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The Role of Dark Carbon Dioxide Fixation in Root Nodules of Soybean[1]

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ABSTRACT

The magnitude and role of dark CO_2 fixation were examined in nodules of intact soybean plants (Harosoy 63 × *Rhizobium japonicum* strain USDA 16). The estimated rate of nodule dark CO_2 fixation, based on a 2 minute pulse-feed with $^{14}CO_2$ under saturating conditions, was 102 micromoles per gram dry weight per hour. This was equivalent to 14% of net nodule respiration. Only 18% of this CO_2 fixation was estimated to be required for organic and amino acid synthesis for growth and export processes. The major portion (75–92%) of fixed label was released as CO_2 within 60 minutes. The labeling pattern during pulse-chase experiments was consistent with CO_2 fixation by phosphoenolpyruvate carboxylase. During the chase, the greatest loss of label occurred in organic acids. Exposure of nodulated roots to Ar:O_2 (80:20) did not affect dark CO_2 fixation, while exposure to O_2:CO_2 (95:5) resulted in 54% inhibition. From these results, it was concluded that at least 66% of dark CO_2 fixation in soybean may be involved with the production of organic acids, which when oxidized would be capable of providing at least 48% of the requirement for ATP equivalents to support nitrogenase activity.

Dark CO_2 fixation is widespread in the root nodules of legumes and has been estimated to recycle 9 to 30% of nodule respiratory carbon in soybean (5). It has been suggested that reassimilation of respired CO_2 may increase the apparent energy use efficiency of legume symbioses (13), and that selection for increased dark CO_2 fixation may be a feasible means of increasing legume productivity (8).

Several CO_2 fixing enzymes are known to occur in nodules, including carbamoyl phosphate synthetase (16) and phosphoribosylaminoimidazole carboxylase (2). Ribulose bisphosphate carboxylase has been demonstrated to occur in free-living *Rhizobium japonicum* but not in bacteroids (25). However, the primary CO_2 fixing enzyme in legume nodules is PEP[2] carboxylase (4, 5, 15, 27). Several anaplerotic functions have been suggested for dark CO_2 fixation by PEP carboxylase in legume nodules, including the provision of: (a) C skeletons for NH_3 assimilation and export and nodule growth (4, 15, 27); (b) organic acids for export in the xylem to counterbalance cation transport and regulate pH (7); and (c) organic acids for metabolism by bacteroids in support of nitrogenase activity (5). Direct evidence for the third role is currently lacking.

A previous study of dark CO_2 fixation in nodules of soybean (5) demonstrated that the pattern of radioactive labeling obtained by feeding $^{14}CO_2$ was consistent with PEP carboxylase CO_2 fixation. The aim of the present study was to investigate the pathway, role, and quantitative importance of dark CO_2 fixation in soybean nodules.

MATERIALS AND METHODS

Plant Growth. Soybean seeds (*Glycine max* L. Merr. cv Harosoy 63) were inoculated with liquid cultures of USDA 16, a *Rhizobium japonicum* strain which lacks measurable uptake hydrogenase activity, and grown in a greenhouse with supplemental metal arc lighting (14–18 h day, 25–31°C). Plants were cultured in silica sand and watered with N-free nutrient solution.

Nodule N increment was determined as described previously (29) from plants harvested at 21, 28, and 35 d (14 plants per harvest). All rate measurements were made on 28 d old plants except where noted.

Measurement of CO_2 Fixation. In the pulse-chase experiments, root systems with shoot attached were sealed into a 50 ml sidearm Erlenmeyer flask or 250 ml Mason jar equipped with fittings to permit gas flow. Humidified air containing 5% CO_2 was passed over the root systems at 200 to 250 ml min^{-1} for at least 5 min; then the gas lines were closed and $^{14}CO_2$ was injected to give a specific activity of approximately 3×10^3 to 6×10^4 MBq L^{-1}. After a 2 min pulse-feed the gas lines were opened and the container flushed with 5% CO_2 for a chase period of 0 to 60 min; then the plants were removed and frozen rapidly in liquid N_2.

