

Activities of the Pentose Phosphate Pathway and Enzymes of Proline Metabolism in Legume Root Nodules¹

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ABSTRACT

Based on localization and high activities of pyrroline-5-carboxylate reductase and proline dehydrogenase activities in soybean nodules, we previously suggested two major roles for pyrroline-5-carboxylate reductase in addition to the production of the considerable quantity of proline needed for biosynthesis; namely, transfer of energy to the location of biological N₂ fixation, and production of NADP⁺ to drive the pentose phosphate pathway. The latter produces ribose-5-phosphate which can be used in *de novo* purine synthesis required for synthesis of ureides, the major form in which biologically fixed N₂ is transported from soybean root nodules to the plant shoot. In this paper, we report rapid induction (in soybean nodules) and exceptionally high activities (in nodules of eight species of N₂-fixing plants) of pentose phosphate pathway and pyrroline-5-carboxylate reductase. There was a marked increase in proline dehydrogenase activity during soybean (*Glycine max*) ontogeny. The magnitude of proline dehydrogenase activity in bacteroids of soybean nodules was sufficiently high during most of the time course to supply a significant fraction of the energy requirement for N₂ fixation. Proline dehydrogenase activity in bacteroids from nodules of other species was also high. These observations support the above hypothesis. However, comparison of pentose phosphate pathway and pyrroline-5-carboxylate reductase activities of ureide *versus* amide-exporting nodules offers no support. The hypothesis predicts that pyrroline-5-carboxylate and pentose phosphate pathway activities should be higher in ureide-exporting nodules than in amide-exporting nodules. This predicted distinction was not observed in the results of *in vitro* assays of these activities.

P5CR² catalyzes the NAD(P)H-dependent reduction of P5C to form proline. The activity of P5CR is substantially higher in soybean nodules than that reported for other plant and animal tissues (11). The apparent K_m of soybean nodule P5CR activity for NADPH was 25 times lower than for NADH. The activity was inhibited 78% in the presence of 4 mM NADP⁺, but was not inhibited by proline (11) or NAD⁺ (our unpublished result). Based on these results, Kohl *et al.* (11) suggested that P5CR might have two major roles in

¹ Supported by grant No. GM38786 from the National Institutes of Health.

² Abbreviations: P5CR, P5C reductase; P5C, pyrroline-5-carboxylate; PPP, pentose phosphate pathway; R5P, ribose-5-phosphate; PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; AAT, aspartate aminotransferase; GS, glutamine synthetase; GO-GAT, glutamate synthase; gfw, g fresh weight; gdw, g dry weight; ProDH, proline dehydrogenase.

addition to the production of the considerable quantity of proline needed for biosynthesis, particularly of cell wall glycoproteins. These possible roles are: the production of reduced carbon skeletons (proline) whose subsequent oxidation contributes to fueling energy-intensive biological N₂ fixation; and the production of NADP⁺ which makes possible, in a manner described below, the assimilation of biologically fixed N₂ into ureides, the major form in which biologically fixed N₂ is transported from soybean root nodules to the plant shoot (19, 21).

Increasing the availability of NADP⁺, as a consequence of the reduction of P5C to proline, should increase flux through the PPP, since this flux is tightly regulated by the intracellular concentration of NADP⁺ (12). P5C has been shown to stimulate the activity of the PPP in human fibroblasts, Chinese hamster ovary cells, rabbit kidney cells (18), human erythrocytes (27), and rat and mice lens (24). Based on analogy with these results in animal systems, Kohl *et al.* (11) proposed that the high P5CR activity in soybean nodules might function to increase the activity of the PPP. This, in turn, would increase the rate of production of R5P. In human erythrocytes the activity of PRPP synthetase, which catalyzes the transfer of the pyrophosphoryl-group of ATP to R5P producing PRPP, is sensitive to R5P levels (27). PRPP is one of the substrates for the synthesis of PRA, the first committed intermediate of purine ribonucleotide biosynthesis. The rates of PRA synthesis, and therefore purine synthesis, are influenced by PRPP levels. Yeh and Phang (27) have shown that P5C increases the synthesis of purine ribonucleotides by enhancing the activity of the PPP, giving rise to higher concentrations of R5P and subsequently PRPP. Since soybean nodules export fixed N primarily in the form of ureides, the synthesis of which requires *de novo* purine ribonucleotide synthesis, a possible role for the high activity of P5CR observed in soybean nodules is the regulation of the PPP by increasing the level of NADP⁺, thereby stimulating formation of R5P, PRPP, purines, and ureides.

Kohl *et al.* (11) also reported the presence of substantial ProDH activity in soybean nodules. About half of this activity was in bacteroids, the symbiotic form of *Bradyrhizobium japonicum* which is responsible for the energy-intensive reduction of dinitrogen. Very little activity was present in plastids or mitochondria (less than 2% of the total activity), the latter being the site of ProDH activity in other plant and animal tissues. The activity of P5CR was localized in the cytosol of the plant cells. The specific activity of ProDH in bacteroids of soybean nodules was comparable to that in rat liver mitochondria. The novel compartmentation of ProDH

in soybean nodules supported the possibility that, together, P5CR and ProDH play a role in the transfer of redox potential (energy) from the plant cytoplasm to the bacteroid (11).

