Plant Physiol. (1983) 72, 469–473
0032-0889/83/72/0473/05/$00.50/0

Alfalfa Root Nodule Carbon Dioxide Fixation[1, 2]

I. ASSOCIATION WITH NITROGEN FIXATION AND INCORPORATION INTO AMINO ACIDS

Received for publication December 27, 1982 and in revised form February 22, 1983

CARROLL P. VANCE, SUSAN STADE, AND CARL A. MAXWELL
United States Department of Agriculture, Science and Education Administration, Agricultural Research Service, and the Department of Agronomy and Plant Genetics, The University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT

In vivo **CO_2 fixation activity and *in vitro* phosphoenolpyruvate carboxylase activity were demonstrated in effective and ineffective nodules of alfalfa (*Medicago sativa* L.) and in the nodules of four other legume species. Phosphoenolpyruvate carboxylase activity was greatly reduced in nodules from both host and bacterially conditioned ineffective alfalfa nodules as compared to effective alfalfa nodules.**

Forage harvest and nitrate application reduced both *in vivo* and *in vitro* CO_2 fixation activity. By day 11, forage harvest resulted in a 42% decline in *in vitro* nodule phosphoenolpyruvate carboxylase activity while treatment with either 40 or 80 kilograms nitrogen per hectare reduced activity by 65%. *In vitro* specific activity of phosphoenolpyruvate carboxylase and glutamate synthase were positively correlated with each other and both were positively correlated with acetylene reduction activity.

The distribution of radioactivity in the nodules of control plants (unharvested, 0 kilograms nitrogen per hectare) averaged 73% into the organic acid and 27% into the amino acid fraction. In nodules from harvested plants treated with nitrate, near equal distribution of radioactivity was observed in the organic acid (52%) and amino acid (48%) fractions by day 8. Recovery to control distribution occurred only in those nodules whose *in vitro* phosphoenolpyruvate carboxylase activity recovered.

The results demonstrate that CO_2 fixation is correlated with nitrogen fixation in alfalfa nodules. The maximum rate of CO_2 fixation for attached and detached alfalfa nodules at low CO_2 concentrations (0.13–0.38% CO_2) were 18.3 and 4.9 nanomoles per hour per milligram dry weight, respectively. Nodule CO_2 fixation was estimated to provide 25% of the carbon required for assimilation of symbiotically fixed nitrogen in alfalfa.

Current estimates with annual legumes suggest that 30% of the carbon gained through photosynthesis in the shoot is used for nodule function and maintenance and that approximately 60% of the carbon partitioned to the nodules is lost as CO_2 through respiration (14, 16). This results in a loss of 18% of the total photosynthate through the nodule to the atmosphere. Recent studies of annual legumes suggest that nonphotosynthetic CO_2 fixation via nodule PEP[3] carboxylase (EC 4.1.1.31) acts as a mechanism for recovery of some of this respired CO_2, thus increasing nodule efficiency and providing an added source of carbon for assimilation of fixed N.

Studies with lupine and soybean suggest different relationships between N_2 fixation and PEP carboxylase. In lupine, manipulations that reduced nitrogen fixation activity caused a concomitant decrease in PEP carboxylase activity (12). Coker and Schubert (7) found that CO_2 fixation activity declined in advance of the decrease in N_2 fixation activity in soybean nodules.

Several investigations on the role of PEP carboxylase in nodule metabolism have involved exposure of either excised or attached nodules to $^{14}CO_2$. Lawrie and Wheeler (13) demonstrated that high levels of radioactivity were initially associated with glutamate and aspartate and later with asparagine in excised nodules of *Vicia faba* after exposure to $^{14}CO_2$. In excised lupine nodules, aspartate was the only amino acid labeled within the first 10 min of exposure to [3,4-^{14}C]glucose (12). Coker and Schubert (6) showed that when intact nodulated roots of soybean were exposed to $^{14}CO_2$, label was initially incorporated into organic acids with subsequent incorporation into aspartate and glutamate. The first detectable labeled product in *Pisum sativum* nodules exposed to $^{14}CO_2$ was malate with subsequent rapid conversion to amino acids (10). Cookson *et al.* (8) showed that amino acids accounted for approximately 25% of the carbon fixed from $^{14}CO_2$ in excised nodules of *Phaseolus vulgaris* and that tricarboxylic acid cycle intermediates accounted for 60 to 70% of the radioactivity recovered in bleeding xylem sap, while the remainder was found in aspartate, arginine, lysine, and allantoin.

