Root Nodule Enzymes of Ammonia Assimilation in Alfalfa (*Medicago sativa* L.)[1]

DEVELOPMENTAL PATTERNS AND RESPONSE TO APPLIED NITROGEN

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ABSTRACT

Nitrogenase-dependent acetylene reduction activity of glasshouse-grown alfalfa (*Medicago sativa* L.) decreased rapidly in response both to harvesting (80% shoot removal) and applied NO_3^- at 40 and 80 kilograms N per hectare. Acetylene reduction activity of harvested plants grown on 0 kilogram N per hectare began to recover by day 15 as shoot regrowth became significant. In contrast, acetylene reduction activity of all plants treated with 80 kilograms NO_3^--N per hectare and harvested plants treated with 40 kilograms NO_3^--N per hectare remained low for the duration of the experiment. Acetylene reduction of unharvested alfalfa treated with 40 kilograms N per hectare declined to an intermediate level and appeared to recover slightly by day 15. Changes in N_2-fixing capacity were accompanied by similar changes in levels of nodule soluble protein.

After an initial lag of 24 hours, specific activities of alfalfa nodule glutamine synthetase, NADH-glutamate synthase, and NAD-glutamate dehydrogenase (oxidative amination) decreased similar to but less rapidly than acetylene reduction activity. Increased specific activities of these nodule enzymes occurred as acetylene reduction activity increased and shoot growth resumed. The observed rates of glutamine synthetase and glutamate synthase were sufficient to assimilate ammonia produced via symbiotic N_2 fixation. Nodule NADH-dependent glutamate dehydrogenase (reductive amination) specific activity was not associated with changes in acetylene reduction activity.

The data indicate that host plant glutamine synthetase and NADH-glutamate synthase function to assimilate symbiotically fixed N and that NADH-dependent glutamate dehydrogenase may function in ammonia assimilation during senescence in alfalfa nodules.

A large portion of total plant N in alfalfa is derived from symbiotic N_2 fixation (9). Sizeable amounts of ammonia are generated in bacteroids as the initial product of N_2 fixation (1). Most of this ammonia (90–95%) is exported from bacteroids into the surrounding nodule plant cell cytoplasm (14), assimilated into organic compounds and transported to the shoots as either ureides or amides (19). Ureides are the predominant organic N compounds transported from soybean and cowpea nodules (19). In contrast,

recent studies indicate that amides are the major organic N compounds transported from alfalfa nodules with little detectable ureide formation (F.D. MacDowall and A.L. Barta, personal communication). Studies of ammonia assimilation have been primarily limited to annual legume species that transport ureides (11, 12, 15, 24).

Ammonia assimilation is thought to involve the plant nodule enzymes GS (EC 6.3.1.2)[3], GOGAT (EC 1.4.1.14), and GDH (EC 1.4.1.2,4) (19). Studies of [^{13}N]N_2 incorporation into soybean nodules have shown glutamine to be the initial organic product of N_2 fixation (12). The observed kinetics of ^{13}N incorporation in these labeling studies and similar studies with [^{15}N]N_2 (15) indicate that the GS/GOGAT enzyme system is the primary route for host plant assimilation of symbiotically fixed N in soybean nodules. Induction of high levels of GS, GOGAT, and GDH activity in the plant fraction of various legume nodules has been observed to parallel the development of N_2 fixation (20, 21, 24). Nodule host plant GS comprised 2% of the total soluble protein in N_2-fixing soybean nodules (11). GDH appears to be ubiquitous in legume nodules but its role in N_2 fixation has not been established (2, 4).

The relative contributions of these enzymes to assimilation of fixed N is not fully elucidated for the perennial legume alfalfa. High activity of plant GS has consistently been observed in alfalfa nodules (2, 4, 6). Host plant nodule GS activity in alfalfa and other legumes has previously been measured by an indirect method involving the reaction of glutamine and hydroxylamine forming γ-glutamylhydroxymate and ammonia (11). This reaction does not always accurately reflect GS affinity for ammonia (5, 11). Reports of alfalfa nodule GOGAT have been inconsistent suggesting this enzyme is either not involved in ammonia assimilation or is unstable and labile under many extraction and assay conditions (2, 4, 6). Reported levels of alfalfa nodule GDH activity also are quite varied (2, 4, 6).

Defoliation and other types of stress induce loss of nodule function in a wide range of legumes (13, 16, 17, 26). Root nodules of alfalfa, a perennial forage legume, have an adaptive capacity to undergo temporary, localized senescence in response to harvesting, with subsequent resumption of nodule growth and function as photosynthate again becomes available (26). Low levels of applied fertilizer N also induce temporary loss of nodule function in alfalfa (25).

