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Carbon Dioxide Fixation by Lupin Root Nodules

II. STUDIES WITH ^{14}C-LABELED GLUCOSE, THE PATHWAY OF GLUCOSE CATABOLISM, AND THE EFFECTS OF SOME TREATMENTS THAT INHIBIT NITROGEN FIXATION

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ABSTRACT

Labeling studies using detached lupin (*Lupinus angustifolius*) nodules showed that over times of less than 3 minutes, label from [3,4-^{14}C]glucose was incorporated into amino acids, predominantly aspartic acid, to a much greater extent than into organic acids. Only a slight preferential incorporation was observed with [1-^{14}C]- and [6-^{14}C]glucose, while with [U-^{14}C]-glucose more label was incorporated into organic acids than into amino acids at all labeling times. These results are consistent with a scheme whereby the "carbon skeletons" for amino acid synthesis are provided by the phosphoenolpyruvate carboxylase reaction.

A comparison of $^{14}CO_2$ release from nodules supplied with [1-^{14}C]- and [6-^{14}C]glucose indicated that the oxidative pentose phosphate pathway accounted for less than 6% of glucose metabolism. Several enzymes of the oxidative pentose phosphate and glycolytic pathways were assayed *in vitro* using the 12,000*g* supernatant fraction from nodule homogenates. In all cases, the specific activities were adequate to account for the calculated *in vivo* fluxes.

Three out of four diverse treatments that inhibited nodule nitrogen fixation also inhibited nodule CO_2 fixation, and in the case of the fourth treatment, replacement of N_2 with He, it was shown that the normal entry of label from exogenous $^{14}CO_2$ into the nodule amino acid pool was strongly inhibited.

In lupin nodules, N_2 fixation and asparagine synthesis proceed in accordance with the over-all stoichiometric relationship:

$$N_2 + \text{oxaloacetate} \xrightarrow{\text{ATP,NADPH}} \text{asparagine (7, 10)}$$

In a previous paper (3) we suggested that the oxaloacetate needed for asparagine synthesis is provided by the PEP[1] carboxylase reaction, and we showed that the *in vivo* CO_2 fixation activity and *in vitro* PEP carboxylase activity of lupin nodules were sufficient to provide oxaloacetate at the rates required (3).

In this paper we report further evidence supporting the PEP carboxylase scheme, based on the short term labeling patterns of detached nodules supplied with ^{14}C-labeled glucose. In addition, we show several treatments that inhibit N_2 fixation by detached nodules also inhibit CO_2 fixation and the incorporation of exogenous $^{14}CO_2$ into nodule amino acids.

MATERIALS AND METHODS

Plant Material. Lupins (*Lupinus angustifolius* L. cv. Bitter Blue) inoculated with *Rhizobium* strain NZP 2257 were grown under controlled environment conditions and harvested as previously described (11).

Assays. The acetylene reduction, *in vivo* CO_2 fixation and respiration activities of intact nodules, and the PEP carboxylase (EC 4.1.1.31) activity of nodule homogenates, were assayed as before (3).

Nodule enzymes were assayed at 20 C, in 1 ml total volume, using 25- to 100-μl portions of the 12,000*g* supernatant fraction from nodule homogenates. Reaction progress was monitored spectrophotometrically, utilizing changes in *A* at 340 nm, for 1 to 5 min. In every case activity was proportional to the volume of 12,000*g* supernatant added. Only pyruvate kinase (EC 2.7.1.40) showed any pronounced loss of activity with time (half-life 90 min) and consequently this was assayed first, within 2 min of centrifugation. Coupling enzymes and substrates were obtained from Sigma Chemical Co. and Boehringer Mannheim Gmb H, and where necessary they were dialyzed against the assay buffer before use to remove ammonium sulfate.

Phosphofructokinase (EC 2.7.1.11) assays contained 1 μmol fructose-6-P, 0.5 μmol ATP, 0.1 μmol NADH, 1 unit aldolase (EC 4.2.1.13), 10 units triose-P isomerase (EC 5.3.1.1), 10 units α-glycero-P dehydrogenase (EC 1.1.1.8), 75 μmol Tris, 5 μmol DTT, 5 μmol $MgCl_2$, adjusted to pH 7.8 with HCl. The reaction was initiated with 25 to 50 μl of 12,000*g* supernatant and the higher control rate (either -ATP or -fructose-6-P) was subtracted.

