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Enzymes of Purine Biosynthesis and Catabolism in *Glycine max*[1]

I. COMPARISON OF ACTIVITIES WITH N_2 FIXATION AND COMPOSITION OF XYLEM EXUDATE DURING NODULE DEVELOPMENT

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ABSTRACT

During the period examined from 12 to 63 days after planting, the ureides, allantoin and allantoic acid, were the predominant nitrogenous solutes in the xylem exudate of soybeans (*Glycine max* [L.]) growing solely on symbiotically fixed nitrogen, accounting for approximately 60% and greater than 95% of the total nitrogen in the xylem exudate before and after the onset of active nitrogen fixation, respectively. For plants between 18 and 49 days of age, the apparent rate of ureide export estimated from concentrations of ureides in xylem exudate collected over a period of one hour was closely related to the rate of nitrogen fixation estimated from measurements of C_2H_2 reduction by nodulated root systems. After this time, the apparent rate of ureide export per plant continued to increase, reaching a maximum value at day 63 of 12 micromoles per plant per hour, even though the rate of C_2H_2 reduction per plant declined approximately four-fold. The most probable pathway for the biosynthesis of ureides involves the catabolism of purines. The levels of phosphoribosylpyrophosphate (*PRPP*) synthetase, which catalyzes the formation of the PRPP required for purine synthesis, increased in parallel with the rates of nitrogen fixation (C_2H_2) from day 18 reaching a maximum value of 13.9 micromoles per plant per hour at day 49, and then both activities declined rapidly. During the period of active nitrogen fixation the ratio of PRPP synthesis estimated from measurements of PRPP synthetase activity in cell-free extracts to the apparent rate of ureide export was between 1 and 2. The activities of the enzymes of purine catabolism, xanthine dehydrogenase, uricase, and allantoinase, increased in parallel with the increases in nodule mass and the export of ureides with maximum activities of 13, 119, and 79 micromoles per plant per hour, corresponding with apparent rates of ureide export in the range of 9.5 to 11.9 micromoles per plant per hour. These results demonstrate that there is a close association between nitrogen fixation, PRPP synthetase activity, and ureide export in soybeans and support the proposal that recently-fixed nitrogen is utilized in the *de novo* synthesis of purines which are subsequently catabolized to produce the ureides.

Based on *in vitro* measurements of the ATP requirement for the nitrogenase-catalyzed reduction of dinitrogen and whole plant studies of the effects of altering photosynthesis on the rates of N_2 fixation in legumes, a number of researchers have suggested that the availability of photosynthetically reduced carbon is the major factor limiting symbiotic nitrogen fixation (9). An estimated 15 to 30% of the net photosynthate of a plant may be required to support the reduction of N_2 and the assimilation of ammonia within the nodule (30). Although it is normally assumed that the major use of photosynthate is as a substrate for energy-yielding metabolism which produces the ATP and low potential electrons needed to drive the reduction of N_2, in actuality more than half of the photosynthate required for nodular activities is utilized in the assimilation and transport of recently-fixed nitrogen (30). For this reason, there is a need to analyze the energetics of the assimilation and transport of nitrogen in N_2-fixing plants.

In general, plants have adopted different "strategies" for assimilating and transporting nitrogen (20). The amide, asparagine, is the predominate form of organic nitrogen in the xylem exudate of many N_2-fixing plants including lupins (20), peas (20) and *Myrica* (13), the ureide citrulline predominates in the N_2-fixing nonlegume, *Alnus glutinosa* (14, 26, 27); and the ureides, allantoin and allantoic acid, are the principal forms of organic nitrogen exported from nodules of soybeans (15, 17, 31), cowpeas (10), and beans (4). The composition of the exudate may vary depending upon environmental conditions, plant age, and the sources of available nitrogen (15, 16, 20). Whether there are advantages to the differences in the form of nitrogen transported is open to investigation. McNeil *et al.* (18) have demonstrated that differences in the form of organic nitrogen being exported from roots and nodules may be one mechanism for selectively partitioning nitrogen to specific sinks. Likewise, there may be differences in the amount of photosynthate required for the generation of the energy and carbon skeletons used in the synthesis of different nitrogenous compounds. Therefore, the differences in carbon requirements may affect the overall efficiency of ammonia assimilation and transport.

In soybeans, recently-fixed nitrogen is incorporated into ureides (21, 26) within the nodule (15, 26) and exported to other parts of the plant. On the basis of the carbon skeleton requirement, the export of ureides, which have a C:N ratio of 1, may reduce the carbon costs of assimilation (12). An estimate of the overall efficiency of transporting ureides, however, requires elucidation of the pathway of ureide synthesis and an analysis of the respective costs of synthesis. Two possible pathways for ureide biosynthesis have been proposed (2, 24). The first involves the condensation of urea and a two-carbon fragment such as glyoxylate. The second involves the oxidative catabolism of purines. The presence of xanthine dehydrogenase, uricase and allantoinase, enzymes of purine catabolism, in nodules of ureide-exporting plants along with results of inhibitor studies (1, 6, 28, 33, 34) support the existence of the latter pathway. One of the problems which remains unresolved is to determine whether the rates of *de novo* purine synthesis and purine catabolism are sufficient to account for the rates of ureide synthesis. To address this problem, the activities of PRPP[2] synthetase, xanthine dehydrogenase, uricase, and allantoinase in cell-free extracts were examined and compared

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to the composition of the xylem exudate and the estimated rates of nitrogen fixation during nodule development.

MATERIALS AND METHODS

Growth of Plants. Soybean seeds (*Glycine max* [L.] Merr. var. Amsoy 71) were inoculated with *Rhizobium japonicum* (USDA Strain 3I1b 110 obtained from D. Weber, USDA, Beltsville, MD) and planted in 20-cm plastic pots filled with Perlite. Plants were irrigated with nitrogen-free nutrient solution (5) and grown in a greenhouse in natural daylight extended to a photoperiod of 16 h with supplemental fluorescent lighting (3, 5). Plants were grown during the period of late May to late July.