The specific radioactivity of the gas phase during the feed period was determined by removing 0.1 ml gas samples for injection into an IR gas analyzer, and 0.1 to 1.0 ml samples for injection into sealed 5 ml glass minivials containing 1 ml of ethanolamine. The vials were allowed to stand overnight, then 4 ml of a dioxane-based scintillation fluid was added and radioactivity was determined by liquid scintillation spectrometry.

As well, $^{14}CO_2$ was fed to nodules of intact plants for varying lengths of time. In these experiments, root systems were sealed in their original pots and exposed to $^{14}CO_2$ in a circulating system (total volume approximately 300 ml), and the specific activity determined as above.

Determination of Acid-Stable Radioactivity. After weighing, frozen samples of nodules or other plant parts were ground in 5 to 50 ml of 80% cold acidified ethanol. The final volume was determined, and a sample of 200 to 500 μl was added to 15 ml ScintiVerse I scintillation fluid (Fisher Scientific Co.) and 7.5 ml of water for liquid scintillation spectrometry. Activities were expressed on a dry weight basis, determined from measured dry to fresh weight ratios.

Fractionation of Nodule Material. The crude nodule extracts were centrifuged to remove insoluble material, and radioactivity was measured in ScintiVerse I scintillation fluid as above. The supernatant was dried *in vacuo*, then the residue was resuspended

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[2] Abbreviations: PEP, phosphoenolpyruvate; DW, dry weight; nod, nodules; nod rt, nodulated root.

in 5 to 10 ml water, and extracted with an equal volume of chloroform to remove lipid material. The aqueous portion was separated into acidic, basic, and neutral fractions by an ion exchange procedure similar to that of Atkins and Canvin (1). The fractions were dried *in vacuo* and resuspended in either water or the appropriate HPLC buffer (1–5 ml). A sample was removed for liquid scintillation spectrometry in dioxane-based scintillation fluid. Recovery was generally 70 to 95% of radioactivity present in the crude 80% ethanol extract.

HPLC Separation of Organic and Amino Acids. Samples were prepared for HPLC by filtering through 0.2 μm filters with Luer lock connections. Organic acids were separated isocratically on an Interaction ORH801 ion exclusion column equipped with Valco C6U injection valve and 25 μl sample loop, Bio-Rad Micro-Guard column and Rheodyne 7335 0.45 μm inline filter, and detected by UV *A* at 210 nm. The eluent was either 0.005 or 0.05 N H_2SO_4, the column temperature 38°C, and the flow rate 0.5 ml min^{-1}. Fractions were collected every 0.2 min during the course of a 16 min run for liquid scintillation spectrometry in 1 ml of dioxane-based scintillation fluid.

Amino acids were separated by ion exchange HPLC with an Interaction AA503 column and step gradient elution with a lithium citrate based Pierce Pico-Buffer system. The amino acids were detected fluorometrically following post-column reaction with *o*-phthalaldehyde. Fractions were collected every 0.3 to 1 min during the course of a 112 min run and radioactivity was measured in ScintiVerse I scintillation fluid (2 × sample volume).

Xylem Sap Analysis. Xylem exudates were collected from decapitated root systems of five plants as previously described (12). Total ureide content was measured in individual samples by the method of Vogels and Van der Drift (28), with absorbance determined at 535 nm. The remaining xylem sap was pooled and assayed for amino N by a ninhydrin method (9).

Gas Exchange Measurements. Acetylene reduction and H_2 production were measured in intact nodulated roots of 12 plants with a flow-through system as previously described (14, 29). Respiration measurements were carried out on 9 plants with a flow-through system equipped with an infrared gas analyzer. Root respiration was distinguished from nodule respiration by the method of Ryle *et al.* (21).