Aldolase assays contained 1 μmol fructose-1,6-diP, and NADH, Tris, DTT, $MgCl_2$, triose-P isomerase, and α-glycero-P dehydrogenase as for phosphofructokinase. The reaction was initiated with 25 to 100 μl of 12,000*g* supernatant and the rates for the -fructose-1,6-diP control and the aldolase contamination of coupling enzymes were subtracted.

Pyruvate kinase (EC 2.7.1.40) assays contained 1 μmol PEP, 1 μmol ADP, 0.1 μmol NADH, 4 units lactate dehydrogenase (EC 1.1.1.27), 100 μmol Tris, 100 μmol KCl, 10 μmol $MgCl_2$, adjusted to pH 7.5 with HCl. The reaction was initiated with 100 μl of 12,000*g* supernatant and the higher control rate (either -ADP or -PEP) was subtracted.

Glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) assays contained 0.5 μmol ATP, 1 μmol 3-P-glyceric acid, 0.1 μmol NADH, 2 units 3-P-glyceric acid kinase (EC 2.7.2.3), 100 μmol Tris, 10 μmol $MgCl_2$, adjusted to pH 7.6 with HCl. The reaction was initiated with 50 to 100 μl of 12,000*g* supernatant and the control rate (-3-P-glyceric acid) was subtracted.

Glucose-6-P dehydrogenase (EC 1.1.1.49) and 6-P-gluconate dehydrogenase (EC 1.1.1.44) assays contained 0.5 μmol $NADP^+$, 100 μmol Tris, 10 μmol $MgCl_2$, and either 1 μmol glucose-6-P or 1 μmol 6P-gluconate, adjusted to pH 7.6 with HCl. Assays were initiated with 100 μl 12,000*g* supernatant and the control rate (-substrate) was subtracted. No activity was detected for either enzyme using NAD^+.

All activities were expressed as nmol h^{-1} mg fresh weight nodule $tissue^{-1}$.

[1] Abbreviations: PEP: phosphoenolypyruvate; PPP: pentose phosphate pathway.

Nodule Culture. Nodules harvested 13 days after inoculation were placed with broken surface downward on 0.7% (w/v) agar containing 5 mM potassium acetate, 5 mM Mes, and 0 to 0.4 M sucrose, adjusted to pH 5.5 with KOH (11). After 16 h in covered Petri dishes at 25 C, separate batches of 60 to 80 mg fresh weight were removed for assaying each activity.

Experiments with Labeled Glucose. [1-^{14}C]Glucose (62 mCi/mmol, 0.2 mCi/ml), [6-^{14}C]glucose (59.6 mCi/mmol, 0.238 mCi/ml) and [U-^{14}C]glucose (260 mCi/mmol, 0.226 mCi/ml) were obtained from The Radiochemical Centre, Amersham, England. [3,4-^{14}C]Glucose (7.27 mCi/mmol) was obtained as an ethanolic solution from New England Nuclear and was evaporated to dryness and redissolved in water at 0.2 mCi/ml prior to use.

Groups of 15 to 20 freshly detached nodules (100–200 mg fresh weight) were placed broken surfaces upward on aluminum foil. A total of 5 μl radioactive glucose solution was spread over the broken surfaces of each group, taking about 30 s. The small droplets were rapidly absorbed, and the nodules were then either placed in vials for measurements of $^{14}CO_2$ release, or incubated in the open for various times before homogenization.

Acetylene reduction assays on groups of nodules treated with 5 μl water instead of glucose solution showed no difference from untreated controls.

Nodules to be incubated in different gas mixtures were placed in 30-ml Erlenmeyer flasks fitted with serum caps, and the flushing gases were prehumidified by bubbling through water.

Analysis of Products of [^{14}C]Glucose Metabolism. The CO_2 released from nodules was removed by continuous flushing and trapped in vials containing 20 ml of a mixture of ethanolamine, ethanol, and water (1:1:8, v/v). One-ml aliquots were taken to determine radioactivity as described (3).