Comparable investigations on the role of nodule PEP carboxylase in perennial legumes are lacking. In addition, there is a large disparity in the estimated carbon input from PEP carboxylase to nodule N assimilation in annual legumes (5, 7, 26), indicating that the contribution of this system to N_2 fixation and N assimilation is poorly understood. The contribution of PEP carboxylase to nodule N and C economy of perennial forage legumes has not been investigated.

Earlier studies on alfalfa nodule metabolism in our laboratory have detailed the changes in nodule structure, the activity of nitrogenase, and the major N-assimilating enzymes following forage harvest and N application (11, 22). The objectives of this study were to identify the major products of CO_2 fixation in alfalfa nodules before and after forage harvest and N application, and to investigate the relationship between PEP carboxylase activity and N_2 fixation.

[1] Contribution No. 13,088 from the Minnesota Agricultural Experiment Station. This research was supported in part by the United States Department of Agriculture, Science and Educational Administration, under Grant 82-CRCR-1-1124 from the Competitive Research Grants Office.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by either the United States Department of Agriculture or approval to the exclusion of other products that may also be suitable.

[3] Abbreviations: PEP, phosphoenolpyruvate; GOGAT, NADH-dependent glutamate synthase; 2,4-DNPH, 2,4-dinitrophenylhydrazine; GS, NH_3-dependent glutamine synthetase; OA, organic acid; AA, amino acid; MDH, malic dehydrogenase; LSC, liquid scintillation counting; AS, asparagine synthetase; AAT, aspartate aminotransferase.

MATERIALS AND METHODS

Plant Material. Adzuki bean (*Vigna angularis* WILLD. Ohioi and Ohashi), navy bean (*Phaseolus vulgaris* L.), and soybean (*Glycine max* L.) nodules were obtained from field-grown plants in the R1 stage. Alfalfa (*Medicago sativa* L.) and birdsfoot trefoil (*Lotus corniculatus* L.) nodules were obtained from vegetative plants grown under conditions previously described by Vance *et al.* (21). Ineffective nodules regulated by host plant factors were produced on alfalfa clones MnPL-480(In), MnNC-3226(In), MnNC-3811(In), MnSa(In), and MnAg(In) (17). Characterization and conditions required for the growth and maintenance of these clones is as described for MnPL-480(In) by Viands *et al.* (24). Bacterially induced ineffective nodules and effective nodules were produced on alfalfa plants (cv Saranac) grown in enclosed culture tubes on agar (23). Seedlings in culture tubes were inoculated either with *Rhizobium meliloti* strain 102F51 (effective) or with one of the following ineffective strains: 1029, 1054, 1064, or 1058 (donated by Dr. S. Long, Stanford University, Palo Alto, CA). Nodules were collected and PEP carboxylase activity was assayed 6 to 8 weeks after inoculation.

Alfalfa plants (cv Saranac) used in NO_3^- and forage harvest (75% shoot removal) experiments were grown in a sand bench as described by Groat and Vance (11). Plants were sampled at 0, 1, 8, 11, 15, and 22 d after treatment. Nodules in all experiments reported in this paper were picked manually, placed in beakers on ice, and used within 30 min for enzyme extraction or for determination of *in vivo* CO_2 fixation activity.

Preparation of Nodule Cell-Free Extracts and Enzyme Assays. Excised nodules of all plant types (100–200 mg) were extracted in a Mes-NaOH buffer system (11) for stabilization of the plant cytosol enzyme activities. The homogenates were centrifuged at 18,100*g* for 20 min and the clear supernatant fraction was placed on ice in capped vials until assayed for plant enzyme activities and soluble protein.

Conditions for the assay of nodule NADH-GOGAT and GS activities were described previously (11). Rates of PEP carboxylase activity were measured spectrophotometrically in a coupled assay system involving MDH as previously described for the soybean nodule enzyme (18). PEP carboxylase activity was assayed at 25°C in 100 mM Bicine, pH 8.5, containing 2 mM PEP, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, and 1.6 mM NADH. Exogenous addition of MDH activity did not increase the *in vitro* PEP carboxylase activity in any of the nodule extracts assayed.

Protein Determination. Soluble protein was measured in nodule extracts by the method of Lowry *et al.* (15) following precipitation of the protein with 7% TCA. A standard curve was constructed using BSA (12.5–87.5 μg protein).

Acetylene Reduction Activity. The acetylene reduction assay described by Vance *et al.* (21) was used to estimate rates of $N_2(C_2H_2)$ fixation by intact alfalfa nodules on excised root systems.

Product Identification. Oxaloacetate was identified as the initial radioactive product of the $^{14}CO_2$ fixation reaction in alfalfa root nodules by 2,4-DNPH derivatization and TLC with authentic standards. The 2,4-DNPH derivative was prepared according to Bachelard (2) and separated by TLC on silica gel plates (Brinkmann Instruments Inc., Des Plaines, IL)[2] in a petroleum ether:ethyl acetate:acetic acid (13:7:2) solvent system for 90 min (19).

