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Glutamine synthetase activity is present in both the cytosol and bacteroid fractions of soybean root nodules. Total activity measurements indicate that at least 90% of the activity in nodules is located in the cytosol. Results reported by McParland, et al. (29), showed that many properties of glutamine synthetase from nodule cytosol are similar to those of the enzyme from other eucaryotic organisms. Glutamine synthetase has been purified to more than 90% homogeneity from both the nodule bacteroids and free-living Rhizobium japonicum. The enzymes from these sources displayed similar behavior in all aspects of purification. Experiments in which a snake venom phosphodiesterase was used to remove adenyl groups from the enzymes from both nodule bacteroids and free-living Rhizobium japonicum showed that both glutamine synthetases

contain adenylyl groups. In contrast, no evidence has been found for adenylylation or deadenylylation of the cytosol glutamine synthetase. On the basis of behavior during purification and mobility in gel electrophoresis experiments, the bacteroid and cytosol glutamine synthetases are distinctly different while the glutamine synthetases from bacteroids and free-living Rhizobium japonicum had similar electrophoretic and other properties. In many aspects the enzyme from Rhizobia is similar to the glutamine synthetases from Escherichia coli and other gram-negative bacteria. The physiologically active, unadenylylated glutamine synthetase from rhizobia showed greater biosynthetic activity with magnesium than with manganese as the divalent cation activator. The adenylylated enzyme showed a preference for manganese as a divalent cation activator for biosynthetic activity.

The Glutamine Synthetases In Soybean Root Nodules And Free-living *Rhizobium japonicum*

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LIST OF ABBREVIATIONS

A	absorbancy
$\overset{\circ}{A}$	angstrom
ALA	δ-aminolevulinic acid
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5-triphosphatase
C	Celsius (degree of temperature)
CTP	cytosine-5-triphosphate
DEAE	diethylaminoethyl
DTT	dithiothreitol
K_m	Michaelis constant
mA	milliampere(s)
P_i	inorganic phosphate
ppt	precipitate
psi	pound per square inch
PVPP	polyvinylpolypyrrolidone
SDS	sodium dodecylsulfate
S.E.M.	standard error of means
SVD	snake venom phospho-diesterase
Tes	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
Tris	tris(hydroxymethyl)aminomethane

The Glutamine Synthetases In Soybean Root Nodules And Free-living *Rhizobium japonicum*

INTRODUCTION

Ammonia produced by nitrogenase or by nitrate and nitrite reductases is initially incorporated into glutamine in the presence of glutamine synthetase (32, 57). Glutamine synthetase activity was first demonstrated by Krebs (21) using slices of kidney, brain, and retina of various vertebrate species. Speck (53) showed that partially purified enzyme preparations from acetone-dried pigeon liver catalyzed a reaction of glutamate, ammonia and ATP yielding glutamine, ADP and inorganic phosphate. In 1950 Stumpf and Loomis (60) reported a plant amide enzyme system in sugar pumpkin seedlings which caused the formation of glutamylhydroxamic acid and ammonia from glutamine and hydroxylamine. Loomis (25) documented the distribution of glutamine synthetase in various species of the phyla *Thallophyta* and *Spermatophyta*. In the 1960's, Stadtman (71) and his co-workers characterized glutamine synthetase from *Escherichia coli*. During the same period, Jaenicke (56) and Meister (46) elucidated the characteristics of mammalian glutamine synthetases.

Approximately 16 percent of all protein in living tissue is composed of nitrogen (39). This emphasizes the role that the amide, glutamine, plays in the incorporation of nitrogen into the metabolic pathways of life. Glutamine is the precursor for several metabolites (15,31,55) and as an amide it serves as the substrate for enzyme systems which catalyze the formation of other amides and amino acids (30,45). In plant systems, glutamine appears to be a major compound involved in the transport of

nitrogen from the roots to the shoots of plants (32). Recent evidence has shown that glutamine in combination with ammonia and other amino acids may regulate the multi-enzyme systems for the fixation and assimilation of dinitrogen (6, 49, 59, 64).

Members of the Leguminosae in symbiosis with various species of the genus Rhizobium contribute greatly to the world-wide biological reduction of dinitrogen to ammonia. In 1972 legumes were estimated to fix about 35×10^6 metric tons of atmospheric nitrogen (8) as compared to 30×10^6 metric tons of ammonia produced by industrial methods. Other biological agents that contribute substantially to the quantities of N_2 fixed over the world include nodulated, non-leguminous woody species, algae and bacteria in rice fields, and associations of bacteria with marine organisms (8). Ozonization and lightning also add to the reduction of dinitrogen from the earth's atmosphere. In spite of the quantities of N_2 fixed, the earth faces an impending food shortage and inadequate supply of fixed nitrogen is a major limiting factor (41).