Host plant ammonia assimilation associated with N_2 fixation and induced senescence of alfalfa nodules is not well understood.

[3] Abbreviations: GS, NH_3-dependent glutamine synthetase; NADH-GOGAT, NADH-dependent glutamate synthase; NAD-GDH, NAD^+-dependent glutamate dehydrogenase (oxidative deamination); NADH-GDH, NADH-dependent glutamate dehydrogenase (reductive animation); PMSF, phenylmethylsulfonyl fluoride.

The objectives of this study were to develop more reliable and accurate assay procedures for GS and GOGAT to assess which plant ammonia-assimilating enzyme activities are most closely associated with symbiotic N_2 fixation and to evaluate the effects of harvest and applied N on ammonia assimilation enzymes in alfalfa root nodules.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L. cv. Vernal) was grown from seed in a glasshouse as described previously (26). The sand bench was divided into nine equal sections, separated by polyethylene film to prevent lateral movement of applied N between sections. Micro- and macronutrients (except for N) and lime ($CaCO_3$) were incorporated into the sand. The sand was inoculated prior to seedling emergence with a commercial preparation of *Rhizobium meliloti* (Nitragin Co., Milwaukee, WI)[4] and with a suspension of pure cultures of several strains of *R. meliloti* (G. Hardarson, University of Minnesota).

Seedlings were grown to approximately 10% flowering, tops were harvested, and the seedlings were allowed to regrow until 10% flowering. At this time (day 0), tops were either harvested (70–80% shoot removal) or left unharvested and an aqueous solution of KNO_3 equivalent to either 40 or 80 kg N ha^{-1} was surface-applied to the bench sections in a complete randomized block arrangement (three replicates per treatment). Plants not treated with KNO_3 were given an amount of potassium (as K_2SO_4) equivalent to that of KNO_3 at 40 kg N ha^{-1}. Controls consisted of plants that were both unharvested and not treated with applied N. Plants were sampled near 8:30 a.m. at 0, 1, 4, 10, and 15 days after treatment. Plant samplings were shaken gently to remove sand and then placed in cold water. Nodules were picked manually and placed in 20-ml beakers on ice until extraction. Root and shoot dry weights were determined after drying to constant weight at 80 C.

Acetylene Reduction Activity. N_2 fixation rates of intact nodules on excised root systems were estimated by the acetylene reduction technique. Triplicate plant samples were gently shaken free of sand, tops were removed at the crown, and were immediately assayed in sealed 1-liter jars as described by Vance et al. (26). After incubation in the glasshouse (20–23 C) for 60 min, acetylene-dependent ethylene production was measured using GLC. Rates per plant were divided by nodule mass per plant and data expressed as nmol C_2H_2 min^{-1} g^{-1} fresh weight nodules.

Preparation of Nodule Cell-free Extracts. Samplings of excised nodules (300–400 mg) were extracted by hand at 0 to 3 C in a ground glass homogenizer (Kontes, Vineland, NJ) with 1 ml (100 mg nodules)$^{-1}$ extraction buffer consisting of 100 mM Mes-NaOH, pH 6.8, 100 mM sucrose, 2% (v:v) 2-mercaptoethanol, 15% (v:v) ethylene glycol. The resulting homogenates were centrifuged at 3,500g for 8 min, PMSF was added to the supernatant fractions to 0.1 mM as a protease inhibitor, and centrifugation at 30,000g for 20 min produced a clear solution of the host plant cell cytoplasm and its organellar contents. This fraction was assayed for plant enzyme activities and soluble protein.

Enzyme Assays. Nodule host plant enzyme activities were determined as initial rates of reaction at 23 to 25 C. Optimum conditions of buffer, pH, substrate concentrations, etc. resulting in maximum enzyme activity were determined for each enzyme to be measured and were subsequently used for assays. Enzyme activities were directly proportional to volume of extract added in the ranges used for assays.

GDH and GOGAT were measured spectrophotometrically, monitoring absorbance due to NADH at 340 nm. NAD-GDH (oxidative deamination) was determined with 500 μM NAD and 50 mM L-glutamate in 100 mM Tris-HCl, pH 8.0. NADH-GDH (reductive amination) and NADH-GOGAT were assayed in 100 mM K^+-phosphate, pH 7.6, containing 0.1% (v:v) 2-mercaptoethanol. Substrate concentrations for NADH-GDH assays were 100 μM NADH, 2.5 mM α-ketoglutarate, and 200 mM NH_4^+ (as SO_4^{2-}). NADH-GOGAT activity was measured using 100 μM NADH, 2.5 mM α-ketoglutarate, 10 mM L-glutamine, and 1 mM aminooxyacetate (to inhibit aminotransferase activities). GDH and GOGAT activities were corrected for endogenous NAD^+ (NADH) reduction (oxidation) in the absence of added substrate.