In Vivo CO_2 Fixation Assay. The *in vivo* CO_2 fixation assay was modified from Christeller *et al.* (5). Alfalfa root nodules (100 mg fresh weight) were placed on moist filter paper at the bottom of a sealed 10-ml reaction flask (Kontes, Vineland, NJ). The assay was initiated by injection of 4 M lactic acid into center wells containing 8 μCi of aqueous $NaH^{14}CO_3$ (42 to 52 mCi $mmol^{-1}$, ICN, Irvine, CA) suspended above the nodules. After incubation for 30 min at 23°C, the reaction was terminated by injection of 1.5 ml of hot 50% ethanol (70°C) onto the nodules and the flasks were opened to release any unreacted $^{14}CO_2$. The nodule samples were homogenized in 50% ethanol, followed by extraction in a 45°C water bath for 20 min and centrifugation at 18,100*g* for 15 min. An aliquot of the supernatant was treated with HCl and the acid-stable radioactivity was determined by LSC.

Separation of Labeled Nodule Extracts. ^{14}C-labeled alfalfa root nodule extracts were separated into AA, OA, and neutral fractions using Dowex ion exchange resins prepared according to Atkins and Canvin (1). Samples were successively passed through Dowex 50W, and Dowex 1 columns followed by 25 ml of 50% ethanol. This eluant was collected as the neutral fraction. The AA fraction was eluted from the Dowex 50W column with 25 ml of 2 M NH_4OH while the OA fraction was eluted from the Dowex 1 column with 25 ml of 6 M formic acid. Fractions were lyophilized to dryness and resuspended in 600 μl of 50% ethanol (OA and neutral fractions) or 20% ethanol (AA fraction). The total radioactivity incorporated into each fraction was determined by LSC.

RESULTS

Legume *in vitro* nodule PEP carboxylase, GOGAT, and GS specific activities are shown in Table I. Alfalfa nodule PEP carboxylase specific activity averaged 644 ± 60 nmol min^{-1} mg^{-1} protein, the highest of any of the nodules investigated, while birdsfoot trefoil nodules, with an average PEP carboxylase specific activity of 296 ± 47 nmol min^{-1} mg^{-1} protein, had the lowest specific activity. Adzuki bean, soybean, and navy bean nodules had levels of PEP carboxylase activity intermediate to those of alfalfa and birdsfoot trefoil. Nodule GOGAT specific activities

Table I. *Phosphoenolpyruvate Carboxylase (PEPC), Glutamine Synthetase, and Glutamate Synthase Activity in Several Legumes*

Each value is the mean ± SE of three replications.

Legume	Nodule Protein	PEPC	GS	GOGAT
	mg protein g^{-1} fresh wt	*nmol min^{-1} mg^{-1} protein*		
Soybean	14.8 ± 0.5	488 ± 26	254 ± 2	23 ± 4
Adzuki bean	14.1 ± 0.3	415 ± 8	198 ± 8	14 ± 1
Navy bean	11.1 ± 1.4	494 ± 54	284 ± 13	22 ± 3
Birdsfoot trefoil	11.5 ± 0.8	296 ± 47	112 ± 17	18 ± 5
Alfalfa	20.1 ± 1.4	644 ± 60	140 ± 20	53 ± 3

Table II. *Comparisons of PEP Carboxylase (PEPC) in Nodules Induced by Various Strains of R. meliloti and in Ineffective Alfalfa Genotypes*

Nodule Source	Nitrogenase[a]	PEPC[b]
		nmol min^{-1} mg^{-1} protein
R. meliloti strain		
1029	−	74 ± 10
1054	−	31 ± 4
1064	−	24 ± 3
1058	−	87 ± 4
102F51	+	309 ± 56
Alfalfa genotype		
MnSa(In)	−	68 ± 20
MnAg(In)	−	119 ± 30
MnPL-480(In)	−	19 ± 4
MnNC-3226(In)	−	34 ± 7
MnNC-3811(In)	−	83 ± 10
Saranac	+	636 ± 50

[a] Nitrogenase is based on acetylene reduction activity after 120 min incubation period. + = activity; − = no activity.

[b] Each value is the mean ± SE of three replications.

were positively correlated to PEP carboxylase, with alfalfa nodules displaying the highest activity and adzuki bean nodules the lowest specific activity. Nodule GS activity of bean species was substantially higher than that of either alfalfa or birdsfoot trefoil. *In vivo* CO_2 fixation assays were also performed on excised nodules of all of these legumes and the ranking of activity was similar to that obtained with the *in vitro* assay.