The development of activities of enzymes involved in the initial assimilation of fixed NH_4^+ [GS, GOGAT, and AAT], as well as most of those involved in ribonucleotide and ureide synthesis, have been shown to parallel that of nitrogenase activity (C_2H_2 reduction) within soybean nodules (20, 21). (The exception is allantoinase, an enzyme essential for the formation of the ureide allantoinic acid, the major ureide exported from soybean nodules. The specific activity of allantoinase is highest in nodules prior to the onset of nitrogen fixation and actually decreases after nodules begin to export ureides even though ureide production increases dramatically during the same period [21]). While the above summary statement is true in a general sense, the exact timing of the induction of GS is in dispute (1, 23). However, all parties agree that GS activity increases substantially either prior to or just after the onset of nitrogenase activity. In contrast, PEP carboxylase, which is essential for nodule metabolism throughout growth, apparently plays some unique role during early nodule development. The specific activity of PEP carboxylase undergoes a pattern of early induction followed by a subsequent decline (5). Essential housekeeping enzymes required for nodule growth and maintenance but not unique to nodules or required for the process of nitrogen fixation might be expected to follow a slightly different pattern. The specific activity of such enzymes would likely be similar in young (nonfunctional) nodules and other tissues with high metabolic activity, such as root meristems, and would remain fairly constant throughout plant growth and development. For nonessential enzymes, the specific activities might be expected to be highest in roots and decline in nodules as the synthesis of essential proteins increased. In this context, it was of interest to determine the developmental profile of activities of enzymes of the PPP and proline metabolism. In addition, it was of interest to determine whether these activities were different in ureide- versus amide-exporting nodules. A finding that PPP and P5CR activities were higher in ureide-exporting nodules would provide additional support for a role of P5CR activity in stimulating purine, and hence ureide, synthesis.

MATERIALS AND METHODS

Biochemicals

The source of all nonradioactive biochemicals was Sigma Chemical Co. $[5\text{-}^3\text{H}]$ Proline was obtained from Du Pont-New England Nuclear. Nuclear magnetic resonance analysis of radioactive proline produced by the method used by this company showed that the tritium was about equally distributed between the two H-atoms attached to the C-5 position of proline. $[1\text{-}^{14}\text{C}]$ Glucose and $[5\text{-}^{14}\text{C}]$ ornithine were purchased from ICN (Costa Mesa, CA) and Amersham, respectively.

Plant Culture

Seeds of soybean (*Glycine max* L. Merr., cv Williams 82) were obtained from Mangelsdorf Seed Co. (St. Louis, MO). Seeds of cowpea (*Vigna unguiculata*; PI 194202), dahl (*Ca-*

janus cajan; PI 399516), and peanut (*Arachis hypogaea*; PI 475851) were supplied by the Southern Regional Plant Introduction Station, Experiment, GA 30212. Seeds of lupin (*Lupinus luteus*; PI 384565), garden beans (*Phaseolus vulgaris*; PI 312079), and peas (*Pisum sativum* cv. Alaska) were supplied by the W-6 Regional Plant Introduction Station, Pullman, WA 99164-6402. Alfalfa seed (*Medicago sativa* cv Saranac) was supplied by Professor D. K. Barnes, University of Minnesota. All rhizobial inocula were gifts from the Nitragin Division of Lipha Chemicals, Milwaukee, WI 53209. Inocula were stabilized on peat. Soybean seeds were inoculated with strain 61A89, also known as USDA 110; cowpea and dahl seeds with a commercial mixture of strains 32H1, 32Z3, 41Z2, 150B1, and 176A22; bean seeds with 127K88; peanut seeds with a commercial mixture of strains 8A50, 8A57 and 8A64; lupin seeds with a mixture of strains 96A5, 96B9, 96B15, 96E3, and 96E7; alfalfa seeds with a mixture of strains 102F34A, 102F51A, 102F66D, and 102F77B; and pea seeds with a mixture of strains 128C56G and 175G10B. After inoculation, seeds were planted in 20-cm pots filled with perlite and grown in the greenhouse (24°C day, 20°C night) with supplemental illumination (800 μE at plant height supplied by 1000 W Sylvania metal halide lamps; 14 h light). After germination, plants were thinned to 5 per pot, except alfalfa, which was thinned to 10 per pot. There were four pots for each harvest. Plants were fertilized with N-free nutrients according to the method described by Fishbeck *et al.* (7). Plants were harvested at the times indicated. Nine plants were used for the acetylene reduction assay for nitrogenase (9). The acetylene assay was done in triplicate using the entire root system of three plants per replicate. The size of the container (275–1000 mL) depended on the size of the root system. After injecting the containers with 10% acetylene and incubating 15 and 30 min, the concentration of ethylene was measured with a Varian Series 2100 gas chromatograph. The change in concentration between 15 and 30 min was used as an index of nitrogenase activity. All of the nodules from the remaining plants were harvested and used for enzyme assays.