Since there is ample evidence to substantiate an important role of glutamine synthetase as the primary step in ammonia incorporation, research efforts have been made to elucidate the characteristics of the enzyme in the soybean root nodules. Both the cytosol (or soluble non-bacteria fraction) and bacteroid fractions have been examined (29). The major objective of this research was to develop a procedure for the purification of glutamine synthetase from Rhizobium japonicum grown in laboratory media and also purification of the enzyme from Rhizobium japonicum bacteroids. Other goals concerned the characterization of the enzymes

from different sources for the purpose of understanding among other things the regulatory role the enzyme might play in controlling nitrogenase synthesis.

REVIEW OF LITERATURE

Role of Glutamine in Nitrogen Metabolism

The amide, glutamine, has been shown to be a primary intermediate in the metabolism of N_2 (25, 69) and nitrate (32, 57). The amide is formed by the enzyme, glutamine synthetase which catalyzes the following reaction:

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \xrightarrow{\text{metal}^{2+}} \text{L-glutamine} + \text{ADP} + P_i$$

Glutamine is important in the biosynthesis of histidine, tryptophan and glutamate (28). It is the amide donor in the glutamine-dependent asparagine biosynthesis in soybean and lupin cotyledons (45). Also, it is the donor in the glutamine: 2-oxoglutarate amino-transferase reaction (GOGAT) which produces glutamate as a product (30). Glutamate may function as an ammonia carrier through a thermodynamically favorable series of specific glutamine-utilizing amido-transferase reactions which occur at the expense of phosphate group transfer (17).

Glutamine is involved in purine biosynthesis via the glutamine phosphoribosyl-pyrophosphate transferase reaction (72). The synthesis of guanosine 5'-phosphate from xanthosine 5'-phosphate requires glutamine as the amide donor (18). Nicotinamide adenine dinucleotide (NAD) synthetase which catalyzes the amidation of desamido-NAD^+ requires ATP and glutamine or ammonia as the amide donor (68). The biosynthesis of pyrimidines and arginine requires carbamyl phosphate as a biosynthetic intermediate. This compound is synthesized by glutamine-dependent

carbamyl phosphate synthetase (63). Carbamyl phosphate synthetases are ubiquitous in living tissues (63).

In legumes, glutamine, asparagine, glutamate and arginine constitute over 80% of the amino acids found in stems, roots and nodule exudates (58). Recently, Robertson, et al. (44), reported that glutamine and asparagine in the xylem sap of nodulated lupins reached levels of 0.6 mM and 3.3 mM, respectively. These two amino acids constituted over 83% of the amino acid content (44) of the xylem sap from the nodule. Seradella nodules exposed to $^{15}N_2$ incorporated this label into aspartate, glutamate and glutamine (19). Wong and Evans (70) reported that the exudates from excised soybean nodules contained asparagine, aspartic acid, glutamine and glutamic acid at concentrations of 125, 29, 20 and 5 mM of nodule xylem sap, respectively.

In addition to its involvement in the synthesis of various metabolites and in nitrogen transport, glutamine regulates its own synthesis by influencing the catalytic ability of glutamine synthetase in Escherichia coli and certain other gram-negative bacteria (28). Ammonia and glutamine also cause the repression of the synthesis of glutamine synthetase and nitrogenase (49). Studies by Shanmugam, et al., have shown that L-aspartate in combination with L-glutamine repressed nitrogenase synthesis in certain strains of Klebsiella pneumoniae (48).

Biochemical Role and Properties of Glutamine Synthetase

Reactions Catalyzed. That glutamine synthetase plays an important role in the assimilation of nitrogen has been well established (32,69).

One important biochemical role of this enzyme is the catalysis of the following biosynthetic reaction:

$$\text{L-glutamate} + NH_4^+ + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{L-glutamine} + \text{ADP} + P_i$$

In addition, the enzyme also catalyzes the following reactions:

$$\text{L-glutamine} + NH_2OH \overset{\text{metal}^{2+}}{\underset{\text{ADP}}{\rightleftharpoons}} \gamma\text{-glutamyl hydroxamate} + NH_3 \qquad \text{(b)}$$

P_i or arsenate

$$\text{L-glutamate} + \text{ATP} \overset{\text{metal}^{2+}}{\rightleftharpoons} \text{pyrollidone carboxylate} + \text{ADP} + P_i \qquad \text{(c)}$$

$$\text{L-glutamine} + H_2O \overset{\text{metal}^{2+}}{\underset{\text{ADP, arsenate}}{\rightleftharpoons}} \text{L-glutamate} + NH_3 \qquad \text{(d)}$$

$$\text{nucleoside}_{(1)}\text{triphosphate} + \text{nucleoside}_{(2)}\text{diphosphate} \overset{\text{metal}^{2+},\ P_i \text{ or arsenate}}{\underset{\text{glutamate, glutamine}}{\rightleftharpoons}} \text{nucleoside}_{(1)}\text{diphosphate} + \text{nucleoside}_{(2)}\text{triphosphate} \qquad \text{(e)}$$