Nodule NADH-GOGAT activity was highly unstable in commonly used extraction buffers (e.g. Tris, phosphate) of pH greater than 7.0 and was completely lost upon freezing and thawing of these extracts. The Mes, pH 6.8, extraction solution used in this experiment, including high concentrations of 2-mercaptoethanol and ethylene glycol, minimized loss of plant NADH-GOGAT activity in alfalfa nodule extracts to 1 to 2%/h without affecting GDH and GS activities. All measurements of NADH-GOGAT activity were made within 3 to 4 h of extraction.

GS activity in nodule extracts was determined by measuring incorporation of radioactivity from L-[^{14}C]glutamate into glutamine using previously described procedures (18). GS assays were conducted with 10 mM NH_4^+ (as SO_4^{2-}), 10 mM ATP, 11 mM $MgSO_4$, 10 mM [^{14}C]glutamate (20 μCi mol^{-1} [U-^{14}C]glutamate; ICN, Irvine, CA), and 100 mM Tricine-NADH buffer, pH 8.3, in a final reaction volume of 250 μl. The reaction was terminated with 1 ml of ice-cold water and small columns (7.5 × 40 mm) of Dowex 1 (acetate form) were used to separate [^{14}C]glutamine from unreacted [^{14}C]glutamate. Columns were eluted with 4 ml water and product formed was measured using liquid scintillation spectrometry. Assay controls consisted of reaction mixtures lacking ATP but were close to zero in all cases. Plant GS activity in nodule extracts was relatively stable at −20 C. Assays were conducted within 48 h at which time GS activities had not changed from values measured within 2 h of extraction.

Protein Determination. Soluble protein was measured by the method of Bradford (3) using a reagent consisting of 0.2% Coomassie Brilliant Blue G-Type in 3.5% (w:v) $HClO_4$. BSA was used for constructing standard curves (20–300 μg protein).

RESULTS

Plant Growth. Shoot dry weight of all harvested plants increased slowly for 10 days (Fig. 1) but increased significantly between days 10 and 15. Treatment with either 40 or 80 kg NO_3^--N ha^{-1} had no effect on shoot regrowth of harvested plants (Fig. 1, B and C).

Shoot dry weight of unharvested alfalfa increased throughout the course of the experiment (Fig. 1). Shoot growth of unharvested plants was not affected by applied nitrogen (Fig. 1, B and C).

Root weight of harvested plants decreased slightly during the experiment while that of control plants increased slightly (not shown). Applied nitrogen had no significant effect on root weight of either harvested or unharvested alfalfa (0.8–1.2 g dry weight $plant^{-1}$). Nodule mass remained relatively constant for both control and treated plants during the experiment (80–120 mg fresh weight $plant^{-1}$).

Acetylene Reduction Activity. Nitrogen fixation potential, estimated by acetylene reduction activity, of harvested plants not treated with NO_3^- declined to 30% of control within 24 h and remained low (20–25% of control) through day 10 (Fig. 2A). As shoot regrowth accelerated between days 10 and 15, acetylene reduction activity began to recover. In contrast to the pattern observed for harvested plants, nodule specific activity of control plants did not change significantly during the experiment.
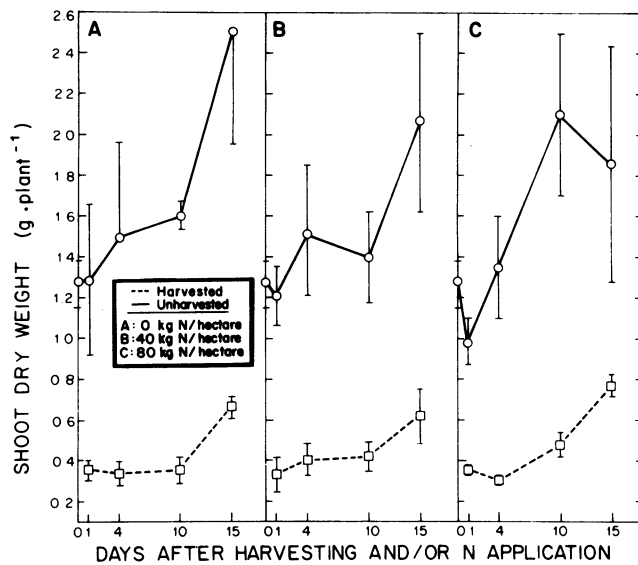
FIG. 1. Shoot growth of harvested and unharvested alfalfa treated with 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