Decarboxylation of Malate. The malate peak from the HPLC separation was assayed for label in the C-4 position by decarboxylation with NADP malic enzyme (EC 1.1.1.40, Sigma Chemical Co.), following the method of Rutter and Lardy (20), modified for assay of ^{14}C. The reaction (in 4 ml total volume) was carried out in 20 ml glass scintillation vials, and was initiated by addition of 0.5 units of enzyme. After 1 h the vials were acidified and allowed to stand overnight. The remaining radioactivity was determined by liquid scintillation spectrometry in dioxane-based scintillation fluid.

RESULTS

C and N Budget. A C and N budget was developed for nodules of 28 d old soybean plants to assess the relative importance of dark CO_2 fixation in providing C skeletons for growth and export processes in nodules (Table I). The rate of dark CO_2 fixation, measured with a 2 min pulse-feed with $^{14}CO_2$ (Table I, item 1) accounted for 14% of net respiration (Table I, item 2). The rate of N fixation (Table I, item 5) was estimated from the C_2H_2 reduction and H_2 evolution rates (Table I, items 3 and 4). The ratio of CO_2 evolved to NH_3 produced was 5:1, a value similar to the results of others (13, 24).

The partitioning of fixed N in nodules (Table 1, item 8) was estimated from the xylem sap ureide and amino acid concentrations (Table I, items 6a and 6b) and the rate of N accumulation in dry matter (Table I, item 7). Ureide export from nodules (Table I, item 8a) accounted for 87% of recently fixed N, while amino acid production and dry matter accumulation (Table I, items 8b and 8c) accounted for 7 and 6%, respectively.

The magnitude of the requirement for CO_2 fixation in support of nodule growth and C and N export was estimated from these data. The sum of the requirements for amino acid synthesis for nodule growth (Table I, item 9b), xylem export of amino acids (Table I, item 9a) and xylem export of TCA cycle organic acids (Table I, item 10) was found to account for only 18% of the measured rate of dark CO_2 fixation (Table I, item 11b). When, in addition, the rate of ureide production (Table I, item 12) was considered, the total estimated requirement for C from CO_2 fixation accounted for only 50% of the measured rate (Table 1, item 13b).

Time Course of $^{14}CO_2$ Labeling. Varying the length of exposure to $^{14}CO_2$ from 2 to 60 min resulted in a 75% decline in the apparent CO_2 fixation rate (Fig. 1A). This was attributed to a loss of previously fixed CO_2 from nodules rather than to a decline in the gross CO_2 fixation rate, since varying the length of 5% CO_2 pretreatment prior to a 2 min pulse-feed with $^{14}CO_2$ did not change the initial CO_2 fixation rate.

When root systems were given a 2 min pulse-feed with $^{14}CO_2$ followed by a 0 to 60 min chase (Fig. 1B), 84% of the label in nodules was lost within 15 min and 92% within 60 min. In a second pulse-chase experiment (Table II) in which plants were exposed to $^{14}CO_2$ for 2 min and then given a 0 or 15 min chase, a less pronounced decline was seen (40% in the acidic and basic fractions).

The label appearing in roots, stems, and leaves 60 min after the 2 min exposure to $^{14}CO_2$ amounted to only 14% of the label which was lost from nodules during the 60 min chase period (data not shown).

Pattern of Labeling over Time. Labeled samples from the first pulse-chase experiment (Fig. 1B) were fractionated into acidic, basic, and neutral fractions (Fig. 2). Immediately after a 2 min pulse-feed with $^{14}CO_2$, the majority of the label (77%) was in the acidic fraction, with 16, 3, and 4% appearing in the basic, neutral, and insoluble fractions, respectively. During the 60 min chase period, loss of label occurred in the acidic fraction (83% of the total loss) and the basic fraction (16%), with the neutral and insoluble fractions accounting for only 1% of the total loss.

HPLC analysis of the acidic fraction (Fig. 2) demonstrated that initially 76% of the total label in the acidic fraction was present in malate, and that malate accounted for 67% of the total loss of label from all four fractions during the first 7 min of a chase period.

Immediately after the 2 min pulse-feed (Table II) the samples labeled with higher specific activity contained label in malate (65% of the amino acid fraction), citrate (17%), and fumarate (7%). During the 15 min chase period, a decline was seen in the labeling of individual organic acids (Table II), including malate (51% decline), citrate (82%), and fumarate (38%), with a small amount of label appearing in succinate.