To evaluate how alfalfa nodule PEP carboxylase activity was related to N_2 fixation capability, *in vitro* PEP carboxylase activity was measured in both bacterial and host plant conditioned-ineffective nodules. In all cases, whether ineffectiveness was the result of bacterial or host genetic factors, the *in vitro* PEP carboxylase activity of ineffective nodules was substantially lower than that of effective nodules (Table II). The mean PEP carboxylase specific activity of *Rhizobium*-induced ineffective nodules on plants grown in tube culture (23) was 54 nmol min^{-1} mg^{-1} protein whereas that of comparably grown effective nodules was 309 nmol min^{-1} mg^{-1} protein. The unusually low activity obtained for effective nodule PEP carboxylase of plants in the *Rhizobium*-induced experiments was probably the result of the plants growing in the tube culture environment. Similarly, the mean PEP carboxylase specific activity of host plant-induced ineffective nodules was 64 nmol min^{-1} mg^{-1} protein whereas that for effective nodules was 636 nmol min^{-1} mg^{-1} protein. Nodule CO_2 fixation was associated with effective N_2 fixation.

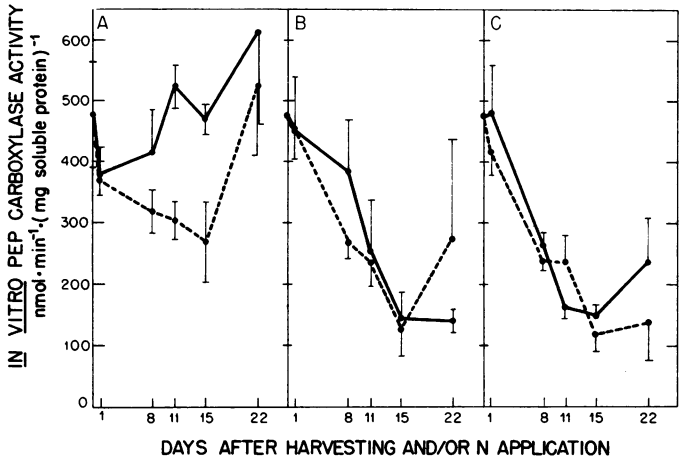

FIG. 1. Patterns of *in vitro* PEP carboxylase specific activity following harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C). Shoots were harvested (- - -) and allowed to regrow for 22 d while shoots of control plants (——) were not harvested. Each point is the mean of three replicates ± SE.

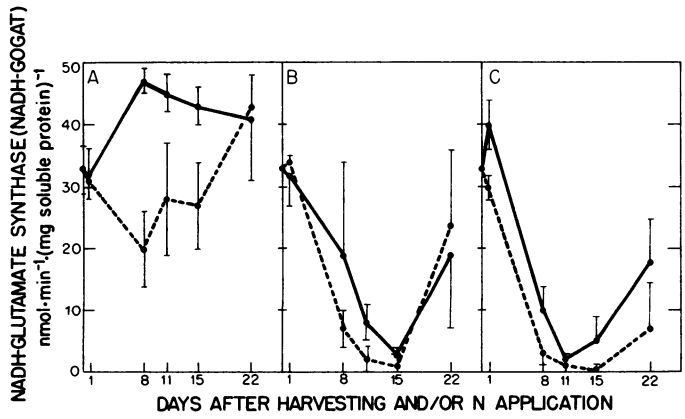

FIG. 2. Effects of harvesting and/or application of 0 (A), 40 (B), and 80 kg NO_3^--N ha^{-1} (C) on alfalfa nodule NADH-GOGAT specific activity. Shoots were harvested (- - -) and allowed to regrow for 22 d while shoots of control plants (——) were not harvested. Each point is the mean of three replicates ± SE.

Forage harvest and NO_3^- reduced *in vitro* PEP carboxylase activity of alfalfa nodules (Fig. 1). PEP carboxylase activity in harvested plants not treated with NO_3^- declined to 58% of the control (unharvested, 0 kg N ha^{-1}) by 11 d after cutting and then recovered to 85% of the control value by day 22. Treatment with either 40 or 80 kg N ha^{-1} resulted in a steady decline in PEP carboxylase specific activity in the nodules of unharvested and harvested plants. By day 15, PEP carboxylase specific activities in nodules from NO_3^--treated plants (harvested and unharvested) averaged 29% of the day 0 value. PEP carboxylase activity in nodules of harvested plants treated with 40 kg N ha^{-1} began to show a slight recovery trend between days 15 and 22 although the differences between harvested and unharvested values were not statistically significant. Nodule soluble protein concentrations substantiated this recovery (data not shown). No recovery of PEP carboxylase activity was evident in nodules from plants treated with 80 kg N ha^{-1}. Patterns of *in vivo* nodule CO_2 fixation activity were similar to *in vitro* nodule PEP carboxylase activity for all treatments.