Collection and Analysis of Xylem Exudate. Xylem exudate was collected for 1 h at mid-day from the cut stems of 15 to 20 plants, the volume was determined, and the sap was stored at −20 C. The concentrations of the ureides in the exudate were measured using the differential analysis of Vogel and Van der Drift (35). Amino acids were quantified with an Aminco fluorescence detector (American Instrument Co., Silver Spring, MD) after derivatization of a sample of the exudate with *o*-phthaldehyde (11, 26) and separation of the derivatized amino acids by reverse-phase chromatography on an Ultrasil-ODS column (26). Based on the absence of detectable amounts of nitrate in the sap and results presented by McClure and Israel (17) for the total nitrogen balance for sap, the total nitrogen was assumed to be equal to ureide N plus amino acid N. The volume of sap and concentrations of the ureides and amino acids collected over this 1-h period were used to calculate an apparent rate of export. This calculation is based on certain assumptions concerning the loading of the xylem after detachment of the shoot and the distribution and concentration of solutes (see McClure and Israel (17) for discussion).

Preparation of Extracts. Extracts of nodules were prepared in the following manner: Approximately 1 to 3 g of nodules were harvested from 3 to 120 plants depending upon the age of the plants, weighed, and gently ground in a cold mortar and pestle in the presence of 2 volumes of 25 mM Tes, 0.3 M sorbitol (pH 7.5) and 0.1 g acid washed PVP per g nodule tissue. The extract was filtered through 8 layers of cheesecloth and the filtrate was centrifuged at 120*g* for 10 min to remove cell debris and PVP. The supernatant fluid was transferred to another tube and centrifuged at 1100*g* for 15 min to remove bacteroids. The supernatant fluid was weighed and stored on ice until used for enzyme assays. The weight and density of the extract was used to calculate the volume of the extract obtained per gram fresh weight of nodules.

Enzyme Assays. Nitrogenase activity was measured using the acetylene reduction assay (5, 29). Nodulated roots from 2 to 4 plants were sealed in a 250-ml flask from which 25 ml of air was removed and 25 ml of C_2H_2 was added (29). Samples were removed and analyzed by GC on a 1-m Porapak R (Alltech Associates, Deerfield, IL) column (3).

PRPP[2] synthetase activity in extracts was measured using the ^{32}P-transfer assay (25, 32). The complete reaction mixture contained 25 μmol Tes (pH 7.5), 12.5 μmol NaF, 2.5 μmol $MgCl_2$, 1.5 μmol ATP (pH 7.0), [γ-^{32}P]ATP (0.1 to 0.2 μCi/assay), and 2.5 μmol ribose-5-P in a final volume of 0.5 ml. The NaF was added to inhibit other ATPase activity.Blanks without ribose-5-P were used to determine the amount of "non-specific" ATPase activity in the extracts. Labeled ATP was synthesized using a modification of the ^{32}P-exchange reaction of Glynn and Chappell (7). The ^{32}P was obtained as a carrier-free solution of Pi from New England Nuclear. The reaction was initiated with the addition of enzyme extract, incubated for 20 min at 37 C and terminated by adding 0.5 ml of 5% cold $HClO_4$. The tubes were placed on ice for 10–15 min at which time 0.3 ml of a suspension of acid washed charcoal was added. Tubes were left on ice for an additional 10 min and 0.2 ml of a BSA-carrier solution (32) was added. The tubes were centrifuged at 1500*g* to remove charcoal and precipitated protein. The non-charcoal absorbable radioactivity in an aliquot of the supernatant fluid was measured by scintillation spectrometry using a Triton-based scintillation fluid (33% Triton: 66% toluene (v/v) with 5 g/l PPO and 0.1 g/l POPOP). Results were calculated after subtracting the amount of ^{32}P-released in the absence of ribose-5-P. Results presented are averages for triplicate determinations for two different levels of extract.

Glutamine synthetase activity was assayed using the ^{32}P-transfer assay as described above (25). The reaction mixture contained 25 μmol Tes (pH 7.5), 10 μmol $MgCl_2$, 2.5 μmol glutamate and 12.5 μmol NH_4Cl, 2.5 μmol ATP, and 0.1 to 0.2 μCi [γ-^{32}P]ATP per assay. Blanks without glutamate, NH_4Cl or both were used to measure nonspecific activities. The NaF inhibited glutamine synthetase activity and was deleted from the reaction mixture. The activities which were not dependent upon both glutamate and ammonia were subtracted to calculate the glutamine synthetase activity. Reaction mixtures were incubated for 20 min at 37 C and terminated as described above. Results presented were based on triplicate determinations at two different levels of enzyme.

Xanthine dehydrogenase and uricase activities were measured spectrophotometrically at room temperature. The reaction mixture for measurement of xanthine dehydrogenase activity contained 75 μmol Tes (pH 8.4), and 5 μmol NAD. Enzyme extract was added to sample and reference cuvettes and the reaction was initiated with the addition of 0.25 μmol xanthine to the sample cuvette. The final volume was 1.5 ml. The change in *A* at 340 nm was monitored for 5 to 7 min for replicated samples. Uricase activity was measured as a change in the *A* at 292 nm due to the loss of uric acid. The reaction mixture contained 75 μmol of CHES (pH 10.0), and 100 μl of a solution of uric acid prepared by dissolving 12.5 mg uric acid in 50 ml of 0.02 M CHES (pH 10). The final volume of the reaction mixture was 1.5 ml. The reaction was initiated with the addition of extract.