Preparation of Plant Extracts

After being weighed, rinsed with tap water, and blotted dry, nodules were stored at -80°C . P5CR and ProDH rates measured with frozen nodules and reported in this paper were comparable to rates determined with freshly picked, chilled nodules (11). PPP rates in preparations from fresh and frozen tissue were comparable (data not shown). Frozen nodules were gently crushed with grinding buffer (2 volumes per gfw nodules) consisting of 100 mM tricine buffer (pH 8.0) with 0.4 M sucrose. The nodule slurry was filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged 20 min at 12,000g to clear the plant cytoplasm of bacteroids. The bacteroid preparation, which was used to assay ProDH activity, was contaminated with starch granules, mitochondria, and plastids from the host plant cells. However, it was established earlier that mitochondria and plastids contained less than 2% of the total ProDH activity in the nodule (11). The procedure used does not result in rupture of bacteroids (11). Soybean roots were harvested from 19-d-old plants and cell-free extracts were prepared as described above for nodules. The root

cytoplasmic fraction was obtained after centrifugation at 12,000g for 20 min.

Protein Determination

The amount of protein in extracts was determined as described by Bradford (2) with bovine serum albumin as a standard. Because the color intensity varies with the nature of the protein, results of the Bradford assay were compared with estimates of protein concentration based on Kjeldahl digestion (3) of washed TCA precipitates of the extracts. The quantity of protein in the plant cytoplasmic fraction estimated from total N in Kjeldahl digests (assuming protein is 16% N) correlated well with the Bradford assay. The correlation coefficient was 0.949, $p < 0.005$ (data not shown). However, based on a paired t test, the difference in the results of the two methods was statistically significant ($p < 0.025$), with the Bradford method overestimating protein content by about 1 mg protein per gfw nodule (or about 10%). In general, the Bradford method grossly underestimated the protein content of the bacteroid fraction (data not shown). For this reason, bacteroid protein estimation was based on the Kjeldahl method.

Enzyme Assays

The cytoplasmic fraction (12,000g supernatant fluid) of the host plant cells was used for measuring GS, PPP, and P5CR activities. ProDH activity was measured in the bacteroid fraction (the pellet formed by centrifugation at 12,000g). All assays were done in triplicate. For measuring the activity of the overall PPP, the assay mixture contained 0.5 μCi [^{14}C] glucose, 10 mM unlabeled glucose, 0.57 units hexokinase type C-300 from baker's yeast (where one unit is defined as the amount which will phosphorylate 1 μmol of glucose per min at pH 8.5 and 15°C), 2.5 mM ATP, 0.8 mM MgCl_2 , 5 mM NADP^+ , 10 μL nodule extract (corresponding to about 5 mgfw nodule), and 100 mM potassium phosphate buffer (pH 7.5), in a total volume of 250 μL . Incubations were carried out for 30 min in 25 mL glass scintillation vials at ambient temperature. After starting the reaction by adding nodule extract, the vials were immediately sealed with serum stoppers, each fitted with a pin to which a filter paper, wetted with 10 μL of 10% (w/v) KOH, was attached. The reaction was stopped by injecting 100 μL of 70% HClO_4 , and incubations were continued for an additional 30 min in order to quantitatively trap CO_2 remaining in solution and in the head space of the vial. The filter paper was then placed in a fresh scintillation vial and counted with 3 mL of 3a70 counting fluid (Research Products International Corp.). Minus enzyme controls were used to correct the counts in experimental samples. Typically, control samples had less than 200 cpm, compared with several thousand in experimental samples. The measured activity was NADP^+ dependent, with NAD^+ being totally ineffective as an electron acceptor (data not shown). Therefore, under the assay conditions used, glycolysis, in combination with the Krebs' cycle, did not contribute to CO_2 production.

GS activity was measured by the transferase hydroxymate assay described by Farnden and Robertson (see p. 285 of ref.

6). P5CR activity was measured by the method of Phang *et al.* (17). DL-P5C was prepared from its 2,4-dinitrophenylhydrazine derivative as described by Mezl and Knox (15). [^{14}C]L-P5C was prepared enzymatically from [^{14}C]ornithine by the method of Smith *et al.* (25). The ProDH assay was based on transfer of ^3H from the C-5 position of proline to the medium (8, 11). As a positive control, ProDH activity was measured with rat liver mitochondria, a tissue with known ProDH activity (10). Rat liver mitochondria were isolated by the method of Morr e (16). In calculating the quantity of proline oxidized, we took into account that only half of the radioactivity of the H-atoms on the C-5 position of proline were available for dehydrogenation, but we did not attempt to correct for the probably higher rate of dehydrogenation of H versus ^3H .

RESULTS

Enzyme Activities during Soybean Nodule Development

In soybeans, nodules are first visible to the eye between 7 and 10 d after seed inoculation. Nitrogenase activity, as measured by the C_2H_2 reduction assay, is readily detectable by the 12th to 15th d. Accordingly, we sampled plants between 14 and 114 d after planting in order to encompass all phases of development and senescence. Plant and nodule fresh weights are presented in Table I. The fraction of the total plant mass in the nodules varied between 0.5% in soybeans harvested 14 d after planting to 12% at 32 d. Nodule mass continued to increase throughout the time course, but after 32 d, the ratio of nodule fresh weight to whole plant fresh weight declined. By 114 d, nodules accounted for less than 4% of the total fresh weight. There was no striking variation during the growing season in the amount of protein per unit nodule fresh weight in the plant cytoplasm or bacteroids. Mean values were 12.0 ± 1.1 (SD) and 16.1 ± 2.6 (SD) mg protein per gfw nodule, respectively. The standard deviations reflect the variation during the time course rather than measurement error.