Similar to previous studies (11), *in vitro* nodule GOGAT activity changed in response to harvest and applied NO_3^-. Nodule GOGAT specific activity decreased sharply as a result of harvest in plants not treated with NO_3^- between days 1 and 8 and then showed a steady increase, with activity recovering to control levels by day 22 (Fig. 2). Nodule GOGAT specific activity of plants treated with either 40 or 80 kg N ha^{-1} in both the harvested and unharvested treatments displayed a steady decline through day 15 and subsequently began to recover between days 15 and 22. Recovery of GOGAT activity was greater in nodules treated with 40 kg N ha^{-1} than in those nodules treated with 80 kg N ha^{-1}.

Statistical analysis of the data revealed that *in vitro* nodule PEP carboxylase specific activity and GOGAT specific activity were significantly correlated ($P = 0.01$) when analyzed by harvest treatment (data for each harvest treatment combined over all NO_3^- levels and all days of the experiment) and by NO_3^- levels (data for each NO_3^- level combined over all harvest treatments and all days of the experiment). Correlation coefficients for *in vitro* PEP carboxylase specific activity and GOGAT specific activity of 0.82 and 0.81 were obtained for unharvested and harvested treatments, respectively. Analysis of the data by NO_3^- levels gave correlation coefficients of 0.69, 0.71, and 0.86 for the 0, 40, and 80 kg N ha^{-1} treatment levels, respectively. Both nodule *in vitro* PEP carboxylase specific activity and GOGAT specific activity were highly correlated ($P = 0.01$) with acetylene reduction activity on a per plant basis when the data were analyzed by harvest treatment. Correlation coefficients of 0.63 and 0.62 were obtained for nodule GOGAT specific activity and acetylene reduction activity while correlation coefficients of 0.59 and 0.58 were obtained for *in vitro* nodule PEP carboxylase specific activity and acetylene reduction activity in unharvested and harvested treatments, respectively. Highly significant correlations ($P = 0.01$) between nodule GOGAT specific activity and acetylene reduction activity (0.78) and between *in vitro* PEP carboxylase specific activity and acetylene reduction activity (0.66) occurred at the 80 kg N ha^{-1} treatment level but not at the 0 or 40 kg N ha^{-1} treatment levels. There was no significant harvest by NO_3^- level interaction.

Both forage harvest and applied NO_3^- caused a shift in distribution of fixed $^{14}CO_2$ into the OA and AA fractions in alfalfa nodules (Table III). The distribution of radioactivity in nodules of unharvested plants not treated with NO_3^- was relatively constant and averaged 73% incorporated into the OA fraction while 27% was incorporated into the AA fraction. By day 8, forage harvest of plants not treated with NO_3^- resulted in 62% of the total radioactivity in the OA fraction and 38% in the AA fraction, with recovery to control (unharvested, 0 kg N ha^{-1} values given above)

Table III. *Distribution of Total Radioactivity into Alfalfa Nodule Organic Acid and Amino Acid Fractions*

Sample	Days after Harvest and/or N Application[a]									
	1		8		11		15		22	
	OA	AA	OA	AA	OA	AA	OA	AA	OA	AA
	dpm × 10^{-3}									
0 kg N ha^{-1}										
Unharvested	78 (79)	21 (21)	51 (72)[b]	20 (28)[b]	103 (83)	21 (17)	105 (74)	37 (26)	81 (66)	42 (34)
Harvested	81 (76)	25 (24)	35 (62)	22 (38)	71 (64)	40 (36)	65 (74)	23 (26)	69 (66)	35 (34)
40 kg N ha^{-1}										
Unharvested	65 (73)	24 (27)	46 (73)	17 (27)	45 (76)	14 (24)	27 (52)	25 (48)	27 (67)	13 (33)
Harvested	79 (81)	19 (19)	21 (57)	16 (43)	22 (63)	13 (37)	19 (53)	17 (47)	34 (67)	17 (33)
80 kg N ha^{-1}										
Unharvested	72 (79)	19 (21)	37 (74)	13 (26)	27 (73)	10 (27)	24 (58)	18 (42)	23 (59)	16 (41)
Harvested	44 (65)	24 (35)	18 (51)	17 (49)	18 (46)	21 (54)	22 (52)	20 (48)	24 (52)	22 (48)

[a] Each value is the mean of at least two replications. Numbers in parentheses, percentage of total.
[b] A portion of this sample was lost during preparation.

distribution by day 15. The distribution of total radioactivity incorporated into the OA and AA fractions in nodules from unharvested plants treated with 40 kg N ha^{-1} remained unchanged until day 15 when 52 and 48% was found in the OA and AA fractions, respectively. By day 22, distribution of radioactivity in nodules of unharvested, 40 kg N ha^{-1} plants approximated the control. This recovery between days 15 and 22 corresponds with the recovery trend observed in *in vitro* PEP carboxylase activity (Fig. 1). A similar pattern was observed in unharvested plants treated with 80 kg N ha^{-1}, but there was no recovery by day 22.