The physiological role of reactions (b), (c), (d) and (e) is not presently known (42). Reaction (b) is used as an assay for glutamine synthetase activity via a colorimetric method (71). This reaction is termed the γ-glutamyl transferase reaction and is characteristic of all glutamine synthetases (15). Reaction (c) is an ATPase reaction and its role in cell metabolism has not been demonstrated. Beale, et al., (3) however, have proposed a pathway for the biosynthesis of δ-aminolevulinic (ALA) acid in green plant tissues in which glutamate serves as a precursor of ALA through an initial reaction as formulated by equation

(c). Reaction (d), an arsenate-dependent glutaminase reaction, has been studied in glutamine synthetases from E. coli (55), peas (24) and certain mammals (31). Reaction (e) is a transphosphorylase reaction and its physiological significance is not yet understood.

Properties of the Bacterial Enzyme. Glutamine synthetase has been purified and characterized from a variety of organisms. Most prominent has been the extensive study by Stadtman and his co-workers of the glutamine synthetase from Escherichia coli (54). This enzyme was purified 18-fold over the crude extract with about 9% recovery in total activity (51). The enzyme was crystallized by two different techniques one of which involved the use of zinc sulfate (33) and the other ammonium sulfate (51).

The glutamine synthetase from E. coli has a molecular weight of 592,000 and is composed of 12 identical subunits each with a molecular weight of 48,500 (15). The subunits are arranged so that two hexamer groups of subunits comprise an enzyme molecule (15).

Glutamine synthetase from Bacillus subtilis is structurally similar to the enzyme from E. coli. For example, it is composed of 12 subunits arranged in two groups of hexameric rings (11) and has a molecular weight of about 600,000. The two enzymes differ in their amino acid compositions. The adenylylation system which incorporates a 5-adenylyl moiety onto the hydroxyl group of a tyrosine residue in glutamine synthetase from E. coli is not present in the B. subtilis. The enzyme from Bacillus stearothermophilus has an amino acid composition similar to the glutamine synthetase from B. subtilis and has a molecular weight of

of 620,000 with 12 subunits of 51,500. The glutamine synthetase from B. stearothermophilus also lacks the capability to bind an adenylyl moiety (66).

Each subunit of the glutamine synthetase from E. coli contains multiple binding sites for a variety of substrates and metabolites (15). This complex situation is not restricted to the enzyme from E. coli but the interactions of metal ligands, substrates, cofactor and inhibitors are different in the various organisms (54). According to Stadtman, et al., each of the enzyme subunits from E. coli has inhibitor binding sites for each of the six end-products of glutamine metabolism. Also, the subunit has two sites for binding divalent cations (Mn^{2+}, Mg^{2+}, Co^{2+}) and one site for binding monovalent cations (Na^{+}, Li^{+}, Cs^{+}, imidazole, K^{+}). Included in each subunit is a site for binding the adenylylating-deadenylylating enzymes which are involved in the hydrolysis of a tyrosine which is the sixteenth residue of the polypeptide. The product has a 5'-adenylyl-O-tyrosyl covalent bond (15).

The adenylylation-deadenylylation of glutamine synthetase is controlled by a variety of factors (47). The process requires a complex cascade of enzymes (1). There are three sites on each subunit for interactions with other subunits. Most important are the substrate binding sites for glutamate and ammonia which interact with the binding site for ATP (10). Wedler has proposed that ATP and glutamate have a synergistic effect on the substrate binding site and that a random order of substrate addition and release takes place with the enzyme from E. coli (67). Recently, Powers and Riordon (40) showed that phenylglyoxal inhibited glutamine synthetase activity and that the compound was

specifically bound to arginine residues. When ATP was added prior to the addition of phenylglyoxal, inhibition did not occur thereby establishing that arginine is the site where ATP is bound to the enzyme.

Divalent cations interact with the two forms of the glutamine synthetase from E. coli. This interaction is dependent on degree of adenylylation of the enzyme, pH of the medium, concentration of ATP and the species of divalent cation (10). In the biosynthetic reaction the adenylylated enzyme is activated by Mn^{2+} and has a pH optimum that is different from the unadenylylated form (15).