Table I. Fresh Weight of Nodules and Entire Plant during Hydroponic Growth of Soybeans with N-Free Medium

Days after Planting	Entire Plant Mean \pm SE	Nodules Mean \pm SE	Total Mass in Nodules
	<i>gfw per plant</i>		<i>%</i>
14	2.1 \pm 0.2	0.01 \pm 0.00	0.50
17	2.7 \pm 0.1	0.09 \pm 0.00	3.33
20	3.1 \pm 0.1	0.16 \pm 0.01	5.16
24	3.9 \pm 0.1	0.24 \pm 0.02	6.15
27	5.1 \pm 0.2	0.32 \pm 0.02	6.27
32	4.5 \pm 0.2	0.54 \pm 0.05	12.00
35	9.8 \pm 1.1	0.72 \pm 0.06	7.35
41	15.1 \pm 1.4	1.12 \pm 0.09	7.42
49	27.1 \pm 2.5	1.60 \pm 0.30	5.90
55	30.7 \pm 2.0	1.73 \pm 0.10	5.64
63	53.0 \pm 3.6	2.70 \pm 0.24	5.09
76	79.9 \pm 4.2	3.45 \pm 0.23	4.32
86	91.5 \pm 5.5	4.01 \pm 0.38	4.38
114	147.2 \pm 8.5	5.69 \pm 0.51	3.87

Activities (per gfw nodule) of nitrogenase, GS, PPP, P5CR, and ProDH in soybean nodules during plant ontogeny are presented in Figure 1. Nitrogenase activity (upper panel) increased from a value that was not significantly different from zero (15 ± 28 (SE) nmol min^{-1} gfw nodule $^{-1}$) to maximum values of about $400 \text{ nmol min}^{-1}$ gfw nodule $^{-1}$ during the period from 24 to 32 d after planting. Thereafter, there was a general three- to four-fold decline in activity in agreement with previous reports (20, 21). GS activity increased six-fold in parallel with nitrogenase activity, reaching maximum activity 49 d after planting and then gradually decreasing indicating that the N_2 -fixing metabolism of the plants used in this experiment were typical for soybeans. Activities of P5CR and the PPP (center panel) were elevated at the earliest harvest (14 d after planting) prior to the onset of active fixation. In 19-d-old seedlings, P5CR activity in nodule compared with root tissue extracts was 11.6 ± 0.3 times higher per gdw and 5.1 ± 0.3 times higher per mg protein. PPP activity on d 19 was 4.9 ± 0.5 times higher per gdw and 2.2 ± 0.3 times higher per mg protein in nodule compared with root extracts. Thus, it would appear that extremely high levels of nodule PPP and P5CR activities are very rapidly induced prior to the onset of measurable C_2H_2 reducing activity.

The activity of ProDH in the bacteroid fractions of soybean nodules (Fig. 1, lower panel) increased with time after planting in parallel with nitrogenase activity (after a lag of several days) from values statistically indistinguishable from zero at the first three sampling dates to a maximum of $88 \pm 2 \text{ nmol min}^{-1}$ gfw nodule $^{-1}$ in bacteroids from nodules harvested at 49 d. Except for the apparent peak of activity of the 49-d sample, there was a general increase in ProDH activity throughout the time course to a value of $80 \pm 6 \text{ nmol min}^{-1}$ gfw nodule $^{-1}$ in the 114-d sample. Using 16 mg as the value of bacteroid protein per gfw nodule (see above), the latter value is equivalent to about 5 nmol min^{-1} mg protein $^{-1}$. This is close to our previously published value of $4.9 \text{ nmol min}^{-1}$ mg protein $^{-1}$ (11) and close to that of ProDH in rat liver mitochondria ($4.3 \text{ nmol min}^{-1}$ mg protein $^{-1}$) assayed under conditions identical to those with soybean nodule bacteroids. This is certainly a minimum estimate of the maximum potential activity of ProDH for the following reasons. (a) The assay is based on tritium loss from C-5 of proline during its dehydrogenation to P5C with the calculation including the explicit assumption that the rates of tritium and proton losses are equal. Rates estimated in this way are understated by a factor related to the tritium isotope effect. For example, the value of the latter is about 7 for the enzymatic reduction of benzyl-alcohol to benzyl-aldehyde mediated by NAD^+ (4). Experiments are in progress to determine the isotope effect for the ProDH reaction. (b) These activities were supported by endogenous electron acceptor concentrations, the initial acceptor presumably being FAD, with O_2 being the terminal acceptor. This leaves open the possibility that much higher rates would be observed if an assay using a saturating concentration of an exogenous electron acceptor were developed.