Other metabolic products were separated by the methods of Atkins and Canvin (1). Nodule tissue was ground in 10 ml of a mixture of 95% ethanol, water, and 90% formic acid (33:7:2, v/v) at 3 C. Homogenates were centrifuged (5 min at 12,000*g*) and the pellets resuspended in water. The supernatants were dried and then redissolved first in 5 ml water (water-soluble fraction) and then in 5 ml methanol (water-insoluble residues). The water-soluble fractions were treated in one of two ways. In experiments where the total metabolized fraction was required, they were passed successively through Dowex 50W and Dowex 1-X8 columns (12 × 10 cm i.d.) and the final water eluate, containing only neutral species, was denoted the unmetabolized glucose fraction. The charged species, eluted from the Dowex columns with 2 M HCl, plus the 12,000*g* pellet and water-insoluble residue were defined as the metabolized fraction.

The fractions termed as amino acid and organic acid fractions were obtained by initially applying the water-soluble fraction to a Dowex 50W column and washing with water. The eluate and washes were combined and applied to a Dowex 1-X8 column, which was also washed with water. The amino acid fraction was eluted from the Dowex 50W with 2 M HCl and the organic acid fraction eluted from the Dowex 1-X8 also with 2 M HCl. Aliquots of the fractions were taken to determine radioactivity as described (3).

Further characterization of the amino acid fraction was carried out by two-dimensional TLC (8). Extracts were chromatographed with and without standard amino acids (aspartate, asparagine, glutamate, glutamine, and alanine), autoradiographed, and stained with ninhydrin.

RESULTS

Studies with Labeled Glucose. When acetyl-CoA is produced from glucose by glycolysis, the C-3 and C-4 atoms of the glucose molecule are lost as CO_2. Thus at early times after feeding [3,4-^{14}C]glucose to nodules, the organic acid fraction, principally tricarboxylic acid cycle intermediates derived from acetyl-CoA, should be unlabeled.

On the other hand, [3,4-^{14}C]glucose metabolized to PEP and then converted to oxaloacetate by PEP carboxylase should not lose any label, since the pyruvate dehydrogenase reaction is bypassed.

We argued that if the PEP carboxylase reaction was a significant source of oxaloacetate for amino acid synthesis, the amino acid fraction should incorporate more label from [3,4-^{14}C]glucose than from [1-^{14}C]- or [6-^{14}C]labeled glucoses, at least at early times. If, on the other hand, the tricarboxylic acid cycle was the only source of oxaloacetate for amino acid synthesis, the amino acid fraction should incorporate label from [1-^{14}C]- and [6-^{14}C]-labeled glucoses and no label from [3,4-^{14}C]glucose.

The results of an experiment using [3,4-^{14}C]-, [6-^{14}C]-, and [U-^{14}C]-labeled glucoses are shown in Figure 1. In order to allow for differing glucose specific activities and extents of catabolism, we expressed these results as the ratio of label in the amino acid fraction to label in the organic acid fraction of the same nodule samples. At labeling times up to 3 min there was a strong preferential incorporation of label from [3,4-^{14}C]glucose into the amino acid fraction. There was a slight preferential early incorporation into the amino acid fraction from [6-^{14}C]glucose, while incorporation of label from [U-^{14}C]glucose favored the organic acid fraction at all labeling times.

Another experiment, using [1-^{14}C]glucose instead of [6-^{14}C]glucose, gave similar results although the preferential incorporation of label from [3,4-^{14}C]glucose into the amino acid fraction was less pronounced (results not shown).

TLC of samples from the amino acid fractions showed that at all times from 1 to 10 min the label from both [3,4-^{14}C]- and [U-^{14}C]labeled glucoses was confined to aspartic acid.