The unadenylylated enzyme is activated by Mg^{2+}, Co^{2+} (15) and Zn^{2+} (55) in the biosynthetic reaction. In the presence of either divalent cation, the enzyme is in a so-called "taut" state and is enzymatically active. In the absence of these cations, the enzyme is reported to be in an inactive "relaxed" state (50). Specific information about the influence of cations on the conformation of the enzyme needs to be verified by electron microscopy (15) and other methods.

Enzyme activity is subject to cumulative inhibition by several of the end-products of glutamine metabolism. These include: AMP, CTP, tryptophan, histidine, carbamyl phosphate and glucosamine-6-phosphate. Also, alanine, glycine and serine, are known inhibitors of the enzyme. Each of these inhibitors may interact at a common site on each of the subunits (15).

Properties of Mammalian Enzyme. Jaenicke and his co-workers have purified and characterized glutamine synthetase from pig brain (56). They report that the molecular weight of the enzyme is 372,000 and that

it is composed of eight subunits. Each subunit has a molecular weight of 47,000. These subunits are arranged in two tetramers which are stacked to form a cubical structure the sides of which are about 100 Å with a 10 Å distance between the two tetramers (56). A similar structure has been reported by Meister, et al., for the glutamine synthetase from the sheep brain (46). Both enzymes are incapable of binding an adenylyl moiety.

Properties of Glutamine Synthetase from Plants. Glutamine synthetase has been reported in several plant systems (25, 32, 34). The most extensive study has been conducted by O'Neal and Joy (36, 37, 38) on the glutamine synthetase from the pea leaves. The enzyme had a specific activity of about 34 umoles of glutamylhydroxamate produced per milligram of protein per minute. The enzyme was unstable in the absence of Mg^{2+} or Mn^{2+}. Magnesium was required for enzyme activity but Mn^{2+} and Co^{2+} were also active. L-Histidine and L-ornithine were the most effective inhibitors of enzyme activity while L-alanine, glycine and L-serine were less effective (37, 38). The physical parameters of glutamine synthetase from the pea leaf have not been established.

Recently, McParland, et al., have purified glutamine synthetase from the cytosol fraction of soybean root nodules (29). This enzyme appears to be similar to glutamine synthetase from pig brain having a molecular weight of 376,000 and a subunit molecular weight of 47,500. The arrangment of the subunits is similar to that reported by Stahl and Jaenicke for the enzyme from pig brain (56).

Glutamine Synthetase Regulatory Mechanisms

The synthesis and activity of glutamine synthetase is regulated by factors that either repress enzyme synthesis or act as feed-back inhibitors. The predominant factor appears to be ammonium (20). Under conditions where high concentrations of ammonium are present, glutamine synthetase in E. coli is altered by adenylylation of a specific tyrosine-O-hydroxyl group on each of the 12 subunits (15). In Klebsiella aerogenes the addition of ammonium "shocked" the cells into an adenylylation shift which was completed after about 8 minutes (28). The adenylylated enzyme is considered the physiologically inactive form of glutamine synthetase and is activated by Mn^{2+}. The physiologically active unadenylylated form is present under ammonium-limiting conditions. This form of the enzyme is activated by Mg^{2+}. The adenylylation-deadenylylation reactions are catalyzed by a complex cascade of enzymes which is regulated by 2-oxoglutarate, glutamine and energy charge (1,2).

The synthesis of glutamine synthetase is controlled by the concentration of ammonium in the cell (20). Repression of enzyme synthesis occurs under conditions of high ammonium in the media. When Klebsiella aerogenes cells were grown in a medium containing higher than 1.0 mM ammonium, the synthesis of glutamine synthetase was repressed and levels of glutamate dehydrogenase increased (28). Cells grown in a medium containing less than 1.0 mM ammonium showed derepressed levels of glutamine synthetase.

In cultures of nitrogen-fixing, free-living cowpea Rhizobia the relative adenylylation of glutamine synthetase was increased as the pO_2

was increased even in cultures supplied with an excess of NH_4^+ or glutamine (4). Bergersen and Turner (4) concluded that the pO_2 effect on the adenylylation state of glutamine synthetase and repression of nitrogenase synthesis may depend on the presence of excess ammonium or glutamine, 34 mM and 2 mM, respectively.

Glutamine Synthetase as Regulator of Enzyme Synthesis

Glutamine synthetase is involved in the regulation of several enzyme systems. Glutamate dehydrogenase levels vary inversely with the levels of glutamine synthetase in Klebsiella aerogenes and respond to high concentrations of ammonium in the medium (28). The repression of glutamate dehydrogenase in an ammonium-limited medium has not been linked directly to derepressed levels of glutamine synthetase. A stimulation in the synthesis of histidase and proline oxidase was shown in nitrogen-starved cells of Klebsiella aerogenes which displayed high levels of active glutamine synthetase (28). This observation was corroborated by the discovery of a Klebsiella aerogenes mutant which was unable to synthesize glutamine synthetase. This mutant also displayed low levels of histidase and proline oxidase. Another mutant exhibiting high levels of glutamine synthetase also contained high levels of both histidase and proline oxidase. Glutamine synthetase has been proposed as a positive control factor in the transcription of the histidine utilization ("hut") DNA (65). Tyler, et al., demonstrated that purified unadenylylated glutamine synthetase enhanced transcription of the "hut" operon in vitro (65). These conditions may be analogous to

ammonium-limited conditions in which glutamine synthetase is unadenylylated.