Enzyme Activities in Different Legumes

Four legumes whose nodules export fixed N as ureides—soybeans (*Glycine max*), cowpea (*Vigna unguiculata*), dahl

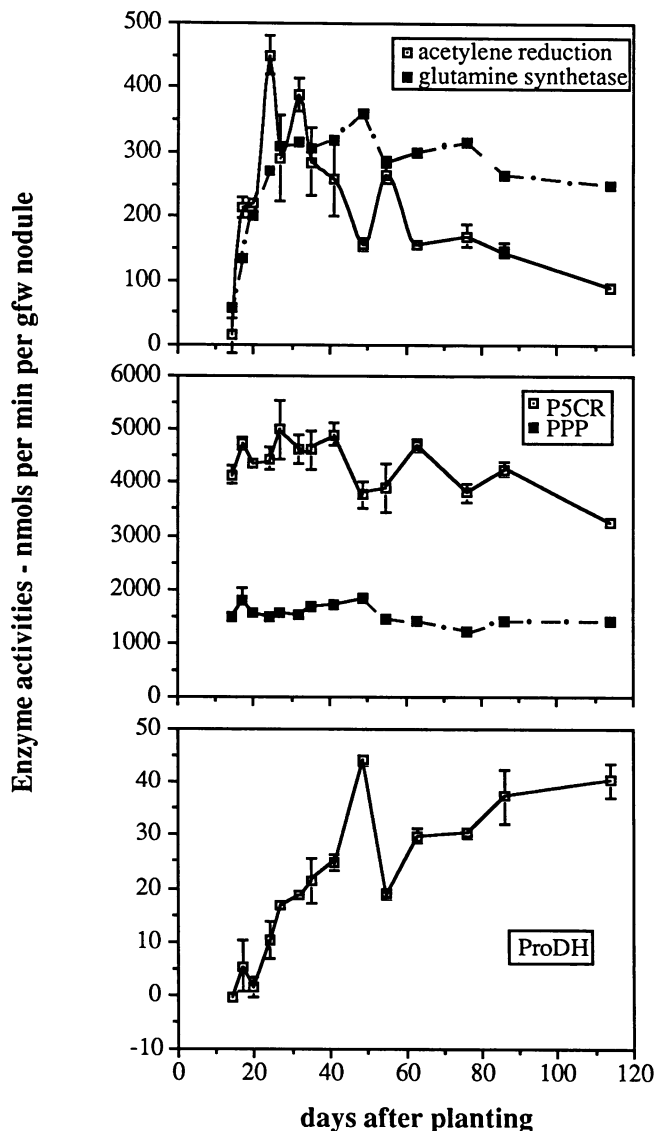


Figure 1. Nitrogenase (C_2H_2 reduction), GS, P5CR, PPP, and ProDH activities in soybean nodules as a function of time during the growing season. Error bars represent the standard error of the mean, based on triplicate assays (except for GS, for which replication was not considered necessary because the ontogeny of its activity in soybean nodules is well known [20, 21]). The standard error of the measurement of P5CR activity was frequently smaller than the size of the symbol. Acetylene reduction was assayed with intact root systems. GS, P5CR, and PPP activities were assayed with the supernatant fraction from a 12,000 g centrifugation. ProDH was assayed with the pellet from that spin.

Table II. Plant Age and Fresh Weight of Nodules and Entire Plant for Several Species Grown Hydroponically with N-Free Medium

Plant	Plant Age	Fresh Weight Entire Plant Mean \pm SE	Fresh Weight Nodules Mean \pm SE	Total Fresh Weight in Nodules
	<i>d</i>	<i>gfw per plant</i>		%
Ureide exporters				
<i>Glycine max</i>	41	15.1 \pm 1.4	1.12 \pm 0.09	7.4
<i>Vigna unguiculata</i>	48	16.3 \pm 0.6	0.74 \pm 0.16	4.5
<i>Cajanus cajan</i>	57	5.1 \pm 0.7	0.61 \pm 0.03	12.0
<i>Phaseolus vulgaris</i>	33	15.6	0.96 \pm 0.05	6.2
Amide exporters				
<i>Arachis hypogaea</i>	31	10.6 \pm 0.3	0.40 \pm 0.04	3.8
<i>Lupinus luteus</i>	50	15.7 \pm 1.0	0.76 \pm 0.15	4.8
<i>Medicago sativa</i>	51	2.5 \pm 0.3	0.03 \pm 0.01	1.2
<i>Pisum sativum</i>	34	9.3	0.79 \pm 0.12	8.5

(*Cajanus cajan*), and garden bean (*Phaseolus vulgaris*)—and four legumes whose nodules export fixed N as amides—peanut (*Arachis hypogaea*), yellow lupin (*Lupinus luteus*), alfalfa (*Medicago sativa*), and peas (*Pisum sativum*)—were used to investigate the generality of patterns of activity of the PPP and enzymes of proline metabolism we observed in soybeans. Table II gives fresh weight of nodules and the entire plant. The percent of total plant fresh weight in nodules ranged from 1.2% (alfalfa) to 12% (dahl).

Figure 2 gives activities of nitrogenase (C_2H_2 reduction), GS, P5CR, the PPP, and ProDH in nodules. On a per gram nodule basis, alfalfa nodules had by far the highest acetylene reduction rate (about 1360 nmol min^{-1} gfw nodule $^{-1}$). Nodules of other plants reduced acetylene at rates ranging from 75 (dahl) to 415 (yellow lupin) nmol min^{-1} gfw nodule $^{-1}$.

Pea nodule P5CR activity was almost half that of soybean nodule P5CR. Other amide-exporting nodules also exhibited high P5CR activities, especially peanut nodules. PPP activity was also no higher in the ureide-exporting nodules than in the amide-exporting nodules.