Pathway of Glucose Metabolism. It was noticed that the percentage incorporation of label from [1-^{14}C]glucose into the combined organic and amino acid fractions of nodule tissue was lower

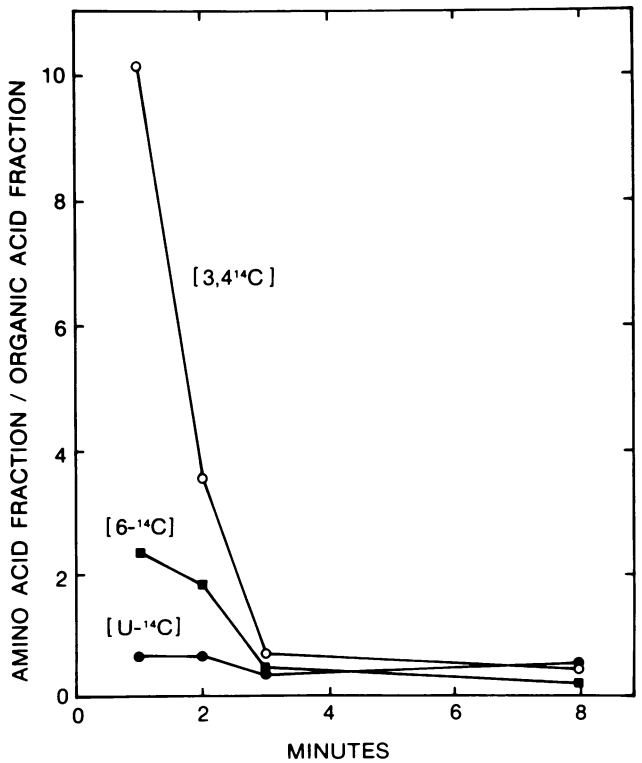

FIG. 1. Incorporation of radioactivity from [^{14}C]glucose into the amino acid and organic acid fractions of intact nodules. The experiment used day 21 nodules incubated in air at 17 C. Maximum glucose metabolized was 5.9% after 8 min. Each point represents a single sample, with measured counts min^{-1} in the amino acid fraction ranging from 5,000 to 11,000 after 1 min: (○), [3,4-^{14}C]glucose; (■), [6-^{14}C]glucose; (●), [U-^{14}C]glucose.

than for any other labeled glucose. This suggested the possibility of a specific loss of label as CO_2 from the C-1 position of glucose, as occurs in the PPP. Since this would complicate the interpretation of the labeling results already described, the contribution of the PPP to nodule glucose metabolism was measured more directly, by comparing the release of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose using the method of Gancedo and Lagunas (4) (Table I). They indicated that the PPP does indeed operate in nodules, but only at a low level.

From the previously measured rates of respiration in detached nodules (3) it was calculated that the flux of glucose through the glycolytic pathway would need to be at least 27 nmol h^{-1} mg^{-1} in day 10/11 nodules and 12 nmol h^{-1} mg^{-1} in day 20/21 nodules.

TABLE I. Contribution of the pentose phosphate pathway to nodule glucose metabolism

Each value represents the mean (± standard error) for at least 3 groups of nodules. The contribution of the pentose phosphate pathway (% PPP) was estimated as described by Gancedo and Lagunas (4)

Nodule age	Duration of experiment	Glucose metabolised		CO_2 released		% PPP
				glucose metabolised		
		1-^{14}C	6-^{14}C	1-^{14}C	6-^{14}C	
days	min	%	%	ratio	ratio	
10	10	38±3	31±3	.085±.01	.009±.002	2.8
17	10	21±3	16±1	.048±.007	.019±.005	1.0
19	10	15±1	10±1	.062±.009	.017±.002	1.6
20	50	38±6	26±4	.222±.007	.092±.009	5.3

The results in Table I suggested that the flux through the PPP would be much lower, less than 1 nmol glucose h^{-1} mg^{-1} at all times. In order to ascertain whether the enzyme levels in nodule tissue were adequate to support these rates, several enzymes from the two pathways were assayed in homogenates from nodules of various ages (Fig. 2).

At all times, the levels of the enzymes assayed *in vitro* were more than adequate to account for the calculated *in vivo* fluxes. Only P-fructokinase resembled PEP carboxylase in showing a marked increase in specific activity with increasing nodule age: the other activities remained essentially constant.

Effects of Inhibiting N_2 Fixation. The *in vivo* CO_2 fixation activity of lupin nodules is sufficient to account for oxaloacetate production at the rates needed for ammonia assimilation (3), however this does not establish that the fixed CO_2 is in fact being used for this purpose. As an independent method of testing the proposed connection between CO_2 fixation and amino acid synthesis, we tested the effects of several treatments known to affect nitrogen fixation.