HPLC analysis of amino acids in samples labeled with high specific activity (Table II) demonstrated the presence of label in aspartate (34% of the total amino acid fraction), glutamine (15%), serine (7%), glycine (16%), and valine (5%) immediately following a 2 min $^{14}CO_2$ pulse. During the 15 min chase, a decline was seen in label within individual compounds, including aspartate (31% decline) and glutamine (27% decline). The amount of label in serine remained relatively constant, whereas detectable label disappeared entirely from glycine and valine.

Position of Label in Malate. Decarboxylation with NADP malic enzyme demonstrated that in the [^{14}C]malate fraction shown in Table II, immediately following the pulse-feed with $^{14}CO_2$ 100% of the label was present in the C-4 position. After a 15 min chase only 54 ± 6% of the label remained in the C-4 position, presumably because of randomization of label.

Table I. *C and N Budget and Estimate of the Requirements for Dark CO_2 Fixation in Nodules of 28 Day Old Soybean Plants*

Item	Value ± SE
C and N budget	
(1) Dark CO_2 fixation (μmol g^{-1} DW nod h^{-1})[a]	102 ± 1
(2) Nodule respiration (μmol CO_2 g^{-1} DW nod h^{-1})	749 ± 89
(3) Acetylene reduction (μmol g^{-1} DW nod h^{-1})	271 ± 18
(4) H_2 evolution (μmol g^{-1} DW nod h^{-1})	46 ± 3
(5) N_2 fixation (μmol N_2 g^{-1} DW nod h^{-1}) ([item 3 − item 4]/3)	75 ± 6
(6) Xylem sap composition	13 ± 1
(a) ureides (mM)	
(b) amino acids (mM)	2.9
(7) Nodule N increment (μmol N g^{-1} DW nod h^{-1})[b]	18 ± 1
(8) Nodule partitioning of fixed N (μmol N g^{-1} DW nod h^{-1})	
(a) exported as ureides[c]	130 ± 14
(b) exported as amino acids[c]	11 ± 1
(c) incorporated into nodule dry matter[d]	8.9 ± 0.5
Theoretical requirements for dark CO_2 fixation	(μmol CO_2 fixed g^{-1} DW nod h^{-1})
(9) Amino acid synthesis[e]	
(a) for export in xylem	7.4 ± 0.8
(b) for nodule growth	8.9 ± 0.5
(10) Organic acid synthesis for export[f]	2.3 ± 0.3
(11) Total organic and amino acid synthesis	
(a) sum of items 9 and 10	19 ± 1
(b) % of measured CO_2 fixation ([item 11a/item 1] × 100)	(18% ± 1)
(12) Ureide production[g]	32 ± 3
(13) Total organic acid, amino acid and ureide production	
(a) sum of items 9, 10, and 12	50 ± 4
(b) % of measured CO_2 fixation ([item 13a/item 1] × 100)	(50% ± 4)

[a] Measured with a 2 min pulse-feed with $^{14}CO_2$ to root systems of intact plants. [b] Based on dry matter increment, and dry matter N content of 5.9%. [c] Calculated from items 5, 6a, and 6b assuming 4 N per ureide and 1.5 N per amino acid exported (12). [d] Calculated from item 7 assuming 50% of the nodule N increment was supplied from recently fixed N (13). [e] Calculated from items 8b and 8c assuming 1 C fixed per amino acid exported incorporated into dry matter, and 1.5 N per amino acid exported (12). [f] Calculated from item 9a assuming an amino acid to tricarboxylic acid cycle organic acid ratio in xylem sap of 3.3:1 (12). [g] Assumes 1 C fixed per molecule (item 8a/4). This C would be released later by uricase in the production of allantoin.

