wall is the substrate of these proteases based on the amino acids released. However, proline, one of the major compounds reported to be released has not been reported as a component of the peptidoglycan layer (15). I have noted that, in their analytical system, proline elutes very close to ammonium (3), and I suggest that ammonium was incorrectly identified as proline in their samples. Thus, the accumulation of ammonium shown in Figure 1 may represent a previously unreported deamination or deamidation of bacteroid cell wall proteins by host-derived enzymes, and the report of Pladys *et al.* (15) is consistent with this suggestion.

The presence of an extremely low concentration of ammonium in the cytosol is reasonable in view of the presence of high levels of glutamine synthetase in the host cytoplasm

of nodules. The apparent K_m values for ammonium of host glutamine synthetases are on the order of 0.2 mM (4, 10), and it has been estimated that, in soybean nodules, the enzyme comprises 2% of the protein in the cytosol (11). Thus, the cytosol constitutes a massive sink for free ammonium coming from bacteroids.

Ammonium has long been known to be the first stable product of N_2 fixation in bacteroids (1). However, only recently has the mechanism for export of ammonium been studied. Cultured bacteria of several *Rhizobium* species have a mechanism for the active transport of ammonium, but, in all cases studied, the carrier could not be detected in bacteroids (5, 6, 12). This has led to the suggestion that transport of ammonium from bacteroids to host cytoplasm is by passive diffusion (6, 12). This concept is not consistent with the high concentrations of ammonium reported previously (7, 21) but is consistent with the very low cytosol ammonium concentrations reported here.

If one assumes that 1 g fresh weight of nodules will yield 0.15 g of bacteroids (JG Streeter, unpublished data) and that bacteroids are 90% water, then a quantity of ammonium in bacteroids of 1.6 μmol/g fresh weight nodule would give an approximate ammonium concentration of 12 mM in the bacteroids. If the 0.85 g of nonbacteroid nodule is also 90% water, the 0.16 μmol ammonium/g fresh weight nodule would give an approximate ammonium concentration of 0.21 mM in the cytosol. These rough approximations suggest a 50-fold concentration gradient between bacteroids and host cytoplasm and are consistent with passive diffusion as a mechanism for the transport of ammonium.

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