Allantoinase activity was determined by measuring the enzymic formation of allantoic acid. Allantoic acid was measured as the diphenylformazan of glyoxylate, which absorbs strongly at 520 nm, after degradation of the allantoic acid by boiling in dilute HCl. The assay procedure was modified from the procedure described by Ory *et al.* (19) to eliminate the formation of a yellow product which interferred with the measurement of the pink-colored diphenylformazan. Because many variables such as buffer used, buffer concentration, length of incubations, timing and order of additions, and the concentration of HCl used to stop the reaction affect the sensitivity, repeatability, and validity of the assay, the assay is described in detail. All reaction mixtures contained 1 ml of 25 mM allantoin and 1 ml of 0.1 M Tes (pH 8.1). Acid (0.5 ml of 0.2 N HCl) was added to tubes to be used for zero-time controls. Extract was added first to the tubes to be incubated for 20 min and then to the controls. All tubes were incubated at 37 C for 20 min and the reaction was terminated by adding 0.5 ml of 0.2 N HCl to the tubes not used as controls. After the acid was added to all tubes they were placed in a water bath at 100 C for exactly 4 min and then immersed in an ice bath for 45 s. Tubes were kept at room temperature while 0.5 ml of a 3 mg/ml solution of phenylhydrazine was added to all tubes and then were incubated for 10 min at 37 C. Tubes were removed and cooled by dipping them into an ice bath for 30 s. Tubes were kept at room temperature while 1.2 ml of concentrated HCl was added and mixed and 0.5 ml of a 16 mg/ml solution of potassium ferricyanide was added. The solution was vortexed several times before and during the subsequent incubation at 37 C for 20–30 min. The *A* at 520 nm, which changes with time, was measured immediately. Tubes which did not have the characteristic pink color of the

[2] Abbreviations: PRPP, phosphoribosylpyrophosphate; CHES, [2-(N-Cyclohexylamino)ethane sulfonic acid]; SEM, standard error of mean.

diphenylformazan of glyoxylate (maximum A at 520 nm) were discarded. Results were based on a standard curve prepared at the same time which was linear over the range of 20 to 400 nmol of allantoic acid.

RESULTS

The apparent rate of export of amino acid and ureide nitrogen in the xylem exudate along with the rates of C_2H_2 reduction and the nodule mass during plant development are presented in Figure 1. Immature nodules were visible on roots 12 days after planting soybean seeds inoculated with a broth culture *R. japonicum*. Nodule mass increased in a linear fashion from days 15–35 after which time the rate of nodule growth doubled (Fig 1C) from days 35 to 49. After day 49, the nodule growth rate declined to approximately the same rate as measured from days 15 to 35.

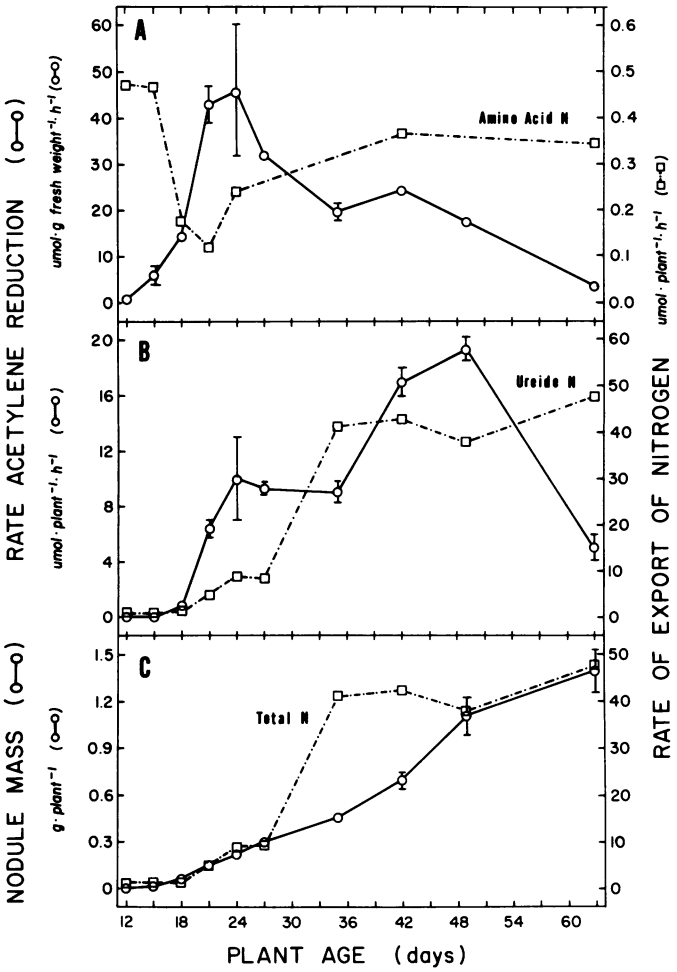

FIG. 1. Comparison of the apparent rate of export of amino acid and ureide nitrogen in the xylem exudate, nodule mass, and the rates of C_2H_2 reduction during plant development. The specific and total activities of C_2H_2 reduction are represented by the solid lines (O—O) in A and B, respectively. The rates of C_2H_2 reduction were determined on nodulated root systems of 4 plants (fewer plants were used at the later samplings) with five replicates. Nodules were harvested from these plants and used to determine the average nodule mass per plant (C). The apparent rate of export of amino acid (A), ureides (B) and total nitrogen (C) in μmol N·plant^{-1}·h^{-1} are presented using the dashed lines (□-·-□). Xylem exudate was collected for 1 h from the cut stems of 15 to 20 plants and pooled. The concentration of nitrogen in amino acids, ureides, and total nitrogen was calculated from the ureide and amino acid composition of the sap. SE bars are presented when the SEM was greater than the size of the symbol. Flowering began between 50 and 54 days of age.

The amount of nitrogen transported in the form of amino acids was highest during the period from 12 to 15 days after sowing after which time the apparent rate of export of total amino acid nitrogen dropped dramatically and then increased again to a level somewhat less than the level present between days 12 and 15 (Fig 1A). This decrease in the export of amino acids in the sap coincided with the rapid increase in nodule mass and the corresponding increase in nitrogenase activity as measured using the C_2H_2 reduction assay. The rate of ureide export increased 7-fold during the period between 18 and 27 days of age but lagged with respect to the increase in C_2H_2-reducing activity (Fig. 1B).