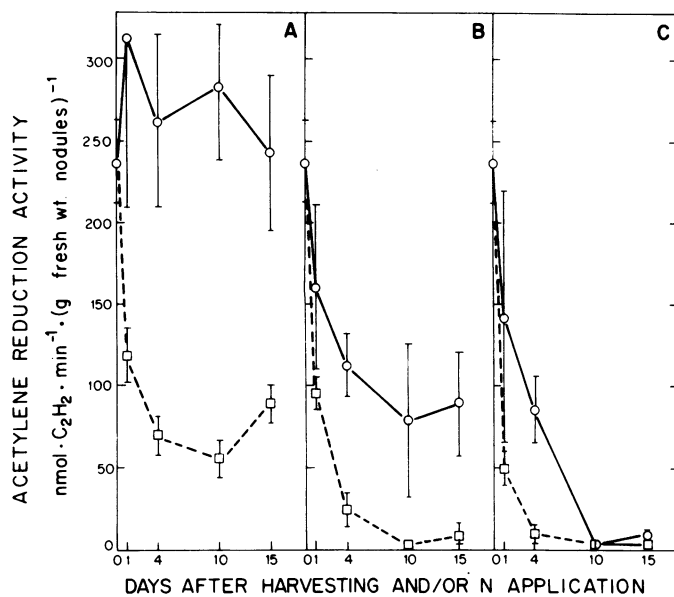

FIG. 2. Acetylene reduction specific activity of harvested and unharvested alfalfa treated with 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

FIG. 3. Changes in nodule-soluble protein following harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

FIG. 4. Patterns of alfalfa nodule GS specific activity following harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

Acetylene reduction activity of unharvested alfalfa declined 50% within 24 h after treatment with either 40 or 80 kg NO_3^--N ha^{-1} (Fig. 2, B and C). Nodule specific activity of uncut plants treated with 40 kg N ha^{-1} (Fig. 2B) further declined to 25% of untreated controls by day 10 and began to recover by day 15. In contrast, specific activity of unharvested alfalfa treated with 80 kg N ha^{-1} (Fig. 2C) declined to essentially zero (1-2% control) by day 10 and showed no recovery by day 15.

Acetylene reduction activity of plants that were both harvested and treated with NO_3^- was at all times less than that of plants receiving either treatment alone (Fig. 2, A, B, and C). The effects of both harvesting and N application on nodule specific activity were approximately additive.

Nodule Soluble Protein. After an initial lag of 24 h, nodule soluble protein (Fig. 3) followed a pattern similar to that of acetylene reduction activity in response to harvesting and/or N
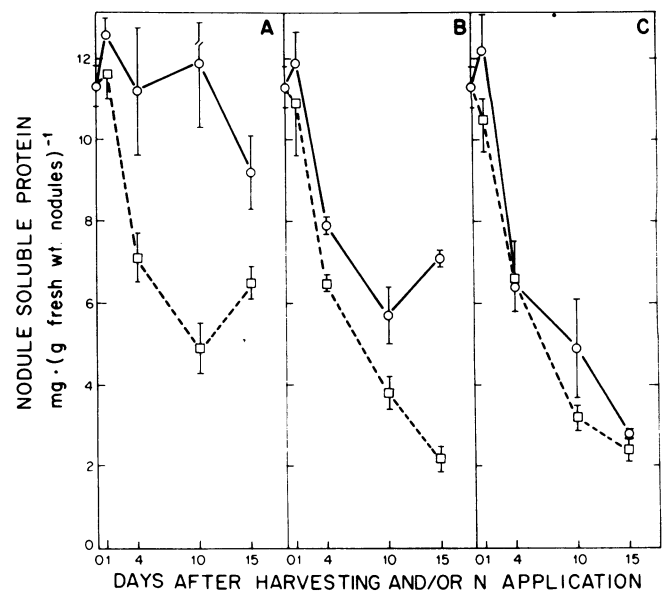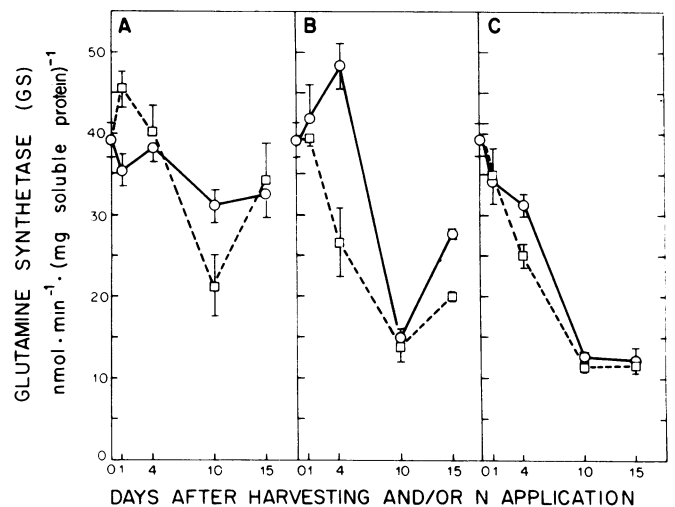
application. Nodule soluble protein of harvested alfalfa not treated with NO_3^- (Fig. 3A) and unharvested plants treated with 40 kg NO_3^--N ha^{-1} (Fig. 3B) decreased initially but began to recover by day 15. Soluble protein in nodules of harvested plants treated with either 40 or 80 kg NO_3^--N ha^{-1} (Fig. 3, B and C) decreased steadily throughout the experiment to 20% of control by day 15. In contrast, nodule soluble protein levels of unharvested controls did not change through day 10 and had decreased slightly by day 15 (Fig. 3A).