Sucrose concentration had been reported to have a strong influence on the acetylene reduction activity of cultured detached nodules (11). We observed strong parallel effects on *in vivo* CO_2 fixation (Fig. 3). Optimal levels of activity were maintained on 0.2 M sucrose, with acetylene reduction in *in vivo* CO_2 fixation levels being very similar, after 16 hr in culture, to those of freshly detached nodules of the same age (results not shown). Sucrose concentrations either lower or higher than 0.2 M strongly inhibited both acetylene reduction and *in vivo* CO_2 fixation, but only slightly

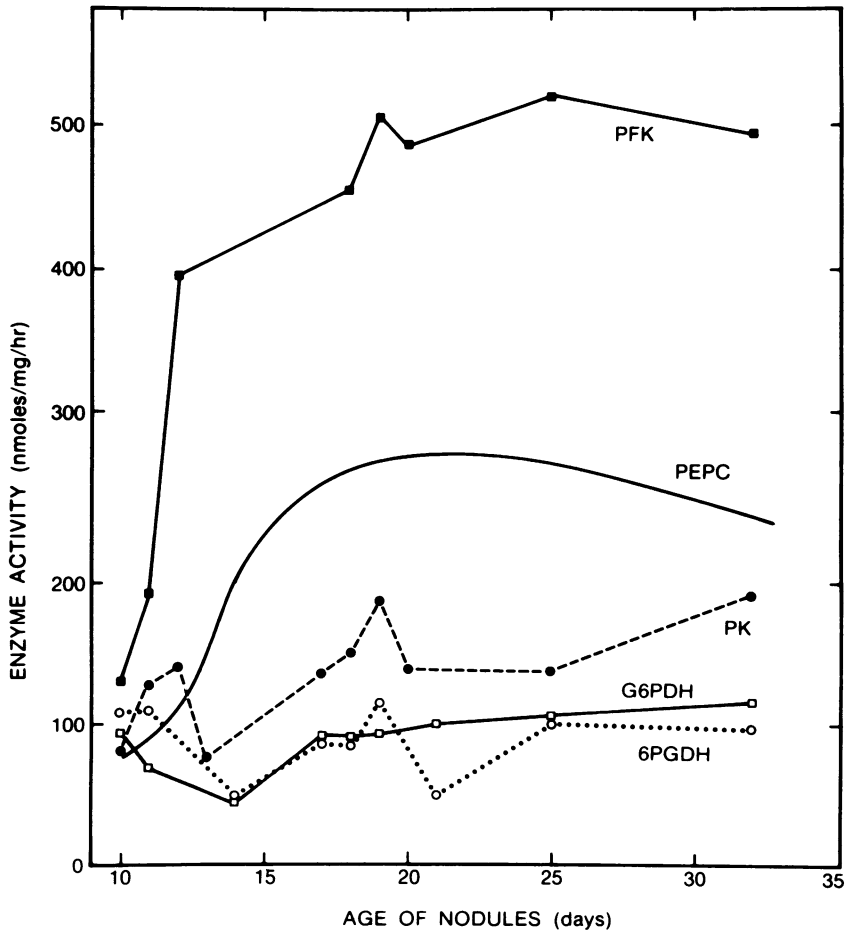

FIG. 2. Activity of enzymes involved in glucose metabolism during nodule development. PEPC (——), PEP carboxylase levels from previous work (3); PFK (■), phosphofructokinase; PK (●), pyruvate kinase; G6PDH (□), glucose-6-P dehydrogenase; 6PGDH (○), 6-P-gluconate dehydrogenase. Other activities, not shown, that were essentially constant were aldolase (217 nmol h^{-1} mg^{-1}) (SE 17, N = 8) and glyceraldehyde-3-P dehydrogenase 180 nmol h^{-1} mg^{-1} (SE 25, N = 9).