Treatments Affecting Nitrogenase Activity and NH_3 Production. Root systems of intact plants were exposed either to an atmosphere of Ar:O_2 (80:20) to interrupt NH_3 production (and NH_3 assimilation), or to 95 to 100% O_2 (balance CO_2) to suppress nitrogenase activity (Table III). Replacing N_2 gas with Ar for 23 h resulted in an increase in H_2 production but CO_2 fixation was not affected. A 30 min treatment with 100% O_2 resulted in complete inhibition of H_2 evolution and no recovery within 90 min after the treatment. Respiration declined to 44% of the original level following the treatment. Dark CO_2 fixation (measured with a 2 min pulse-feed with $^{14}CO_2$) declined to 46% of the control rate following a 30 min treatment with 95% O_2.

DISCUSSION

Dark CO_2 Fixation in the C and N Budget. The rate of dark CO_2 fixation, measured with a 2 min $^{14}CO_2$ pulse-feed, accounted for 14% of net respiration (Table I, items 1 and 2). This was comparable to the results of Coker and Schubert (5), who obtained estimates amounting to 9 to 30% of nodule respiration in soybean. When rates were considered for amino acid export and incorporation into dry matter (Table I, item 9), and tricarboxylic acid cycle organic acid export (Table I, item 10), it was estimated that these requirements could account for only 18% of the observed CO_2 fixation rate (Table I, item 11b). When the rate of ureide synthesis was also taken into account (Table I, item 12), the combined requirements could only account for 50% of the observed rate (Table I, item 13b). These results suggested that dark CO_2 fixation must play another major role, possibly the production of organic acids to serve as C substrate for respiration in support of nitrogenase activity.

Loss of Fixed CO_2. Coker and Schubert (5) reported that in soybean, 70% of the label fixed during a 2 min pulse-feed with $^{14}CO_2$ was lost from nodules within 18 min of a chase period. Similar results were obtained in this study; 92% of the label was lost from nodules within 60 min following a 2 min pulse-feed with $^{14}CO_2$ (Fig. 1B) and a 75% decline was observed in the apparent CO_2 fixation rate when the length of exposure to $^{14}CO_2$ was varied for up to 60 min (Fig. 1A).

The initial CO_2 fixation rate, based on a 2 min exposure to $^{14}CO_2$ (Fig. 1A), was considered the closest approximation to gross CO_2 fixation. However, it most likely underestimated the true rate, given the rapid loss of label. The CO_2 fixation rate measured with a 30 to 60 min pulse-feed with $^{14}CO_2$ (Fig. 1A) was relatively stable, and appeared to represent net fixation.

The estimated rates of amino acid and organic acid export (Table I, items 9a and 10) and the quantity of label detected in other plant parts 60 min following a 2 min pulse-feed with $^{14}CO_2$ accounted for only 14% of the observed loss of label. It was concluded that a more likely cause of the observed loss was release of fixed label to the atmosphere as CO_2.

An attempt was made to measure the $^{14}CO_2$ released from nodules into the gas phase during a chase period. Although

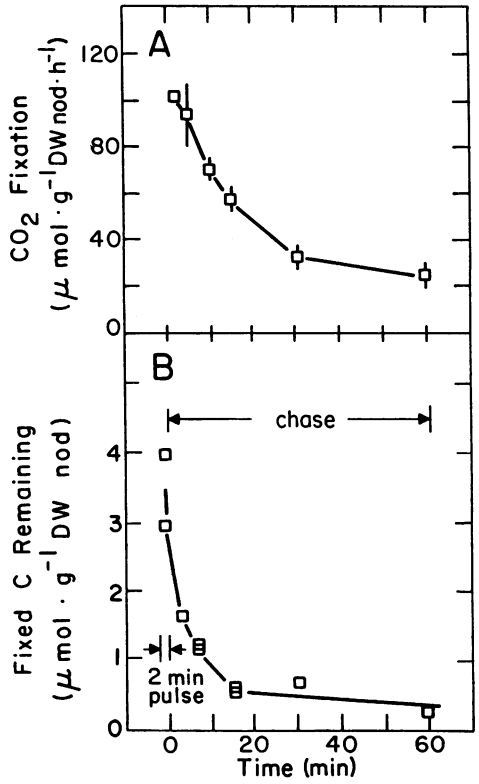

FIG. 1. A, The apparent dark CO_2 fixation rate in nodules of 28 d old soybean plants measured following various periods of exposure to $^{14}CO_2$. Data points represent means ± SE of triplicate samples. B, Fixed C remaining in nodules of 28 d old soybean plants at various times following a 2 min exposure to $^{14}CO_2$. Data points represent individual samples. The initial fixation rate (0 min) was 102 μmol g^{-1} DW nod h^{-1}.