Nodule GS and GOGAT. Initial in vitro activities of nodule host plant GS and NADH-GOGAT (at peak acetylene reduction activity) were 870 ± 20 and 1100 ± 60 nmol min^{-1} g^{-1} fresh weight nodules. Corresponding specific activities were: GS = 78 ± 5; NADH-GOGAT = 97 ± 2 nmol min^{-1} mg^{-1} protein.

Nodule GS specific activity of harvested plants not treated with NO_3^- (Fig. 4A) did not change relative to that of unharvested controls until day 10, whereas nodule NADH-GOGAT specific

activity decreased steadily during this period (Fig. 5A). Both GS and NADH-GOGAT specific activities recovered to levels equal to those of the control by day 15.

Treatment of unharvested alfalfa with 40 kg NO_3^--N ha^{-1} slightly increased nodule host plant GS specific activity (Fig. 4B) and slightly decreased that of NADH-GOGAT (Fig. 5B) through day 4. Specific activities of both enzymes decreased sharply between day 4 and day 10 (to 40–50% control) and then recovered to 85–95% of control by day 15. Specific activities of nodule GS and NADH-GOGAT decreased steadily in response to applied NO_3^- at 80 kg N ha^{-1} (Figs. 4C and 5C).

Nodule GS specific activity of plants that were both harvested and treated with 40 kg NO_3^--N ha^{-1} decreased to 45% of control between day 1 and day 10 and increased slightly by day 15 (Fig. 4B). Nodule NADH-GOGAT specific activity of plants both harvested and treated with NO_3^--N declined steadily during this period, and stabilized at a level 15% of that of the control (Fig. 5B). Specific activities of these enzymes in nodules of untreated controls remained relatively unchanged for the duration of the experiment (Figs. 4A and 5A).

Nodule GDH. Nodule NADH-GDH (reductive amination) activity expressed on a fresh weight basis (Fig. 6) followed a pattern similar to that of acetylene reduction activity in response to either harvesting and/or N application. In contrast, NADH-GDH specific activity expressed on a unit-soluble protein basis (Fig. 7) remained the same or increased during the period of rapidly declining acetylene reduction activity, compared to that of untreated controls. Application of either 40 or 80 kg NO_3^--N ha^{-1} significantly increased nodule NADH-GDH specific activity, particularly of harvested alfalfa (Fig. 7, B and C).

Nodule NAD-GDH (oxidative deamination) specific activity declined in response to either harvesting or applied NO_3^- (Fig. 8). Nodule NAD-GDH specific activity of harvested plants not treated with NO_3^- declined to a minimum level by day 10 (45% control) and then recovered to 135% control by day 15 (Fig. 8A). For all plants treated with 80 kg NO_3^--N ha^{-1} nodule NAD-GDH specific activity decreased steadily throughout the experiment to 20% of control by day 15 (Fig. 8B). NAD-GDH specific activity in nodules of untreated controls declined slightly during the experiment (Fig. 8A).