Plants that were both harvested and treated with N showed the most rapid and pronounced shift in distribution. Nodules from harvested plants treated with 40 kg N ha^{-1} showed a decrease in per cent radioactivity in the OA fraction with a concomitant increase in per cent radioactivity in the AA fraction by day 8 after treatment. This pattern was retained through day 15 and again approached control values by day 22. In comparison, nodules from harvested plants treated with 80 kg N ha^{-1} showed an immediate and persistent response to treatment. The distribution of total radioactivity on day 1 was 65% into the OA fraction and 35% into the AA fraction. By day 8 and through the end of the experiment, nearly equal distribution of radioactivity was observed in the OA (52%) and AA (48%) fractions.

DISCUSSION

Alfalfa root nodules actively fix CO_2 via the enzyme PEP carboxylase and root nodule CO_2 fixation is closely associated with nodule effectiveness and N_2 fixation capacity. Specific activity for *in vitro* PEP carboxylase of effective alfalfa nodules was significantly greater than that of ineffective alfalfa nodules, regardless of whether ineffectiveness was the result of bacterial or of host genetic factors. Treatments that reduced acetylene reduction (*i.e.* applied N and forage harvesting) reduced *in vitro* PEP carboxylase activity, and recovery of acetylene reduction was accompanied by increased PEP carboxylase activity. Identical results were obtained for *in vivo* CO_2 fixation (data not shown). Highly significant positive correlations were observed between alfalfa nodule *in vitro* PEP carboxylase specific activity, GOGAT specific activity, and acetylene reduction activity. Christeller *et al.* (5) reported significant positive correlations between both *in vitro* and *in vivo* PEP carboxylase activity and acetylene reduction activity during the development of lupine nodules. In contrast, Coker and Schubert (7) reported CO_2 fixation was high as soybean nodules started to fix N_2 but then declined prior to attainment of maximum N_2 fixation rates. The decline in soybean nodule CO_2 fixation rates corresponded to a time of decreased amide transport and increased ureide transport in the xylem sap.

The transport form of fixed N in legume species may determine the nature of the relationship between nodule CO_2 fixation activity and N_2 fixation activity. Nodule CO_2 fixation and N_2 fixation appear to be positively correlated in species that transport N primarily as amides (lupine, alfalfa) (5). No correlation was observed between nodule CO_2 fixation and N_2 fixation in two species that transport fixed N as ureides (soybean, broad bean) (7, 26). The initial carbon skeleton of the amide asparagine can be derived directly from oxaloacetate, the initial product of PEP carboxylase. The pathway for incorporation of fixed C from nodule CO_2 fixation into ureides is less clear (4, 8). Coker and Schubert (7) reported incorporation of $^{14}CO_2$ into soybean nodules decreased with the onset of N_2 fixation but they did not demonstrate a role for CO_2 fixation in ureide biosynthesis. Cookson *et al.* (8), however, suggested that the ^{14}C label in dwarf French bean ureides originated from ^{14}C incorporation into oxaloacetate via PEP carboxylase. In any event, additional research is needed to verify the extent and pathway of ^{14}C incorporation from $^{14}CO_2$ into ureides.

Our observations that PEP carboxylase activity is highly correlated with GOGAT activity and that PEP carboxylase and GOGAT activities are higher in alfalfa than in bean species support the interpretation that alfalfa nodule CO_2 fixation and N_2 fixation are directly linked through oxaloacetate. Boland *et al.* (3) and Groat and Vance (11) have previously noted that nodule GOGAT/GS ratios are consistently lower in ureide-transporting legumes as compared to amide-transporting legumes. We again confirm that observation here. Inasmuch as GOGAT tends to be higher in amide transporters and since glutamine amide N may be directly utilized for purine biosynthesis (4), nodule CO_2 fixation is probably more directly related to N_2 fixation and assimilation in amide-transporting legumes than in ureide-transporting legumes.