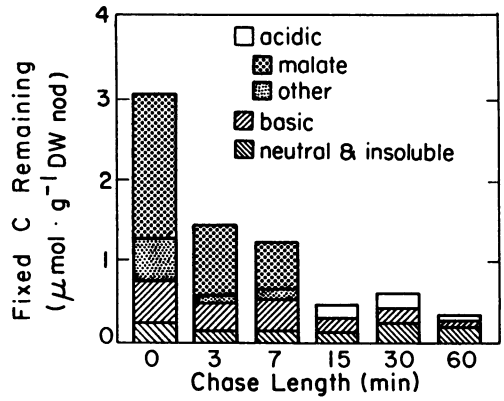

FIG. 2. Fixed C remaining in the acidic, basic, neutral, and insoluble fractions of nodules of 28 d old soybean plants at various times after a 2 min exposure to $^{14}CO_2$. Nodule material was obtained from the pulse-chase experiment shown in Figure 1B. Data points represent one replicate or the mean of two replicates. The acidic fractions of the 0, 3, and 7 min samples were analyzed further to determine the amount of fixed C remaining in malate and other organic acids.

Table II. *Distribution of Fixed C Present in the Organic Acid and Amino Acid Fractions of Nodules of 41 Day Old Soybean Plants at 0 and 5 Min after a 2 Min Exposure to $^{14}CO_2$*

For each time, nodules from 12 plants were pooled for analysis.

Compound or Fraction	Fixed C Present		Percent Decline[a]
	0 min	15 min	
	nmol g^{-1} DW nod		
Malate	485	236	51
Citrate	129	24	81
Fumarate	54	35	36
Succinate	0	17	
Other	124	126	
Total organic acid	792	438	45
Aspartate	200	147	27
Glutamine	87	59	32
Glycine	93	0	100
Serine	41	39	5
Valine	30	0	100
Other	132	146	
Total amino acid	583	391	33
Total organic and amino acid	1375	829	40

[a] Not shown for compounds or fractions in which label increased.

precise measurement of release of fixed label was not possible due to the much larger efflux of dissolved inorganic ^{14}C from the experimental system during the first 10 min of a chase (data not shown), by subtracting a control rate based on steam-killed root systems, a rate of release was obtained which was comparable to the loss in acid stable radioactivity shown in Figure 1. While not conclusive, this result was consistent with the hypothesis that label fixed into organic compounds was released as CO_2.

Pattern of Labeling from CO_2 Fixation. Release of $^{14}CO_2$ from nodules could result from oxidation of organic acids produced by PEP carboxylase (5) or from the fixation and release of CO_2 by the action of phosphoribosylaminoimidazole carboxylase and uricase in the pathway of *de novo* purine synthesis leading to ureides (2). The magnitude of the latter process (Table I, item 12) would be sufficient to account for 42% of the observed loss of ^{14}C from nodules shown in Figure 1A.

The pattern of labeling following a 2 min pulse-feed with $^{14}CO_2$ was similar to that observed by Coker and Schubert (5), and was consistent with CO_2 fixation by PEP carboxylase, since the labeled compounds included tricarboxylic acid cycle intermediates (malate, citrate, fumarate, and succinate) and amino acids derivable from the tricarboxylic acid cycle (glutamine and aspartate). The label in malate immediately following a 2 min $^{14}CO_2$ pulse-feed was located in the C-4 position, and subsequently became randomized, consistent with fumarase activity. Label was not detected in oxaloacetate, the immediate product of PEP carboxylase activity. However, this compound is unstable and its pool size in cells is considered to be small.