The specific activity of C_2H_2 reduction increased rapidly after day 18 reaching a maximum value of 45 μmol C_2H_2 reduced·g nodule fresh weight^{-1}·h^{-1} by day 24 (Fig. 1A). The total C_2H_2-reducing activity per plant peaked at 24 days of age, declined slightly over the next 11 days and then, as a direct result of the rapid increase in nodule mass per plant, increased to a maximum value of 19 μmol C_2H_2 reduced·plant^{-1}·h^{-1} at day 49 (Fig. 1B). Although nodule mass continued to increase, the total C_2H_2-reducing activity declined rapidly thereafter corresponding to the rapid decrease in the specific activity as nodule size increased. The apparent rate of ureide export increased steadily after day 15 to a rate 70-times the rate at day 15 by the end of the period examined and did not decrease with the decreasing rates of C_2H_2 reduction per plant after day 49 (Fig. 1B).

The relative distribution of nitrogenous compounds in the exudate and their apparent rates of export are presented in Figure 2. Even 12 days after planting, the ureides were the predominant form of nitrogen in the xylem exudate of soybeans accounting for 58% of the total nitrogen. Asparagine was the major amino acid present in the exudate at all times examined except day 21. The maximum transport of asparagine occurred prior to the onset of active nitrogen fixation. The transport of asparagine as well as total amino acids reached a minimum value 21 days after planting and increased somewhat thereafter. The aspartate, glutamate, and glutamine concentrations changed very little during ontogeny with the exception of a two-fold increase in the aspartate concentration on day 21. The fraction of the total nitrogen in the form of amino acids was the greatest before the onset of active nitrogen fixation. After nodules were actively reducing dinitrogen, the amount of ureides exported from nodulated roots rose continuously representing 98% or more of the total nitrogen in the exudate after day 21. The maximum estimated rate of ureide export in the xylem was 11.9 μmol ureides·plant^{-1}·h^{-1}.

According to current concepts, the primary assimilation of recently-fixed nitrogen occurs through the action of glutamine synthetase. The glutamine formed in the process can serve as an amino-group donor for the synthesis of glutamate, aspartate, asparagine, other amino acids as well as for the synthesis of purines. The results of measurements of glutamine synthetase activity are given in Figure 3. The specific activity of glutamine synthetase followed a pattern similar to the pattern observed for the changes in the specific activity of C_2H_2 reduction, increasing 30-fold from days 12 to 42 and then declining. The levels of glutamine synthetase per plant, however, increased continuously throughout the period examined and did not decline after day 49 as was the case for the total rate of C_2H_2 reduction per plant.

The first reaction of purine biosynthesis is catalyzed by PRPP synthetase. The PRPP formed is also required for the synthesis of pyrimidines, pyridine nucleotides, histidine, and tryptophan. The specific activity of this enzyme in nodule tissue increased a little over two-fold during nodule development and then dropped rapidly (Fig. 3B). On a per plant basis, the levels of PRPP synthetase activity increased until day 49 at which time the activity declined in parallel with the decrease in C_2H_2 reduction per plant. The maximum estimated rate of PRPP synthesis per plant was 14 μmol

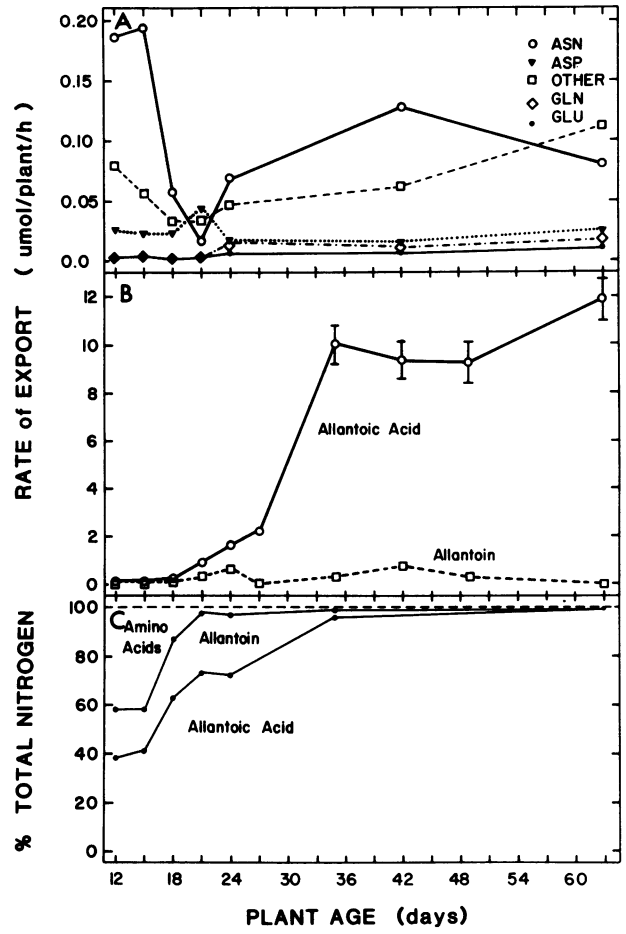

FIG. 2. Apparent rates of transport of amino acids and ureides in the xylem at different stages of nodule development. The concentration of asparagine (○), aspartate (▼) glutamine (◇), glutamate (●) and other amino acids (□) in the xylem sap (*A*) was analyzed by HPLC. The concentration of allantoin and allantoic acid ± SEM in the exudate is presented in B. The total nitrogen in the sap was calculated on the basis of the nitrogen in the amino acids and ureides; levels of nitrate in the sap were not measurable. The percent of the total nitrogen in the form of amino acids, allantoin and allantoic acid are given in C and are represented by the areas between the curves.