Forage harvest and applied NO_3^- reduced *in vitro* PEP carboxylase activity (and *in vivo* CO_2 fixation activity) and shifted the distribution of radioactivity incorporated into the OA and AA fractions of nodules exposed to $^{14}CO_2$. Although less CO_2 was fixed and the distribution of radioactivity shifted as a result of treatments, the total counts incorporated into the AA fraction remained relatively constant as the counts in the OA fraction decreased substantially (Table III). This suggests there is a buffering effect associated with the amino acid fraction that may reflect maintenance of a steady state pool of amino acids in the nodule. This observation and the presence of PEP carboxylase in ineffective nodules suggests that CO_2 fixation may be involved in several pathways of metabolism throughout the ontogeny of the nodule and the fate of the products of CO_2 fixation change as plant

conditions change (7, 9). Malate appeared to be the primary compound in the OA fraction while the neutral fraction consistently contained less than 1% of the total radioactivity incorporated into the nodules.

Although total radioactivity in the nodule AA fraction remained relatively constant with treatments, applied N may induce changes in the distribution of that radioactivity among specific amino acids. Addition of NO_3^- appeared to reduce the amount of radioactivity incorporated into asparagine and alanine while the radioactivity incorporated into aspartate increased (data not shown). This indicates that applied NO_3^- alters nodule N assimilation. Our observations of GOGAT activity support this interpretation. Previous studies have shown GS is less affected than GOGAT by forage harvest and applied NO_3^-. These observations imply a more crucial role for AS and AAT in nodule metabolism than previously noted. Both AS and AAT have been detected in legume nodules; however, regulation of their activity is not well understood (20). Because a major portion of the fixed N is transported from alfalfa nodules as asparagine, it seems apparent we need to evaluate critically the regulation of AAT and AS with respect to constraints associated with the transport of fixed N.

Although the *in vivo* technique was used in the forage harvest and NO_3^- application experiment to measure both quantitative differences in the CO_2 fixation activity between treatments and qualitative changes in the labeling patterns resulting from the treatments, we believe that this assay can also be used to give a reliable estimate of the true CO_2 fixation rate in alfalfa nodules. We have found that alfalfa nodule CO_2 fixation, measured using excised nodules, is linear for at least 20 min. Maximum CO_2 fixation rates obtained in 20-min assays for excised alfalfa nodules were 4.4 ± 0.6 and 32.2 ± 2.1 nmol CO_2 mg^{-1} nodule dry weight h^{-1} for 0.38% (subsaturating) and 4.9 to 8.4% (saturating) CO_2 concentrations, respectively. The CO_2 fixation rate observed for alfalfa nodules on roots was 18.3 ± 0.3 nmol CO_2 mg^{-1} nodule dry weight h^{-1} at a CO_2 concentration of 0.13% while the acetylene reduction rate was 178 nmol ethylene produced mg^{-1} nodule dry weight h^{-1}. If it is assumed that three acetylene molecules are reduced for each dinitrogen fixed and that all of the fixed carbon and nitrogen is used in the synthesis of asparagine, the major N transport compound in alfalfa, then CO_2 fixation by nodule PEP carboxylase is calculated to provide at least 25% of the carbon required for the assimilation and transport of symbiotically fixed N in alfalfa.

Incorporation of acid stable radioactivity into nodules exposed to $^{14}CO_2$, as an accurate estimate of CO_2 fixation, should be viewed with caution (5, 7, 9, 25). Therefore, our standard *in vivo* assay technique was performed under ambient CO_2 conditions for 30 min to allow maximum label incorporation into the nodules and to be ensured of reaching steady state conditions in the nodules. The final CO_2 concentration of the *in vivo* assay ranged from 0.3 to 0.6% due to nodule respiration during the assay period.

Estimates of the carbon input from PEP carboxylase to N assimilation in legumes vary widely (5, 7, 26). These variations may be due in part to the differing methods of calculation of the estimates. However, it also seems feasible that these estimates vary greatly because the contribution of PEP carboxylase to nodule N and C economy likely varies with: (a) ontogeny, (b) environment, (c) genotype, (d) species, and (e) primary transport product.

These studies show that nonphotosynthetic CO_2 fixation catalyzed via the enzyme PEP carboxylase plays a major role in the metabolism of alfalfa root nodules and that a substantial portion of the carbon fixed by this enzyme in alfalfa nodules is utilized for the assimilation of symbiotically fixed N.