Fixed label resulting from carboxylation reactions other than that of PEP carboxylase was not detected. HPLC analysis of the basic fraction showed no incorporation of label into xanthine or hypoxanthine, since all of the labeled peaks corresponded to known amino acids. Uric acid, which emerges in the acidic fraction, was not detectable by mass or radioactivity. The absence of detectable label in these intermediates may be because their pools are small and turn over rapidly (2). No evidence was seen for the incorporation of label into ureides (which emerge primarily in the neutral fraction), although in a previous study using *Phaseolus* (6), label from CO_2 fixation was reported to appear in ureides. The origin of the ^{14}C appearing in serine and glycine is not known, since randomization of label had not yet occurred in malate, precluding incorporation of label from malate into glycolytic intermediates.

Role of CO_2 Fixation in Soybean Nodules. To distinguish

Table III. *Effect of an Ar:O_2 Treatment or a High O_2 Exposure on the Rates of CO_2 Fixation and H_2 Evolution in Nodules and on the Evolution of CO_2 in Nodulated Roots of Soybeans*

A, Gas exchange rates, following a 23 h exposure to an atmosphere of Ar:O_2 (80:20), in nodules of 41 d old soybean plants. B, Gas exchange rates following 30 min exposure of nodulated roots of 30 d old plants to high O_2 concentration (100% O_2 for H_2 evolution and CO_2 evolution; O_2:CO_2 (95:5) for CO_2 fixation). Values represent means ± SE of 2 to 3 plants.

Treatment	CO_2 Fixation[a]	H_2 Evolution	CO_2 Evolution
	$\mu mol\ g^{-1}\ DW\ nod\ h^{-1}$		$\mu mol\ g^{-1}\ DW\ nod\ rt\ h^{-1}$
A. Ar:O_2 treatment			
Air control	63 ± 10	88 ± 11	n.d.[b]
Ar:O_2 (80:20)	57 ± 1	246 ± 33	n.d.
B. High O_2 treatment			
Air control	99 ± 8	49 ± 4	197 ± 24
High O_2	46 ± 5	0	86 ± 12

[a] Measured with a 2 min pulse-feed with $^{14}CO_2$. [b] Not determined.

between the possible association of dark CO_2 fixation by PEP carboxylase with nitrogenase activity and with NH_3 production (and assimilation), an attempt was made to inhibit these processes differentially (Table III). Replacing N_2 with Ar in the gas phase surrounding intact root systems results in diversion of electron flow to H_2, allowing nitrogenase activity to continue but preventing NH_3 production and assimilation. A 23 h Ar treatment resulted in an increase in H_2 evolution, as expected (Table III), but did not have a significant effect on CO_2 fixation. A previous study (17) has demonstrated that xylem sap amino acid concentration drops markedly within 3 to 6 h of exposure to Ar:O_2, implying depletion of the pool of NH_3 available for assimilation.

A 30 min treatment with supraoptimal [O_2] (95 or 100%) resulted in complete inactivation of nitrogenase, blocking both N_2 fixation and H_2 evolution, and hence NH_3 production. The treated root systems demonstrated a 56% decline in respiration, and the nodules a 54% inhibition of dark CO_2 fixation.

From the results of the two treatments, it was concluded that a major portion of dark CO_2 fixation was associated more closely with nitrogenase activity than with NH_3 production and assimilation. Similar results were obtained by Laing *et al.* (11) with He:O_2 and 100% O_2, and Coker and Schubert (5) with acetylene. From the results of the present study, it is suggested that the excess CO_2 fixation provides organic acids for oxidation (presumably in bacteroids) to provide ATP and reductant in support of nitrogenase activity.