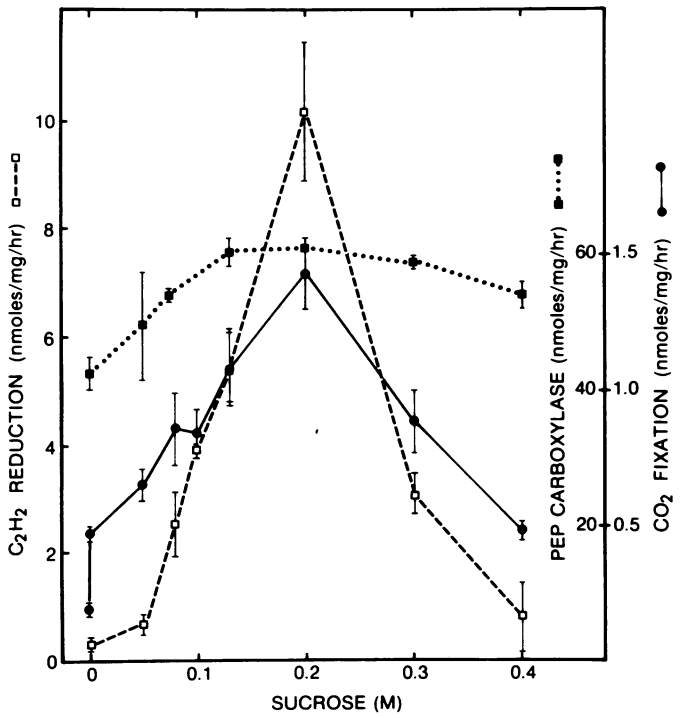

FIG. 3. Effect of sucrose concentration on acetylene reduction, CO_2 fixation, and *in vitro* PEP carboxylase activities of cultured detached nodules. Each point represents the mean (± SE) for two or more groups of nodules: (□), acetylene reduction activity; (●), *in vivo* CO_2 fixation activity (assayed in 0.33% CO_2); (■), *in vitro* PEP carboxylase activity.

inhibited *in vitro* PEP carboxylase activity.

Treating detached nodules with 100% O_2 had been reported to stimulate respiration but strongly inhibit nitrogen fixation (2). The effects of 100% O_2 pretreatments on nodule acetylene reduction activity, respiration, *in vivo* CO_2 fixation, and *in vitro* PEP carboxylase activity were as shown in Table II. Acetylene reduction was rapidly inhibited, respiration rates were reduced to 60 to 70% of the control levels, and there was a progressive decline in *in vivo* CO_2 fixation activity to about 40% of the control level after a 30-min pretreatment. *In vitro* PEP carboxylase activity was stimulated by O_2 pretreatment in one experiment, and slightly inhibited in another.

As an alternative method of inhibiting nitrogenase-catalyzed ammonia production, we treated detached nodules with 10% acetylene in air. The effect was to inhibit *in vivo* CO_2 fixation by 33% after 20 min and by 60% after 60 min (results not shown).

Yet another method of inhibiting nodule ammonia production is to replace N_2 in the atmosphere surrounding the nodules with an inert gas such as He. To our surprise we found that even a 90-min flush of detached nodules with a mixture of He and O_2 (80:20, v/v) resulted in no significant inhibition of subsequent *in vivo* CO_2 fixation activity (results not shown). In spite of this continuing CO_2 fixation, the subsequent incorporation of $^{14}CO_2$ label into amino acids was strongly inhibited, the effect being comparable with that of a 20-min pretreatment with 100% O_2, and even greater than the effect of 10% acetylene in air (Table III). Control treatments showed 53 to 61% of the incorporated label from $^{14}CO_2$ in the amino acid fraction.

DISCUSSION

Labeling Patterns. The experiments with specifically labeled glucose showed a strong preferential incorporation of label from [3,4-^{14}C]glucose into the amino acid fraction at early times (Fig. 1). This is in agreement with our proposal that oxaloacetate for nodule amino acid synthesis is generated by the PEP carboxylase reaction. The rapid decline in the preferential labeling of amino acids by [3,4-^{14}C]glucose was expected, and we attribute it to exchange of oxaloacetate between a cytoplasmic pool used preferentially for amino acid synthesis and another (mitochondrial) pool involved in the reactions of the tricarboxylic acid cycle.

Our finding that the ^{14}C label in the amino acid fraction at early times was concentrated in aspartic acid is in agreement with the proposed pathway for ammonia assimilation (7, 10), although some labeled asparagine might also have been expected.