ProDH activities in the bacteroid fraction of nodules from ureide- and amide-exporting nodules are given in the lower panel of Figure 2. In general, these activities were higher in bacteroids from ureide- than from amide-exporting nodules. Activities ranged from 18% (*Arachis hypogaea*) to 240% (*Phaseolus vulgaris*) of that of bacteroids of soybean nodules, the activity of which is comparable to that of rat liver mitochondria.

DISCUSSION

Our previous observation that the activity of P5CR was approximately four times higher in ureide-exporting soybean nodules than in amide-exporting pea nodules (11) was consistent with our hypothesis that an important function of P5CR activity in soybean nodules was production of $NADP^+$ needed for R5P production by way of the PPP. R5P would then serve as precursor for PPRP needed for processing fixed N into ureides for export from the nodule to the rest of the plant. Since pea nodules export their fixed N as amides, there would be no such role for P5CR in pea nodules. But in this study, pea nodule P5CR activity was almost half that of

soybean nodule P5CR (Fig. 2, center panel), and other amide-exporting nodules also exhibited high P5CR activities, especially peanut nodules, where the P5CR activity was higher than in any of the ureide-exporting nodules. Nor was PPP activity consistently higher in the ureide-exporting nodules than in the amide-exporting nodules (Fig. 2, center panel). Thus, while P5CR activity may generate $NADP^+$ which stimulates carbon atom flux through the PPP, as hypothesized, there is no correlative evidence in this study to support a special role of P5CR in regulating ureide synthesis. The relatively constant values of the activities of P5CR and PPP throughout the development of soybean nodules (Fig. 1, center panel) makes it unlikely that the comparative observations among species of this study are the result of the choice of sampling time. However, such a result cannot be rigorously excluded in the absence of measurements of the phenological variation of the relevant activities for all eight species. Although the protein content (mg protein/gfw nodule) of the nodule cytoplasmic fractions varied by as much as a factor of four among species (data not shown), plotting enzyme activities on the basis of mg cytoplasmic protein, rather than fresh weight, did not reveal any regularities in P5CR and PPP activities when comparing ureide and amide exporters. However, the rank order of the activities was markedly different for activities calculated on a "per gfw" versus "per mg protein basis."

A correlation might be expected between nitrogenase activity and the activity of any enzyme intimately associated with N_2 -fixation. Such a correlation was observed between activities of nitrogenase and GS (Fig. 1, upper panel), an enzyme known to be required for the assimilation of fixed N, during soybean development. But these activities were not correlated across species (Fig. 2, upper panel). We therefore do not consider the lack of correlation of nitrogenase with P5CR and PPP activities as evidence against a possible connection of these activities with N_2 -fixation. A correlation between these P5CR and PPP activities might be expected if an important function of P5CR activity in soybean nodules were production of $NADP^+$ needed for synthesis of precursors of ureide synthesis by way of the PPP. Down regulation *in vivo* of excess capacity (a common occurrence) revealed by *in vitro* assay would result in an exception to this expectation. For example,

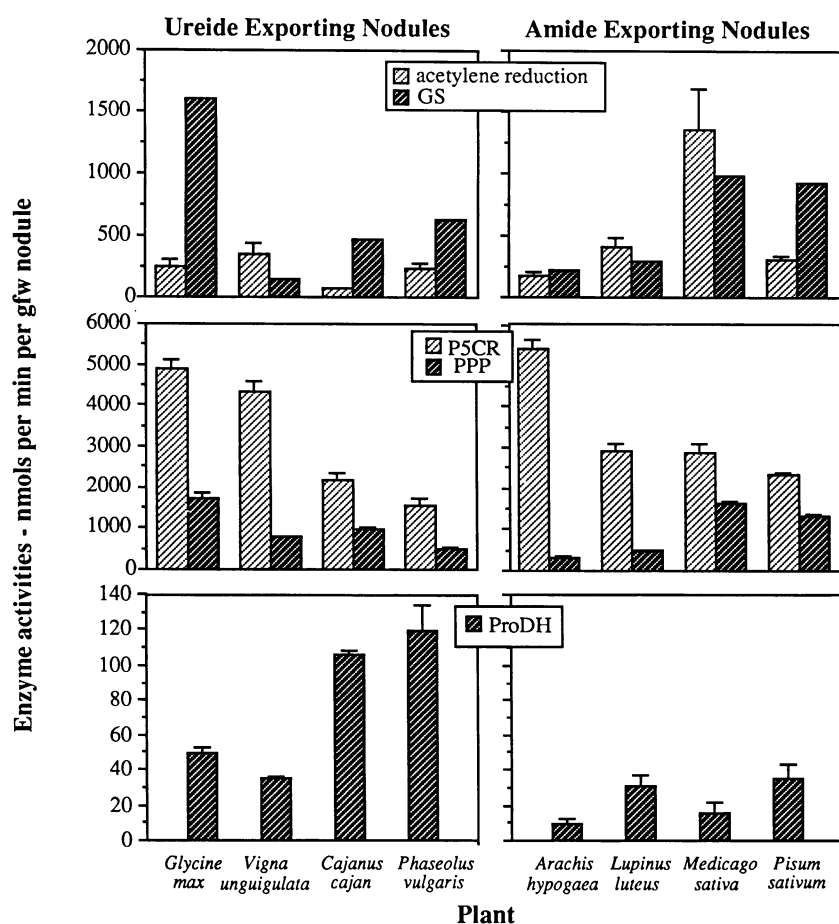


Figure 2. Nitrogenase, GS, P5CR, PPP, and ProDH activities in nodules of four species with ureide exporting nodules and four species with amide exporting nodules. Error bars represent the standard error of the mean. Plants were harvested at approximately midbloom. All measurements are for only one point in time.

the flux through the PPP *in vivo* is known to be only a few percent of the maximum *in vitro* rate, with rate limitation being imposed by the availability of NADP⁺ (12). Thus, the absence of a correlation between *in vitro* rates of P5CR and PPP does not rule out a close intercalation of the two activities *in vivo*. The data of Figure 2 (center panel) suggest a correlation between these two activities in ureide, but not amide-exporting nodules. However, nodules examined were from an insufficient number of plant species to establish the significance of this relationship.