Table IV. *Theoretical Estimate of the Ability of Dark CO_2 Fixation to Supply Sufficient Organic Acids to Meet the Energy Requirements for N_2 Fixation in Soybean Nodules*

Item	Value ± SE
(1) N_2 fixation rate ($\mu mol\ N_2\ g^{-1}$ DW nod h^{-1})[a]	75 ± 6
(2) Nitrogenase energy requirement	
(a) ATP equivalents per N_2 fixed[b]	24
(b) μmol ATP equivalents per g^{-1} DW nod h^{-1}	1800 ± 144
(3) Rate of organic acid use ($\mu mol\ g^{-1}$ DW nod h^{-1})[c]	67 ± 11
(4) Energy production from malate oxidation	
(a) ATP equivalents per malate oxidized[d]	13
(b) μmol ATP equivalents g^{-1} DW nod h^{-1}[e]	871 ± 143
(5) Percentage of the nitrogenase energy requirement accounted for by malate oxidation[f]	48% ± 18

[a] From Table I, item 5. [b] Calculated assuming 1 H_2 produced per N_2 fixed (total of $8e^-$), 16 ATP per N_2 fixed, and P/O = 2.0 (24). [c] From Figure 2, difference in acidic fraction between 0 and 60 min. [d] Assumes malate oxidation via malic enzyme and tricarboxylic acid cycle and a P/O ratio of 2.0 (24). [e] Assumes item 5 (organic acid use) is all malate. [f] Calculated as (item 4b/item 2b) × 100.

There is good evidence for an organic acid requirement in cultured *Rhizobium*, and indirect evidence for a similar requirement in bacteroids. For example, *R. japonicum* requires a dicarboxylic acid as well as a sugar to fix N_2 effectively in culture (30). Free-living *R. japonicum* demonstrates active uptake of succinate and other organic acids (22), and isolated soybean bacteroids can metabolize organic acids (26). Mutants of *R. trifolii* deficient in dicarboxylic acid uptake form ineffective symbioses (19). In soybean bacteroids, crucial enzymes required for sucrose catabolism appear to be absent (18).

Assuming that dark CO_2 fixation in nodules provides organic acids for bacteroids in support of nitrogenase activity, it is of interest to compare the potential supply of energy from organic acids to the requirement for nitrogenase activity (Table IV). When theoretical estimates of the cost of nitrogenase activity were applied to the results of the present study (Table IV, items 1 and 2) it was determined that 1800 μmol of ATP equivalents were required per g DW nod h^{-1}. If malate is assumed to be the C substrate which is oxidized to support nitrogenase activity, and is assumed to account for the entire decrease in labeling of the acidic fraction, the observed rate of catabolism would produce 871 μmol of ATP equivalents per g DW h^{-1} (Table IV, items 3 and 4). Therefore, CO_2 fixation into malate and its subsequent oxidation could provide 48% of the energy requirement for nitrogenase activity (Table IV, item 5). This percentage most likely represents an underestimate for two reasons: (a) underestimation of the gross CO_2 fixation rate with a 2 min pulse-feed with $^{14}CO_2$; and (b) the possibility that substrates with higher energy yields than malate, such as succinate or glutamate (10), may be oxidized by bacteroids.

It is also possible that malate produced in the plant cytosol through PEP carboxylase and malate dehydrogenase is rapidly decarboxylated in the mitochondria by malic enzyme, resulting in the observed loss of label. This cycle has been observed in *Arum* spadices and pea roots by Bryce and ap Rees (3), who suggest that it may be widespread in plant tissues as a possible means of transporting reducing equivalents into mitochondria. Therefore, based on the results of the present study, the exact pathway of organic acid oxidation in soybean nodules remains uncertain.

The results of this study are in contrast to the results of Christeller *et al.* (4) for lupin, Vance *et al.* (27) for alfalfa, and Maxwell *et al.* (15) for alfalfa and birdsfoot trefoil, which suggested that in these amide-exporting legumes the rate of dark CO_2 fixation is comparable to the requirement for C skeletons for NH_3 assimilation. The reason for the difference is not clear. There are some indications that fast-growing rhizobia, such as *Rhizobium leguminosarum*, may be more capable of catabolizing sugars than slow-growing species such as *R. japonicum* (23).

Further work is required to determine the nature of the C requirement for nitrogenase activity in bacteroids of various legumes.

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