Valentine and other researchers have demonstrated the repression of nitrogenase synthesis in Klebsiella sp. grown in the presence of excess ammonia (28). Glutamine synthetase has been proposed as a positive regulator of the nif operon(s) in its unadenylylated form in Klebsiella pneumonia (59). The mechanism proposed involved the repression of nitrogenase synthesis by the adenylylated form of glutamine synthetase. The adenylylated form of the enzyme occurs under high-ammonium conditions. Under nitrogen-starved conditions the unadenylylated, physiologically active glutamine synthetase is postulated to cause derepression of nitrogenase by its interactions with the nif operon(s). Mutant strains of Rhizobium 32H1 auxotrophic for glutamine showed diminished levels of nitrogenase activity in free-living cultures (27). Revertant glu^+ mutant strains of 32H1 isolated from Macroptileum atropurpureum nodules however failed to reduce acetylene.

Shanmugam, et al., have described mutant strains of Klebsiella pneumoniae in which the nitrogenase levels were high both in presence and absence of ammonium (49). The nitrogenase-derepressed strains included mutants (strains SK-25 and 26) which were auxotrophic for glutamine but failed to synthesize an active glutamine synthetase or an immunologically cross-reactive enzyme protein. These studies suggest that glutamine synthetase is not necessarily the principal regulator of the synthesis of enzymes involved in nitrogen fixation and ammonia assimilation.

In another class of mutants (SK-24, 28 and 29) both nitrogenase and

glutamine synthetase were constitutive in the presence of NH_4^+. The activity of glutamine synthetase was markedly decreased when the cells were grown in a medium containing a mixture of L-amino acids as compared to the same strains grown in the presence of NH_4^+. Glutamine synthetase activities of the parental strains were similar regardless of whether L-amino acids or NH_4^+ were supplied. The addition of L-amino acids to the parental strains repressed the induction of nitrogenase synthesis. Consequently, it was shown that L-aspartate in combination with L-glutamine repressed nitrogenase synthesis from 95 to 99% in the nitrogenase-derepressed strains SK-26, SK-27, SK-37, SK-55 and SK-56. In certain strains L-glutamine repressed nitrogenase biosynthesis by 45%. In strains SK-24, SK-28 and SK-29, L-aspartate alone repressed nitrogenase biosynthesis by 75%, 90%, respectively. L-glutamine and NH_4^+ together repressed nitrogenase synthesis significantly in several of the nitrogenase-derepressed strains.

The regulation of nitrogen fixation has been linked to glutamine synthetase (64) and its response to ammonia concentration in various organisms (6). Bacterial mutant studies in which glutamine synthetase is structurally changed or nitrogenase biosynthesis is constitutive and unaffected by ammonium concentrations have promoted these ideas (59). It may be that glutamine synthetase along with its substrates, products and perhaps by-products, are synergistically involved in the control of nitrogen metabolism.

EXPERIMENTAL AND RESULTS

Materials

Chemicals. Reagents purchased from Sigma Chemical Company (St. Louis MO) included γ-glutamylhydroxamate, dithiothreitol, streptomycin sulfate, imidazole, Tris(hydroxymethyl)aminomethane, and Tes(N-tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid). Snake venom phosphodiesterase (1.0 unit hydrolyzed 1.0 umoles bis(p-nitrophenyl)phosphate per minute at pH 8.8 at 37 C) was purchased from Sigma Chemical Company. The imidazole grade I from Sigma Chemical Company was washed with ethyl acetate and petroleum ether and then recrystallized from benzene. The residual benzene was removed in vacuo. Polyvinylpolypyrrolidone (PVPP) was obtained from the GAF Corporation (New York NY) and was prepared by the method of Loomis (26). The Amberlite resin, XAD-4, was a gift from Dr. W. D. Loomis. Polypropylene glycol-400 (PPG) was purchased from Matheson, Coleman and Bell (Los Angeles CA). Polyethylene glycol-4000 (PEG) was purchased from Union Carbide Corporation (New York NY). Diethylaminoethyl cellulose (Whatman DE-32) was purchased from H. Reeve Angel, Incorporated (Clifton NJ). Bio-Gel A-5m and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Richmond CA). Acrylamide and N',N'-methylenebisacrylamide were purchased from Eastman Kodak Company (Rochester NY). All other chemicals used were reagent grade or of the highest quality commercially available.