The ratio of nodule GDH reductive amination and oxidative deamination activities, (NADH-GDH to NAD-GDH), varied considerably during the experiment. Values of (NADH-GDH to
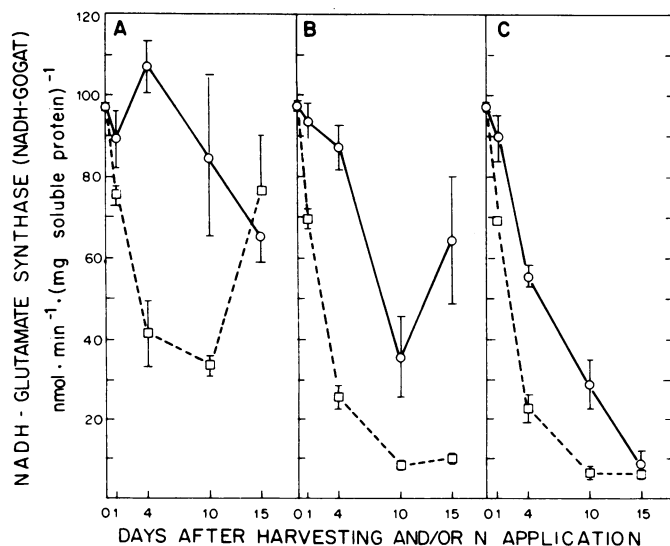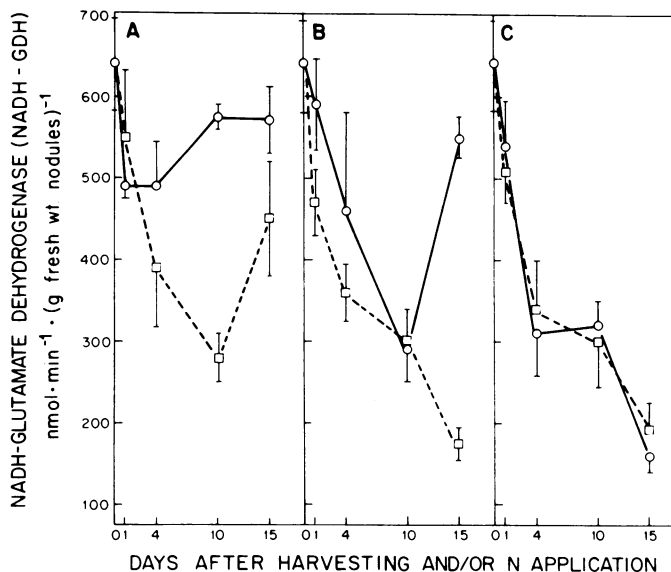

FIG. 6. Effects of harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C) on fresh weight based activity of alfalfa nodule NADH-GDH (reductive amination). Each point is the mean of three replicates ±SE.
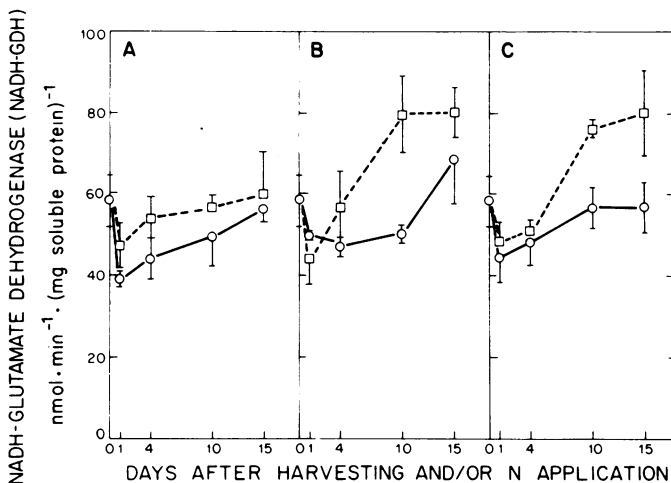

FIG. 7. Time course of specific activity of alfalfa nodule NADH-GDH (reductive amination) following harvest and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

NAD-GDH) near 1.0 were associated with highest acetylene reduction activities and this ratio increased as acetylene reduction activities declined in response to harvesting and/or applied NO_3^-. (NADH-GDH to NAD-GDH) increased to 6.5, for example, by day 15 following treatment of harvested alfalfa with 80 kg NO_3^-- N ha^{-1}.