The pattern of release of $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]glucose appeared to justify our initial assumption that glycolysis is the main pathway for nodule glucose metabolism. The contribution of the PPP was assessed as being less than 6%. Levels of both glycolytic and PPP enzymes found in nodule extracts (Fig. 2) were many-fold greater than required for the calculated *in vivo* fluxes. The Entner-Doudoroff pathway, reported to occur in some strains of *Rhizobium* (6), would require the absence of 6-P-gluconate dehydrogenase activity if it were to operate in the nodule cytoplasm.

Parallel Effects on N_2 and CO_2 Fixation. Three out of four diverse treatments that reduced nodule N_2 fixation also reduced nodule CO_2 fixation.

Sucrose concentration (Fig. 3) would be expected to have many direct and indirect effects on nodule metabolism (4). The measured 7-fold effect of sucrose on the *in vivo* CO_2 fixation activity of cultured nodules may represent an underestimation of the true effect because respiration, which would also be expected to respond to sucrose, dilutes the pool of radioactive CO_2 and can lead to an underestimation of real CO_2 fixation (3).

O_2 pretreatment represents a more severe and possibly more selective method of inhibiting nitrogenase than removal of sucrose. The failure of 100% O_2 to inhibit CO_2 fixation after short treatments (Table II) was not unexpected, since the CO_2 fixation process would be expected to continue normally until the nodule pools of ammonia and amino acids had been metabolized. Significant inhibition of *in vivo* CO_2 fixation was observed after longer O_2 treatments, and since respiration was also inhibited the real

TABLE II. Effect of pre-treatment with 100% O_2 on nodule CO_2 metabolism

Nodules from day 18-19 plants were flushed with humidified 100% O_2 for various times, then immediately assayed in air. Acetylene reduction values represent the mean of 2 samples, all other values represent the mean of 4 or more. Significance assessments (ns, not significant; *, significant at 5% level; ***, significant at 0.1% level) refer to the difference between O_2-treated and air-treated control samples within each experiment.

Experiment No.	Time of O_2 flush	Acetylene reduction activity	CO_2 fixation activity	PEP carboxylase activity	Respiration rate
	min	% air control	% air control	% air control	% air control
1	10	5.6	96 ns	138 ***	60 ***
2	15	3.8	72 *	not measured	70 ***
3	30	0.5	43 *	88 *	67 ***

TABLE III. Effect of some treatments that inhibit N_2 fixation on incorporation of label from $^{14}CO_2$ into nodule amino acids

All experiments used day 24 nodules.

Experiment No.	Treatment	Relative incorporation of label (counts min^{-1} mg^{-1}) into amino acid fraction
		% air-flushed control
1	90 min flush with He/O_2 (80/20)	48[a]
2	" "	50[a]
3	20 min flush with 100% O_2	46[a]
3	10% C_2H_2/90% air	65[b]

[a] Assayed in air at end of flush.

[b] Assayed in 10% C_2H_2/90% air.

effects on CO_2 fixation would again have been underestimated.

Either inactivation of nitrogenase by O_2 or inhibition by treatment with 10% acetylene in air would be expected to inhibit nitrogenase-catalyzed hydrogen evolution (9) as well as ammonia production. Displacement of N_2 with He, however, would allow nitrogenase-catalyzed hydrogen evolution to continue, probably at an enhanced rate. This may perhaps explain why no over-all inhibition of CO_2 fixation was observed despite the inhibition of $^{14}CO_2$ incorporation into amino acids (Table III) after a He/O_2 pretreatment: during rapid hydrogen evolution, PEP carboxylase-catalyzed CO_2 fixation might continue at a high rate merely in order to provide a continuing supply of protons for hydrogen evolution. Such fixation would increase the cytoplasmic pools of oxaloacetate and malate, and lead to a subsequent dilution of $^{14}CO_2$ transfer into the amino acid fraction as observed.

With this single proviso both the inhibitor results and the labeling data are in agreement with our previous suggestion (3) that the CO_2 fixed by mature lupin nodules is incorporated into a pool of oxaloacetate used preferentially for amino acid synthesis.

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