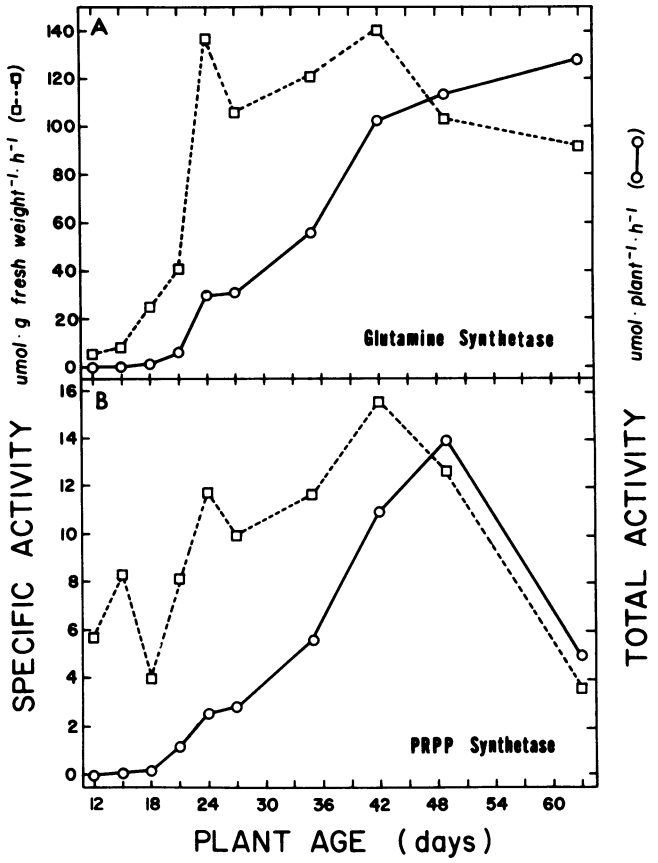

FIG. 3. Glutamine synthetase and PRPP synthetase activities in extracts prepared from nodules at different stages of development. Extracts were prepared from nodules harvested from 126 (day 12) to 3 plants (day 63). Glutamine synthetase (A) and PRPP synthetase activities (B) were measured under standard assay conditions and the values are reported for the specific activity (□--□) and the total activity per plant (○—○) for triplicate determinations.

PRPP formed·h^{-1}. For the period after the onset of active nitrogen fixation, the relative change in the total C_2H_2-reducing activity per plant was closely coordinated with the change in total PRPP synthetase activity. As presented in Figure 4, the ratio of the relative rate of change with time of total activity for C_2H_2 reduction and PRPP synthetase were relatively constant after day 21 approaching a ratio of one.

A number of reports (1, 6, 8, 28, 33, 34) indicate the presence of several of the enzymes of purine catabolism in the nodule tissue of ureide-exporting plants. Xanthine dehydrogenase, uricase, and allantoinase activities were measured during plant development and are reported in Figure 5. The specific activities of xanthine dehydrogenase and uricase increased 6 and 7-fold, respectively, from days 18 to 35 and decreased slightly thereafter. In contrast, the specific activity of allantoinase was greatest in immature nodules (150 μmol allantoic acid formed·g nodule fresh weight^{-1}·h^{-1}) and decreased during nodule development. Except for a peak of activity which occurred between 21 and 24 days of age, the specific activity was relatively constant at 50–70 μmol·g fresh weight^{-1}·h^{-1}. The rise in specific activity coincided with the peak in the specific activity of C_2H_2 reduction. The levels of these three

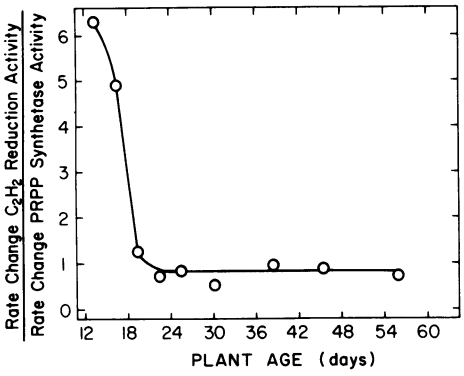

FIG. 4. Comparison of the changes in C_2H_2 reduction and PRPP synthetase activity during development. The ratio of the rate of change in C_2H_2 reduction activity from one sampling to the next to the rate of change in PRPP synthetase activity during the same period was calculated from values taken from Figures 1 and 3.

enzymes per plant increased in parallel with the increase in nodule mass and glutamine synthetase activity with the exception that xanthine dehydrogenase activity dropped slightly on the last sampling date. The maximum values of xanthine dehydrogenase, uricase, and allantoinase activities were 13.4, 119 and 79 μmol·plant^{-1}·h^{-1}, respectively, corresponding to rates of ureide export in the range of 9.5 to 11.9 μmol·plant^{-1}·h^{-1} (Fig. 2).