The clearest distinction, with respect to activities of enzymes related to proline metabolism, between ureide- and amide-exporting nodules is that of ProDH activity in bacteroids (Fig. 2, lower panel). We have no working hypothesis for explaining the significance of this distinction. However, the magnitude of ProDH activity in nodules of all species examined is sufficiently high to supply a significant fraction of the energy requirement for N₂ fixation. This conclusion is based on a comparison of the energy needed to support the observed nitrogenase rates with the energy produced by the oxidation of proline to CO₂ at a rate given by the ProDH assay. For this calculation, we assumed (a) that the rate of N₂ fixation is one-fourth that of acetylene reduction (the former requires two electrons, the latter eight; six to N₂ and two additional electrons for the associated reduction of H⁺); (b) that 24 molecules of ATP are used per molecule of N₂ fixed (22); (c) that 30

molecules of ATP are produced per proline oxidized to 5 molecules of CO₂; and (d) an increase by a factor of 3 in the measured ProDH rate to reflect the underestimation caused by the tritium isotope effect, the latter being a conservative adjustment and consistent with preliminary data (our unpublished data). With these assumptions, in bacteroids from 32-d-old soybean plants, proline oxidation to CO₂ is capable of supplying about 1.4 times the ATP and reducing power needed to support the calculated rates of N₂ fixation, while, in bacteroids from 49-d-old plants, the energy yield of oxidizing the proline is more than eight times that needed to support the calculated N₂ fixation rate. The minimum activity of bacteroids from any other species examined was 18% of that of soybean nodules. Both the localization in bacteroids and its potential ability to contribute to the energy needs of the bacteroid suggest a role for ProDH activity in nitrogen fixation. The marked increase in activity during soybean ontogeny and the high levels of ProDH support a proposed role for ProDH in supplying at least a part of the energy needs of the bacteroids, especially late in growth.

Kohl *et al.* (11) reported that nodule P5CR activity was considerably higher than that reported for most other tissues. Values of as high as 281 and 47 nmols min⁻¹ mg protein⁻¹ were reported for the cytoplasmic fractions from soybean and pea nodules, respectively. Soybean nodule P5CR activities (on a "mg protein" basis) were 27, 10, and 23 times higher

than in pumpkin cotyledons, human erythrocytes, and rat liver, respectively. In this study, the activities of soybean nodule P5CR varied between 3240 and 5000 nmols min⁻¹ gfw nodule⁻¹ (Fig. 1), corresponding to a range of 280 to 470 nmols min⁻¹ mg protein⁻¹. Thus, soybean nodule P5CR activities reported here are as high or higher than those reported by Kohl *et al.* (11). Activities of P5CR in other nodules ranged between 1560 and 5410 nmols min⁻¹ gfw nodule⁻¹ (Fig. 1), corresponding to a range of 186 to 444 nmols min⁻¹ mg protein⁻¹. Extraordinary nodule P5CR activity is therefore confirmed in this study. P5CR activities reported here are higher, even, than in water-stressed barley seedlings (64 nmols min⁻¹ mg protein⁻¹, calculated on a crude extract basis from the measured value for the purified enzyme divided by 35,000 to reflect the degree of purification) (13).

The levels of nodule PPP activity are also quite high. Activities reported in Figures 1 and 2 range from 326 to 1790 nmols min⁻¹ gfw nodule⁻¹, corresponding to 15 to 180 nmols min⁻¹ mg protein⁻¹. These activities are much higher than those reported for mouse lens, where glucose-6-phosphate dehydrogenase activities fell in the range of 2 to 6 nmols min⁻¹ mg protein⁻¹ (24). Since two molecules of NADP⁺ must be reduced for each molecule of CO₂ produced, these rates correspond to 1 and 3 nmols CO₂ produced min⁻¹ mg protein⁻¹. Nodule PPP activities were of the same magnitude as those reported for crude homogenates of ascites tumor cells (750 nmols min⁻¹ gfw tissue⁻¹) (26). Using values of 22% dry weight for tumor cells (14) and 18.2% dry weight for soybean nodules (our unpublished data), PPP activity on a dry weight basis is about 1800 to 9800 nmols min⁻¹ gdw nodule⁻¹ compared with about 3400 nmols min⁻¹ gdw nodule⁻¹ in ascites tumor cells.

Although the activities of P5CR and the PPP in ureide and amide-exporting nodules offer no evidence in support of our original hypothesis concerning their role in promoting ureide synthesis, the very rapid induction of extremely high levels of PPP and P5CR activities supports the premise that these activities play an important role in nodule development and metabolism, perhaps in the initial events involved in nodule formation, and in production of proline (which requires NAD(P)H, possibly provided by the PPP) for energy transfer to bacteroids.