Source of Bacteroids. The bacteroids were extracted by the method described by Bishop, et al. (5) from root nodules that were obtained

from 28-day-old soybean plants (Glycine max Merr., var. Chippewa) and stored at -80 C. The plants were grown in an N-free nutrient medium as described previously by Evans, et al., (14).

Preparation of Bacteria. Rhizobium japonicum was cultured in 2.8 liter Fernbach flasks containing 1.5 liters of the medium as described in table 1. Each flask was inoculated with a 5% (v/v) aliquot of a three day-old culture and incubated on a rotary shaker at 30 C for 80 hrs. The cells were harvested by centrifugation and washed in 10 mM imidazole-HCl, pH 7.3 and 1.0 N NaCl (20% of culture medium volume). The cell pellets were stored at -80 C.

Enzyme Assays

Transferase Reaction. The γ-glutamylhydroxamate transferase assay described by Shapiro and Stadtman (51) was used routinely to determine glutamine synthetase activity throughout the purification procedure. The components of the transferase reaction are shown in table 2. Unless otherwise indicated one unit of glutamine synthetase activity is defined as that amount of enzyme that will catalyze the formation of 1.0 umole of γ-glutamylhydroxamate per minute at 37 C using the procedure of Shapiro and Stadtman (51). Specific activity is therefore defined as units of activity per milligram of protein.

Biosynthetic Reaction. The biosynthetic assay system reported by Shapiro and Stadtman (51) was modified after determining optimum concentrations for each component for the glutamine synthetases from

free-living Rhizobium japonicum and from soybean nodule bacteroids. Each reaction, in a final volume of 0.2 ml, was initiated by the addition of enzyme and incubated at 37 C for 30 minutes. The reaction components are described in table 3. Each reaction assay was terminated by the addition of 1.8 ml of 0.8% $FeSO_4$ in 0.015 N H_2SO_4 followed by 0.15 ml of 6.6% $(NH_4)_6Mo_7O_{27}\cdot 4H_2O$ in 7.5 N H_2SO_4. Color was allowed to develop for at least 2 min and the optical density determined at 660 nm. When excess protein caused turbidity the ammonium phosphomolybdate complex was extracted into 2.2 ml of n-butyl alcohol (52).

Protein Determination. Protein was routinely determined by the method of Lowry as described by Layne (23). In some instances protein content was determined by the Biuret method of Gornall, et al. (16).

Electrophoresis of Proteins

Samples of glutamine synthetase from free-living Rhizobium japonicum and nodule bacteroids were analyzed for purity by gel electrophoresis. Gels were prepared according to the method described by Davies (9) except the polyacrylamide concentration was routinely 5%. Fifteen to 30 ug of glutamine synthetase samples were applied to gels and were subjected to electrophoresis at 5-7 C at 2 mA per tube. The electrolyte buffer contained 25.0 mM Tris-glycine, pH 8.3. Gels were polymerized in 0.5 x 12.5 cm Bio-Rad glass tubes at room temperature. The electrophoresis apparatus was purchased from Canalco Company (Bethesda MD). The current was provided by a constant current power supply unit purchased from Buchler Instruments, Incorporated (Fort Lee NJ). The gels

Table 1. Culture-medium for Rhizobium japonicum.

Major Components	Concentration grams/liter
K_2HPO_4	0.69
$MgSO_4 \cdot 7H_2O$	0.30
Na glutamate	3.30
glycerol	12.00
Minor Components	**milligrams/liter**
$CaCl_2$	15.00
H_3BO_3	0.44
$FeSO_4 \cdot 7H_2O$	0.38
$CoSO_4 \cdot 7H_2O$	0.21
$CuSO_4 \cdot 7H_2O$	0.02
$MnCl_2 \cdot 4H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.32
Na_2MoO_4	0.38
nitrilo-triacetate	21.00
inositol	0.36
Vitamins	**micrograms/liter**
riboflavin	20.00
p-aminobenzoic acid	20.00
nicotinic acid	20.00
biotin	20.00
thiamine-HCl	20.00
pyridoxine-HCl	20.00
calcium pantothenate	20.00

Table 2. Components of the γ-glutamylhydroxamate transferase assay. Reactions, at pH 7.0, were initiated by the addition of enzymes to the transferase assay components in a final volume of 1.0 ml. The reactions were incubated at 37 C for 2.5 minutes unless otherwise indicated and were terminated by the addition of 2.0 ml of 10% $FeCl_3$ in 2.0% trichloroacetic acid and 0.25 N HCl to each tube. The optical density was measured at 540 nm (51).