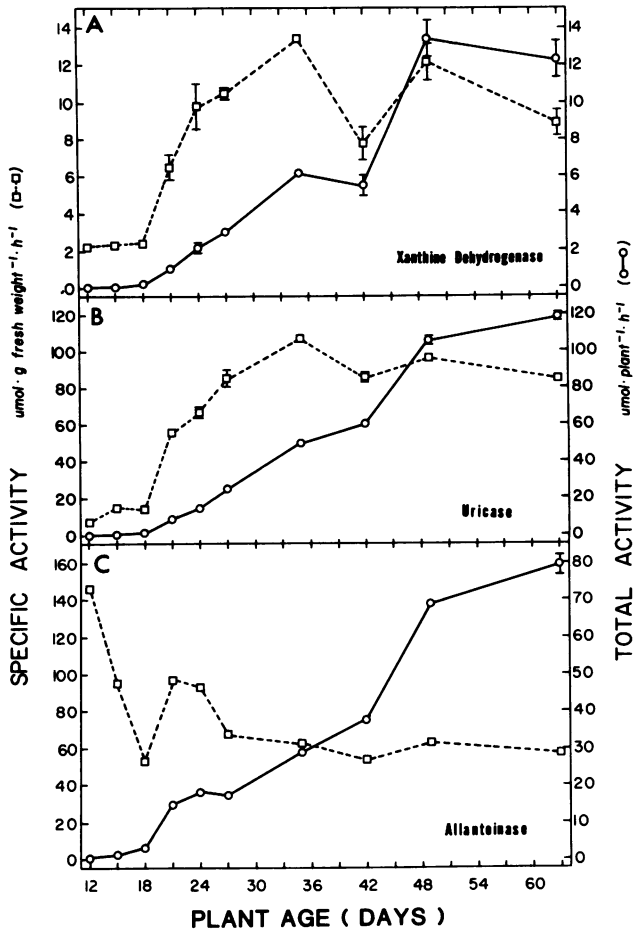

FIG. 5. Xanthine dehydrogenase, uricase and allantoinase activities in extracts prepared from nodules during plant development. Extracts were prepared and assays for xanthine dehydrogenase (A), uricase (B) and allantoinase (C) were carried out under standard assay conditions. Values for specific activity (□—□) and total activity per plant (○—○) for triplicate determinations are presented. SE bars are included when larger than the symbols used.

To examine the relationship between ureide synthesis, nitrogen fixation, and *de novo* purine biosynthesis, the relative rates of nitrogen export as ureides and estimated rates of nitrogen fixation and PRPP synthesis were compared (Fig 6). The rates of nitrogen fixation were estimated using a conversion factor of 2 C_2H_2 reduced per NH_4^+ produced based on relative efficiency measurements (29) for this combination of host cultivar and strain of *R. japonicum*. The ratio of the apparent rate of export of ureide nitrogen per plant per hour to the estimated rate of NH_4^+ production was approximately 2 from the onset of nitrogen fixation (day 18) through day 27 and increased gradually up to day 49. The results of a comparison of the estimated rates of PRPP synthesis based on enzymic assays in cell-free extracts to the rates of NH_4^+ production were similar. Because 4 mol of NH_4^+ are incorporated into each mole of purine synthesized from PRPP, the rates of NH_4^+ production were divided by 4. In a final analysis, the apparent rates of ureide export to the estimated rates of PRPP synthesis were compared. The ratio was approximately 1 or slightly less throughout this same period of plant development in which plants were actively fixing nitrogen.

DISCUSSION

The rapid decline in the concentration of amino acids, predominantly asparagine, in the xylem exudate of soybeans signaled the

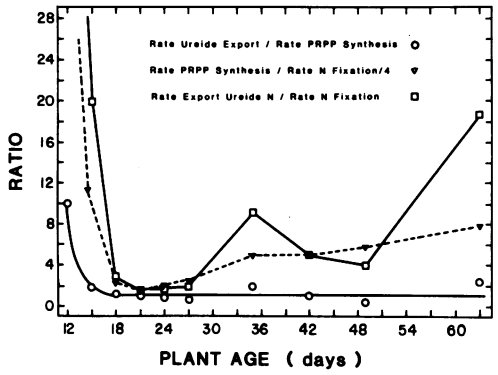

FIG. 6. Comparison of the relative rates of ureide export, nitrogen fixation and PRPP synthesis during nodule development. The ratio of the apparent rate of export of ureide nitrogen to the estimated rate of nitrogen fixation in μmol N (□), the ratio of the apparent rate of export of ureides to the *in vitro* rate of PRPP synthesis (○) and the ratio of the rates of PRPP synthesis to the rate of fixation ÷ 4 (▽) were calculated from results presented in Figures 1 and 3.

onset of active nitrogen fixation and a linear increase in nodule mass. Over the next 9 days of growth, the specific activity of C_2H_2 reduction increased to a maximum value. This increase in nitrogen fixation was not reflected by a corresponding increase in the total nitrogen exported, either as amino acids or ureides. Atkins *et al.* (1) observed an overall decline in the concentration of nitrogenous solutes in the xylem sap collected from nodulated cowpea plants during the early phase of rapid nodule growth. During this period of nodule development, nodule growth may be nitrogen limited. For this reason, recently-fixed nitrogen may be preferentially incorporated into nodule components thereby decreasing the amount of nitrogen available for export to other plant parts. This interpretation is consistent with the common observation that plants totally dependent upon N_2 fixation undergo a period of nitrogen limitation during early nodule development. Through the addition of a solution of "starter" nitrogen during this period, it is possible to alleviate this deficiency which affects N_2 fixation directly by retarding nodule development and indirectly through the affects of nitrogen stress on whole plant photosynthesis.

After soybean nodules started actively fixing nitrogen, the rate of ureide export increased, whereas, in cowpeas (1) there apparently was a 5-day lag. The close correlation between the apparent rates of nitrogen export as ureides and the estimated rates of NH_4^+ production in nodules during this time (Fig. 6) is consistent with results from tracer studies using $[^{15}N]N_2$ (21) and $[^{13}N]N_2$ (26) which indicate that ureides are formed from recently-fixed nitrogen. Values for the ratio of ureide nitrogen exported to NH_4^+ produced equal to approximately 2 may reflect a decrease in the rate of C_2H_2 reduction upon detachment of shoots, as has been noted previously (3, 29), or differences between the theoretical conversion factor used in the calculations and the actual conversion factor. On day 35, there was a rapid increase in ureide export which occurred in parallel with the doubling of nodule growth rate and an increased rate of nitrogen fixation (C_2H_2) per plant. One interpretation of these observations is that nodules are no longer nitrogen limited and are providing sufficient reduced nitrogen to sustain the rapid expansion of photosynthetic leaf tissue. As a direct result of the increased photosynthetic capacity of the plants, there is an increased supply of photosynthate available to support the rapid increase in nodule mass and to sustain the higher rates of nitrogen fixation per plant.