LITERATURE CITED

- Atkins CA, Shelp BJ, Storer PJ (1984) Nitrogen nutrition and the development of biochemical functions associated with nitrogen fixation and ammonia assimilation of nodules on cowpea seedlings. *Planta* **162**: 327-333
- Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254
- Bremner JM (1965) Total nitrogen. In CA Black, DD Evans, LE Ensminger, JL White, FE Clark, RC Dinauer, eds, *Methods of Soil Analysis, Part 2. Chemical and Microbiological Processes*. American Society of Agronomy, Madison, WI, pp 1149-1178
- Cha Y, Murray CJ, Klinman JP (1989) Hydrogen tunneling in enzyme reactions. *Science* **243**: 1325-1330
- Coker GT III, Schubert KR (1981) Carbon dioxide fixation in soybean roots and nodules. *Plant Physiol* **67**: 691-696
- Farnden KJF, Robertson JG (1980) Methods to studying enzymes involved in metabolism related to nitrogenase. In FJ Bergersen, ed, *Methods for Evaluating Biological Nitrogen Fixation*. John Wiley & Sons, New York, pp 265-314
- Fishbeck K, Evans HJ, Boersma LL (1973) Measurement of nitrogenase activity in intact legume symbionts *in situ* using the acetylene reduction assay. *Agron J* **65**: 429-433
- Hagedorn CH, Phang JM (1986) Catalytic transfer of hydride ions from NADPH to oxygen by interconversion of proline and Δ^1 -pyrroline-5-carboxylate. *Arch Biochem Biophys* **248**: 166-174
- Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N₂ fixation: Laboratory and field evaluation. *Plant Physiol* **43**: 1185-1207
- Johnson AB, Strecker JH (1962) The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. *J Biol Chem* **237**: 1876-1882
- Kohl DH, Schubert KR, Carter MB, Hagedorn CH, Shearer G (1988) Proline metabolism in N₂-fixing root nodules: energy transfer and regulation of purine synthesis. *Proc Natl Acad Sci USA* **85**: 2036-2040
- Krebs HAG, Eggleston LV (1974) The regulation of the pentose phosphate cycle in rat liver. *Adv Enzyme Res* **12**: 421-434
- Krueger W, Jager H, Hintz M, Pahlisch E (1986) Purification to homogeneity of pyrroline-5-carboxylate reductase of barley. *Plant Physiol* **80**: 140-142
- Levinson C (1990) Regulatory volume increase in Ehrlich ascites tumor cells. *Biochim Biophys Acta* **1021**: 1-8
- Mezl V, Knox W (1976) Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal Biochem* **74**: 430-440
- Morré DJ (1973) Isolation and purification of organelles and endomembrane components from rat liver. In MJ Crispeels, ed, *Molecular Techniques and Approaches in Developmental Biology*. John Wiley & Sons, New York, pp 1-27
- Phang JM, Downing SJ, Valle D (1973) A radioisotope assay for Δ^1 -pyrroline-5-carboxylate reductase. *Anal Biochem* **55**: 266-271
- Phang JM, Downing SJ, Yeh GC, Smith RJ, Williams JA, Hagedorn CH (1982) Stimulation of the hexose-monophosphate pentose pathway by Δ^1 -pyrroline-5-carboxylic acid in human fibroblasts. *J Cell Physiol* **110**: 255-261
- Reynolds PHS, Boland MJ, Blevins DG, Randall DD, Schubert KR (1982) Ureide biogenesis in leguminous plants. *Trends Biochem Sci* **7**: 366-368
- Reynolds PSH, Boland MJ, Blevins DG, Schubert KR, Randall DD (1982) Enzymes of amide and ureide biogenesis in developing soybean nodules. *Plant Physiol* **69**: 1334-1338
- Schubert KR (1981) Enzymes of purine biosynthesis and catabolism in *Glycine max.* I. Comparison of activities with N₂ fixation and composition of xylem exudate during nodule development. *Plant Physiol* **68**: 1115-1122
- Schubert KR (1982) The Energetics of Biological Nitrogen Fixation. Workshop Summaries—I. American Society of Plant Physiologists, pp 1-30
- Sengupta-Gopalan C, Pitas JW (1986) Expression of nodule-specific glutamine synthetase genes during nodule development in soybeans. *Plant Mol Biol* **7**: 189-200
- Shiono T, Kador PF, Kinoshita JH (1985) Stimulation of the hexose monophosphate pathway by pyrroline-5-carboxylate reductase in the lens. *Exp Eye Res* **41**: 767-775
- Smith RJ, Downing SJ, Phang JM (1977) Enzymatic synthesis and purification of 1-pyrroline-5 carboxylic acid. *Anal Biochem* **82**: 170-176
- Wenner CE, Hackney JH, Moliterno, J (1958) The hexose monophosphate shunt in glucose catabolism in Ascites tumor cells. *Cancer Res* **18**: 1105-1114
- Yeh GC, Phang JM (1988) Stimulation of phosphoribosylpyrophosphate and purine nucleotide production by pyrroline-5-carboxylate reductase in human erythrocytes. *J Biol Chem* **263**: 13083-13089