DISCUSSION

Harvesting shoots and applying NO_3^- caused a rapid decline in N_2 fixation potential of alfalfa nodules. As shoots began to regrow and as N was depleted from the rooting medium nodule function also began to recover. Changes in nodule N_2-fixing capacity were accompanied by similar changes in the specific activities of nodule host plant GS, NADH-GOGAT, and NAD-GDH. Harvesting of shoots and application of NO_3^- did not cause a large scale shedding of nodules. This observation is consistent with previous reports and supports the concept that alfalfa nodules can undergo

FIG. 5. Effects of harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C) on alfalfa nodule NADH-GOGAT specific activity. Each point is the mean of three replicates ±SE.
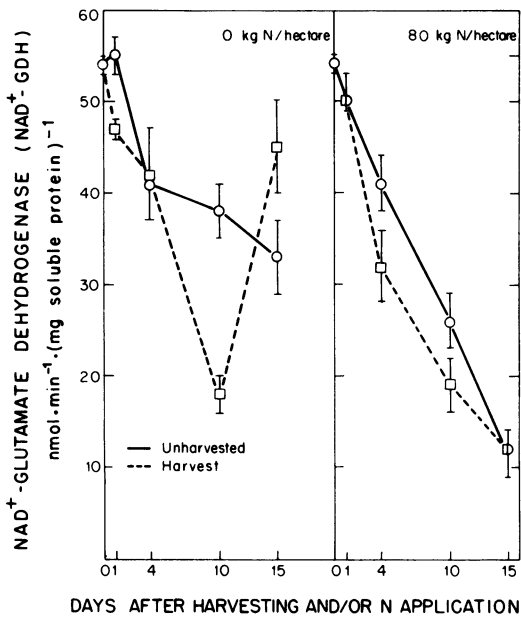
FIG. 8. Time course of alfalfa nodule NAD-GDH (oxidative deamination) specific activity following harvest and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Each point is the mean of three replicates ±SE.

temporary localized senescence and subsequently recover as shoots regrow or soil N is depleted (25, 26).

Kinetics of shoot regrowth following harvest are similar to those reported previously (22, 26). Applied N did not affect shoot dry matter accumulation suggesting that N supply may not be a major limitation to growth and development of new shoot tissue following harvest.

The rapid decline in acetylene reduction activity in response to harvesting and N application has been noted previously in perennial legumes (13, 16, 25, 26). This response is generally attributed to reduced photosynthate supply to nodules. The decline in acetylene reduction activity of unharvested alfalfa caused by combined N (Fig. 2, B and C) may involve factors other than reduced photosynthate supply. Concentrations of NO_3^- or NH_4^+ necessary for repression of acetylene reduction activity are higher, and the kinetics of this repression are slower for alfalfa nodules than for free living, N_2-fixing R. meliloti (10). Concentrations of applied N that result in loss of nodule function are well below levels that are toxic to plant root tissue (7). The mechanism of combined N-induced loss of N_2-fixing capacity of legumes is obscure, but it appears to be a means by which symbiotic N_2 fixation is shut down in favor of utilization of soil N. The presence of combined N in the soil inhibits alfalfa nodulation by R. meliloti (8). Such effects of combined N on symbiotic N_2 fixation suggest utilization of soil N may be energetically a less expensive means of obtaining N for plant growth than symbiotic N_2 fixation.

In vitro specific activity of nodule NADH-GOGAT closely paralleled the changes observed in acetylene reduction activity. This observation suggests a close association between N_2 fixation and nodule GOGAT activity. The presence of plant GOGAT in alfalfa nodules has been the subject of controversy. Duke et al. (6) found no GOGAT in alfalfa nodule extracts and suggested the GS/GOGAT pathway of ammonia assimilation may not operate in alfalfa nodules. In contrast, Boland et al. (2) reported NADH-GOGAT in a wide variety of legume nodules including alfalfa. We found alfalfa nodule NADH-GOGAT activity to be very labile and extracts that have been frozen overnight display little or no GOGAT activity in most common extraction buffers. The extraction buffer described in this manuscript minimized loss of

NADH-GOGAT activity in nodule extracts to 1 to 2% h^{-1}. This buffer also was satisfactory for extracting NADH-GOGAT activity from a variety of other legume nodules (Groat and Vance, unpublished). Azaserine (a glutamine analog) at 1 mM caused 90% inhibition of NADH-GOGAT activity, but had no effect on NADH-GDH activity. A radioisotopic assay involving conversion of [^{14}C]glutamine to glutamate gave NADH-GOGAT activities similar to spectrophotometric assays. These results indicate previous reports of the absence of plant GOGAT in alfalfa nodules (4, 6) reflect unsuitable extraction and/or assay conditions. The apparent K_m for glutamine of alfalfa nodule NADH-GOGAT was 0.3 mM, similar to that reported for other legume nodules (2).