At all the times examined in this study, the activities of xanthine dehydrogenase, uricase, and allantoinase per plant were equal to or several times greater than the apparent rates of ureide export per plant. The rates were greater than those reported previously

for soybeans (34) and for cowpeas (1). The highest specific activities (and total activities) for xanthine dehydrogenase, uricase, and allantoinase measured in these studies were 13 (13), 107 (119), and 146 (79) μmol·g fresh weight^{-1}·h^{-1} (μmol·plant^{-1}·h^{-1}), respectively. The corresponding maximum values in cowpeas (1) were 10 (1.4), 54 (8) and 5 (0.8). In soybeans, the levels of these three enzymes increase in parallel with nodule mass, glutamine synthetase activity, and ureide transport, whereas, in cowpeas the levels of the enzymes increased several days before the beginning of nitrogen fixation and preceded the increase in ureide content in the sap by more than a week. Because of the very short period examined in the case of cowpeas, possible differences in plant growth patterns, and differences in the presentation of results, it is not possible to compare directly the results of both studies. Results of both of these studies provide additional support to the proposal (2, 24) that the ureides originate from purines.

The question as to whether or not purines, which are synthesized *de novo*, serve as direct precursors of the ureides and the exact nature of the pathway still remains to be answered. In preliminary tracer studies, Atkins *et al.* (1) demonstrated that cowpea nodule slices incorporated [^{14}C]glycine into ureides in a manner consistent with the *de novo* synthesis of purines. We have reported (28) that the levels of PRPP synthetase, which catalyzes the first step leading to *de novo* purine synthesis is elevated in soybean nodules above the levels required for nucleic acid synthesis. In addition PRPP amido transferase, which catalyzes the first committed step of *de novo* purine synthesis, has been detected in soybean nodule extracts [(22), Matia and Schubert, unpublished results)]. The level of measured activity, however, is not sufficient to account for the synthesis of ureides. This may be explained by the instability of the enzyme in cell-free extracts. The results of the present study help to establish the role of PRPP synthetase and *de novo* purine synthesis in the production of ureides.

During the period of active nitrogen fixation, the estimated rates of synthesis of PRPP were sufficient to support the flux of fixed nitrogen exported from nodules in the form of ureides. This is reflected in the developmental pattern observed as well as the correlation between the estimated rates of NH_4^+ production, ureide export, and the synthesis of PRPP. From days 18 through 35 there were approximately 1–2 mol of PRPP synthesized (under standard assay conditions) per mole of ureide exported from nodules. These findings support a primary role of PRPP synthetase and *de novo* purine synthesis in the synthesis of allantoin and allantoic acid.

The PRPP synthesized in nodules is also needed for the synthesis of pyrimidines, pyridine nucleotides, tryptophan, and histidine. By using measurements of aspartate transcarbamylase which catalyzes the first committed reaction of pyrimidine biosynthesis as an indicator of general nucleic acid synthesis we have demonstrated that the levels of PRPP synthetase activity are several orders of magnitude greater than those of aspartate transcarbamylase during the period of active N_2-fixation (K. R. Schubert, unpublished results). In comparison to the changes in the specific activities of glutamine synthetase, xanthine dehydrogenase and uricase, there was only a two-fold increase in the specific activity of PRPP synthetase after the onset of nitrogen fixation. This observation is consistent with the most important role for this enzyme in the synthesis of nucleic acids and other essential metabolites during this period of rapid cell growth.

At early stages of nodule development (12–15 days of age), the ratios of ureide export to nitrogen fixation or PRPP synthetase activity were very high which indicates that the ureides present in the exudate at this time may arise from seed reserves of ureides or from the catabolism of nucleic acids. The specific activity of allantoinase was also the highest during this time and is extremely high in germinating seedlings (Chia and Schubert, unpublished results). The catabolism of nucleic acids could also account for at least part of the ureides in the exudate in the later stages of development when the central nodule tissue is becoming senescent and the ratios of ureide export to nitrogen fixation are again rising. The total nitrogen of the nodule tissue, however, is not sufficient to sustain the observed levels of ureide transport. Alternate explanations include substantial decreases in the rates of C_2H_2 reduction of nodulated roots upon removal of the shoots in the older plants, differences between the actual conversion factor and the conversion factor used to estimate the rate of nitrogen fixation, and possible recycling of ureides via the phloem. A similar situation may occur in cowpeas (1) since the rates of ureide export and the levels of the enzymes of purine catabolism continued to rise even though the rates of nitrogen fixation were declining.

Assuming that the pathway of purine biosynthesis in nodules is the same as the pathway in bacteria and animals, one can estimate the energy cost of synthesizing purines and ureides and compare these values to the theoretical values for the synthesis of other nitrogen-containing compounds transported from roots and nodules of nitrogen-fixing plants. Expressing the energy costs in terms of ATP-equivalents (*i.e.* NADH = 3 ATP-equivalents) the cost of purine biosynthesis starting with NH_4^+, CO_2, formate, and P-glycerate (as a source of glycine and Cl unit via serine transhydroxymethylase reaction; Boland and Schubert, unpublished results) is 8 mol of ATP per mol of inosine produced. The range of values reflects differences in the pathways used for the generation of the C1 and C2 carbon fragments required for purine biosynthesis and excludes the costs of the carbon skeletons (*i.e.* the moles of ATP that could be generated upon the complete oxidation of the carbon skeleton to CO_2). Including the equivalent cost of the carbon skeleton, the value is 27 mol ATP per mol of inosine.