LITERATURE CITED

1. Atkins CA, DT Canvin 1971 Photosynthesis and CO_2 evolution by leaf discs: gas exchange, extraction, and ion-exchange fractionation of ^{14}C-labeled photosynthetic products. Can J Bot 49: 1225–1234
2. Bachelard HS 1965 Glucose metabolism and α-keto acids in rat brain and liver *in vivo*. Nature 205: 903–904
3. Boland MJ, AM Fordyce, RM Greenwood 1978 Enzymes of nitrogen metabolism in legume nodules: a comparative study. Aust J Plant Physiol 5: 553–559
4. Boland MJ, KR Schubert 1982 Purine biosynthesis and catabolism in soybean root nodules: incorporation of ^{14}C from $^{14}CO_2$ into xanthine. Arch Biochem Biophys 213: 486–491
5. Christeller JT, WA Laing, WD Sutton 1977 Carbon dioxide fixation by lupin root nodules. I. Characterization, association with phosphoenolpyruvate carboxylase, and correlation with nitrogen fixation during nodule development. Plant Physiol 60: 47–50
6. Coker III GT, KR Schubert 1979 The role of dark CO_2 fixation in amino acid biosynthesis in soybean root nodules. Plant Physiol 63: S-621
7. Coker III GT, KR Schubert 1981 Carbon dioxide fixation in soybean roots and nodules. I. Characterization and comparison with N_2 fixation and composition of xylem exudate during early nodule development. Plant Physiol 67: 691–696
8. Cookson C, H Hughes, J Coombs 1980 Effects of combined nitrogen on anapleurotic carbon assimilation and bleeding sap composition in *Phaseolus vulgaris* L. Planta 148: 338–345
9. Davies DD 1979 The central role of phosphoenolpyruvate in plant metabolism. Annu Rev Plant Physiol 30: 131–158
10. DeVries GE, P In't Veld, JW Kijne 1980 Production of organic acids in *Pisum sativum* root nodules as a result of oxygen stress. Plant Sci Lett 20: 115–123
11. Groat RG, CP Vance 1981 Root nodule enzymes of ammonia assimilation in alfalfa (*Medicago sativa* L.). Developmental pattern and response to applied nitrogen. Plant Physiol 67: 1198–1203
12. Laing WA, JT Christeller, WD Sutton 1979 Carbon dioxide fixation by lupin root nodules. II. Studies with ^{14}C-labeled glucose, the pathway of glucose metabolism and the effect of some treatments that inhibit nitrogen fixation. Plant Physiol 63: 450–454
13. Lawrie AC, CT Wheeler 1975 Nitrogen fixation in the root nodules of *Vicia faba* L. in relation to the assimilation of carbon. II. The dark fixation of carbon dioxide. New Phytol 74: 437–445
14. Layzell DB, RM Rainbird, CA Atkins, JS Pate 1979 Economy of photosynthate use in nitrogen fixing legume nodules. Plant Physiol 64: 888–891
15. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
16. Minchin FR, RJ Summerfield, P Hadley, EH Roberts, S Rawsthorne 1981 Carbon and nitrogen nutrition of nodulated roots of grain legumes. Plant Cell Environ 4: 5–26
17. Peterson MA, DK Barnes 1981 Inheritance of ineffective nodulation and nonnodulation traits in alfalfa. Crop Sci 21: 611–616
18. Peterson JB, HJ Evans 1979 Phosphoenolpyruvate carboxylase from soybean nodule cytosol. Evidence for isoenzymes and kinetics of the most active component. Biochim Biophys Acta 567: 445–452
19. Platt SG, L Rand 1979 Thin-layer chromatographic separation of ^{14}C-labeled metabolites from photosynthate. J Liquid Chromatogr 2: 239–253
20. Rawsthorne S, FR Minchin, RJ Summerfield, C Cookson, J Coombs 1980 Carbon and nitrogen metabolism in legume root nodules. Phytochemistry 19: 341–355
21. Vance CP, GH Heichel, DK Barnes, JW Bryan, LEB Johnson 1979 Nitrogen fixation, nodule development and vegetative regrowth of alfalfa (*Medicago sativa* L.) following harvest. Plant Physiol 64: 1–8
22. Vance CP, LEB Johnson, AM Halverson, GH Heichel, DK Barnes 1980 Histological and ultrastructural observations of *Medicago sativa* root nodule senescence after foliage removal. Can J Bot 58: 295–309
23. Vance CP, LEB Johnson, G Hardarson 1980 Histological comparisons of plant and *Rhizobium* induced ineffective nodules in alfalfa. Physiol Plant Pathol 17: 167–173
24. Viands DR, CP Vance, GH Heichel, DK Barnes 1979 An ineffective nitrogen fixation trait in alfalfa. Crop Sci 19: 905–908
25. Walker DA 1962 Pyruvate carboxylation and plant metabolism. Biol Rev 37: 215–256
26. Wheeler CT 1978 Carbon dioxide fixation in the legume root nodule. Ann Appl Biol 88: 481–484