Component	Concentration (mM)
Hydroxylamine-HCl	60.0
NaOH	60.0
Glutamine (pH 7.0)	30.0
Imidazole-HCl (pH 7.0)	40.0
ADP, monosodium, (pH 7.0)	0.4
Na_2HAsO_4 (pH 7.0)	20.0
$MnCl_2$	0.3

Table 3. Components of the biosynthetic reaction assay. The pH was adjusted with NaOH. The reactions were initiated by the addition of enzyme to 0.1 ml of reaction mixture in a final volume of 0.2 ml and incubated at 37 C for 30 minutes. Each reaction assay was terminated by the addition of 1.8 ml of 0.8% $FeSO_4$ in 0.015 N H_2SO_4 followed by 0.15 ml of 6.6% $(NH_4)_6Mo_7O_{27}.4H_2O$ in 7.5 N H_2SO_4. Color of the $(NH_4)_3(PMo_{12}O_{40})$ complex was allowed to develop for at least 2 minutes and the optical density determined at 660 nm within 10 minutes (51).

Component	Concentration (mM)
Tes	50.0
Acetic acid	50.0
L-glutamate	50.0
ATP, disodium	7.5
NH_4Cl	10.0
$MgCl_2$	20.0

were stained with 0.2% Coomassie Brillant Blue in 7.5% acetic acid, 2.0% trichloroacetic acid and 20% methanol for at least 12 hours and destained for 24 hours by diffusion in 7.5% acetic acid, 2.0% trichloroacetic acid and 20% methanol.

Further analysis of the enzyme samples was made by SDS-polyacrylamide gel electrophoresis method by Laemmli (22). The electrolyte buffer contained 25.0 mM tris-glycine, pH 8.3 and 0.1% SDS. The 8% gels were polymerized in a buffer of 0.3 M tris-HCl, pH 8.8 and 0.1% SDS. The protein samples were heated at 100 C for 5 minutes in 60.0 mM tris-HCl, pH 6.8, 2.0% SDS and 5.0% mercaptoethanol. Fifteen to 30 ug of SDS-treated samples were applied to gels and subjected to electrophoresis at room temperature at 2 mA per tube. The gels were stained and destained as before.

Relative Adenylylation of Glutamine Synthetase

The relative adenylylation of glutamine synthetase at various stages of purification was approximated by a modification of the snake venom phosphodiesterase (SVD) method described by Tronick, et al. (62), and also by the absorbances obtained by the glutamine synthetase transferase reaction in the presence and absence of 60.0 mM Mg^{2+} (51). Other components of this assay are described in table 2.

Aliquots of glutamine synthetase each of which contained approximately 0.1 unit of activity were incubated in the presence of 30.0 ug of SVD at 37 C for 90 minutes. The SVD was dissolved in a 50.0 mM tris-acetate buffer, pH 8.8, containing 15.0 mM magnesium acetate. Control

assays without SVD were incubated in a similar manner. Glutamine synthetase activity in the transferase reaction was then determined in the presence of 60.0 mM Mg^{2+} and 0.3 mM Mn^{2+}. The absorbances of the transferase reactions for samples treated with SVD divided by the absorbances for the untreated control samples resulted in a value which approximated the relative adenylylation of glutamine synthetase (62). A value of 1.0 to 2 is indicative of a low relative adenylylation while a ratio greater than 3.0 indicates a high relative adenylylation.

Relative adenylylation ratios were also obtained from the absorbance values of the transferase reaction conducted with 0.3 mM Mn^{2+} divided by the absorbance values of the transferase reaction conducted with 0.3 mM Mn^{2+} and 60.0 mM Mg^{2+} using samples of untreated glutamine synthetase (62). Assuming that the rhizobial enzyme is composed of 12 subunits each with an adenylylation site, the relative adenylylation number can be estimated using an equation presented by Ginsburg (15):

$$n = 12 - \frac{(\text{activity in 0.3 mM } Mn^{2+} + 60.0 \text{ mM } Mg^{2+})}{\text{activity in 0.3 mM } Mn^{2+}} \quad 12$$

where n represents the number of adenylylated subunits per mole of enzyme.

Purification and Properties of Glutamine Synthetase from Soybean Roct Nodule Bacteroids

Purification of the Enzyme. The bacteroids were washed in 20 ml per g of cells in 10.0 mM imidazole-HCl, pH 7.3, containing 1.0 N NaCl. The bacteroids were suspended in an equal volume of 0.1 M imidazole-HCl, pH 8.0, and disrupted in the French pressure cell at 3.5×10^7 newtons per meter^2 (5000 psi). The broken cells were collected in a beaker containing a volume equal to the suspension buffer of 0.1 M imidazole-HCl, pH 7.0, and 3% (wt/vol) streptomycin sulfate. After disruption was completed the total volume was determined and sufficient streptomycin sulfate was added to attain a 3% (wt/vol) concentration. The mixture was then stirred for 10 minutes. Sufficient $MnCl_2$ and dithiothreitol was added to make 1.0 mM concentration of each and the solution was centrifuged at 40,000 g for 60 minutes. All steps were conducted between 0 and 4 C unless otherwise stated.