In the catabolic sequence of reactions leading to the synthesis of ureides, part of the energy invested in purine biosynthesis can be recovered through the coupling of NAD^+ reduction to the oxidation of hypoxanthine and xanthine catalyzed by xanthine dehydrogenase. For this reason, the occurrence of xanthine dehydrogenase in nodules, (1, 28, 34) and not xanthine oxidase as

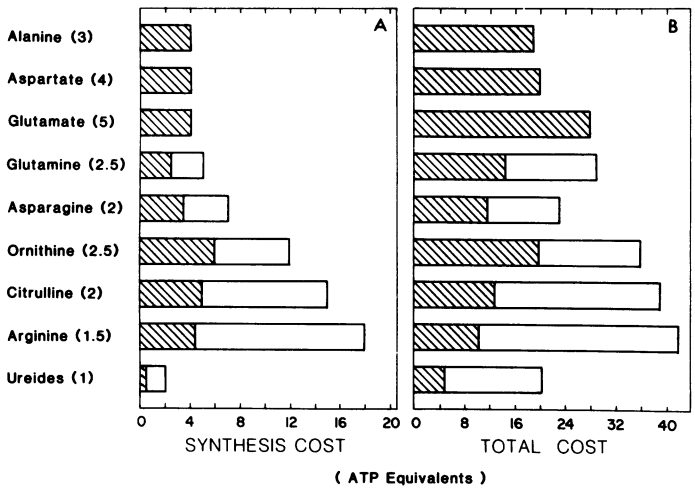

FIG. 7. Comparison of the theoretical costs of synthesis of ureides, asparagine and other amino acids. The theoretical costs of synthesizing various nitrogenous compounds were calculated based on known biochemical pathways and are expressed in terms of ATP-equivalents (NADH = 3 ATP's). The open bars represent the cost per mol of compound and the hatched bars represent the cost on a per nitrogen basis. Values for the C:N ratio for each compound are given in parentheses. The biochemical costs of synthesis are presented in A and the total estimated cost of synthesis including the equivalent cost of the carbon skeletons are given in B. The estimated costs for ureide synthesis assumes that IMP is the ultimate product of *de novo* purine synthesis and IMP is converted to xanthine in two reactions coupled to NAD reduction.

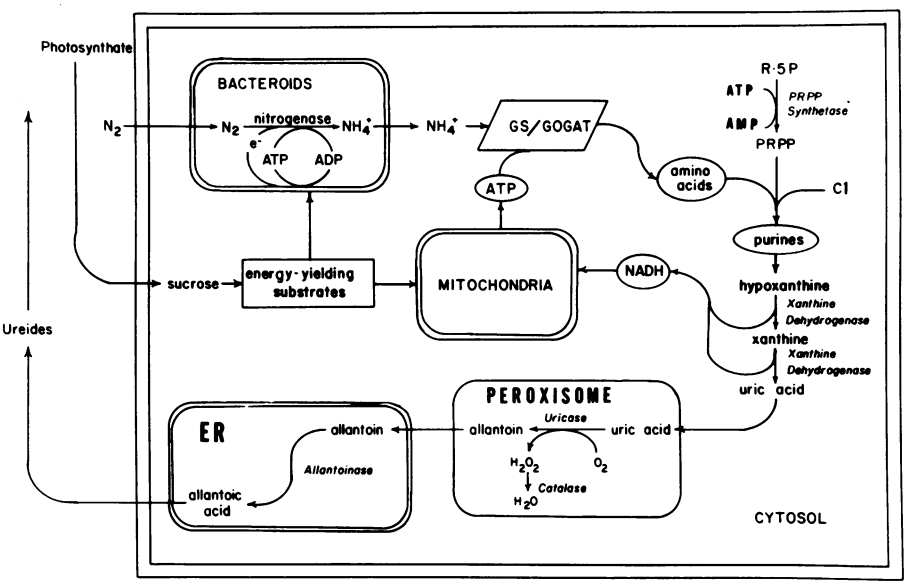

FIG. 8. Proposed pathway and intracellular location of ureide biosynthesis in soybean nodule cells. The proposed pathway for ureide biosynthesis and the intracellular location within nodule cells of the enzymes of purine biosynthesis and catabolism are based on results of these and other studies (8, 28).

reported previously (6, 33), is extremely important in terms of the respective energy costs of ureide production (1, 23, 28, 34). Recent results indicate that xanthine used for ureide synthesis may not be formed directly from hypoxanthine (personal communication Atkins and Blevins; and Boland and Schubert, unpublished results). This possibility is relevant to the question concerning estimated costs and elucidating the exact nature of the pathway(s) of purine and ureide synthesis including possible regulatory sites. Taking into account the NADH produced, then the apparent cost of ureide biosynthesis is 2 ATP-equivalents per mol of ureide or 0.5 per nitrogen transported as allantoin or allantoic acid. This compares to a theoretical cost of asparagine synthesis of 7 ATP-equivalents per mol of asparagine (3.5 per nitrogen). Theoretical estimates of the relative cost of synthesis of ureides, asparagine, and other amino acids are summarized in Figure 7. Based on these estimates, the export of fixed nitrogen in the form of ureides is apparently more efficient than the transport of nitrogen in asparagine or other amino acids and an even greater advantage is apparent if one includes the relative cost of the carbon skeletons.

These estimates, however, do not consider the costs associated with the synthesis of essential enzymes, the transport of ureides across membranes or the costs involved in the metabolism of ureides, the reassimilation of NH_4^+ and the synthesis of amino acids. The latter costs may or may not be "free" when occurring in photosynthetic tissues (30). Transport costs may be extremely important to the overall cost of synthesis but reliable values for transport within nodules are not available but may be similar to those for transport of the other nitrogenous solutes in the xylem stream.

Transport across membranes may also be important because we have demonstrated in previous studies (8, 28) that the enzymes of purine biosynthesis and catabolism are in different intracellular compartments. The proposed pathway and intracellular location of ureide biosynthesis in soybean nodule cells is presented diagrammatically in Figure 8. Although all of the reactions of purine biosynthesis and catabolism are apparently located in the plant cytosol, purine biosynthesis occurs in the soluble fraction of the cytosol; the oxidation of uric acid and the degradation of the H_2O_2 formed in the uricase reaction take place in peroxisomes; and allantoic acid is formed from allantoin in the microsomes which apparently arise from the ER. The ER may also be involved in the excretion of allantoic acid (8).

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