Alfalfa nodule GS specific activity declined upon harvesting and application of NO_3^-, but the decline lagged behind decreasing acetylene reduction activities. The decline in GS specific activity was not as great as that of NADH-GOGAT or acetylene reduction. The recovery of nodule GS in harvested plants grown on 0 N and in unharvested plants treated with 40 kg NO_3^--N ha^{-1} coincided with increased shoot growth, acetylene reduction, and GOGAT specific activity. These data suggest nodule GS is associated with N_2 fixation in alfalfa and that there may be residual pools of ammonia in nodules or other factors that buffer against rapid changes in GS specific activity. Plant GS and GOGAT are proposed to function in a cyclic manner, the product of each serving as substrate for the other (19). Data in this experiment indicate nodule GS and GOGAT may not be tightly coupled. The coupling of GS to GOGAT may not be required as long as glutamate could be supplied as substrate for GS by some other mechanism (e.g. via NADH-GDH or transamination).

Some newly assimilated glutamine amide N is exported from nodules, either directly or as asparagine amide N formed by means of a glutamine-dependent asparagine synthetase. In legume nodules with ureide-based N metabolism, glutamine amide N may be utilized directly for purine biosynthesis leading to ureide production (19). Legumes which export symbiotically fixed N as ureides, consistently have lower host plant NADH-GOGAT/GS ratios than are found for legumes with amide-based nodule N metabolism (Groat and Vance, unpublished).

High levels of nodule GS have been reported previously in alfalfa (2, 4, 6). It should be noted, however, that previous measurements of GS in alfalfa nodules and other plant tissues have been conducted using an indirect assay involving reaction with hydroxylamine (instead of ammonia) to form γ-glutamyl hydroxamate. This "transferase" assay greatly overestimates the physiological activity of nodule GS (11) and has other potentially misleading features (5). The radioisotopic assay used in our experiments permits accurate measurement of GS activity with ammonia. The apparent K_m for NH_4^+ of GS in alfalfa nodule extracts was 0.2 mM suggesting this enzyme has a high affinity for NH_4^+ and is involved in nodule ammonia assimilation.

High activity of plant NADH-GDH in N_2-fixing legume nodules is well documented but its role in symbiotic N_2 fixation remains obscure (2, 4, 6, 19). Alfalfa nodule NADH-GDH on a fresh weight basis responded to harvesting and applied N similarly as acetylene reduction activity. When expressed on a unit protein basis specific activity of NADH-GDH (Fig. 7) remained constant or increased slightly. This difference reflects a constant or increasing level of functional enzyme with changing levels of nodule soluble protein. These data suggest nodule plant NADH-GDH activity is not closely associated with N_2 fixation and that NADH-GDH may be more associated with ammonia assimilation during induced nodule senescence.

Nodule NAD-GDH activity has not been reported previously in alfalfa. NADPH-linked GDH activity in our nodule extracts was negligible. Plants with high acetylene reduction activity also had high levels of nodule NAD-GDH activity resulting in observed ratios of (NADH-GDH to NAD-GDH) near 1.0. Increas-

ing values of (NADH-GDH to NAD-GDH) observed as a result of harvest and/or applied N reflect rapidly declining NAD-GDH specific activity. High relative levels of plant NAD-GDH activity may be unique to N_2-fixing nodules. In other plant tissues, NADH-GDH activity generally exceeds NAD-GDH activity by a factor of 10 or more (7). A K_m for NH_4^+ of 80 mM was determined for alfalfa nodule NADH-GDH in the present study. K_m (NH_4^+) values in the range of 40 to 100 mM are commonly found for plant GDH (2, 23) suggesting to many authors that GDH does not participate in ammonia assimilation in higher plant tissues under normal conditions (2, 7, 19).

Expressed as nmol min^{-1} g^{-1} fresh weight nodules, day zero *in vitro* activities of either GS (870 ± 20) or NADH-GDH (640 ± 50) were sufficiently high to account for theoretical rates of ammonia production by nitrogenase (acetylene reduction activity = 240 ± 30). Nodule GS and NADH-GOGAT specific activities varied along with acetylene reduction activity following harvest and/or N application; but NADH-GDH specific activity did not.

The results of this study indicate plant GS and NADH-GOGAT function to assimilate symbiotically fixed N in alfalfa root nodules. The observed changes in specific activities of ammonia-assimilating enzymes relative to one another are evidence of a major shift in alfalfa nodule N metabolism during senescence induced by harvesting and/or applied NO_3^-. Levels of nodule GS, GOGAT, and GDH are useful indicators of host plant metabolism associated with symbiotic N_2 fixation in alfalfa.

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