The supernatant was used as the crude preparation and the pellet was discarded. Sufficient polyethylene glycol-4000 (50%, wt/vol) was added dropwise to the crude extract to obtain 10% saturation. The mixture was stirred in an ice-bath for 10 minutes and centrifuged. All centrifugations were carried out at 15,000g for 15 min unless otherwise stated. Glutamine synthetase activity was recovered by suspending the pellet in 20 to 30 ml of 10.0 mM imidazole-HCl, pH 7.3, 1.0 mM $MnCl_2$ and 1.0 mM dithiothreitol. The supernatant did not contain enzyme activity and was discarded.

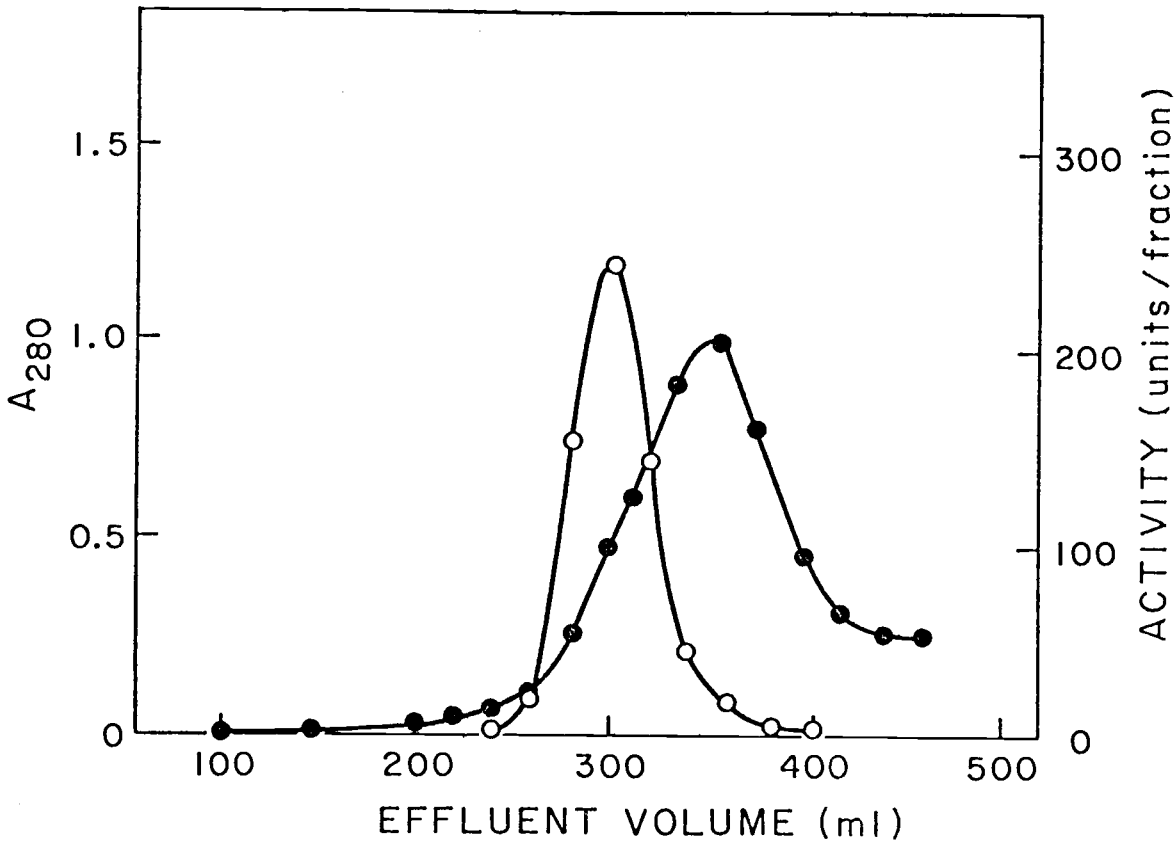
The 0 to 10% polyethylene glycol fraction was applied to a column of DEAE-cellulose which had been previously equilibrated with 10.0 mM imidazole-HCl, pH 7.3, 1.0 mM $MnCl_2$ and 1.0 mM dithiothreitol. The column was then washed with 6 bed volumes of the equilibrating buffer. Glutamine synthetase was eluted from the column with 0.2 N NaCl in equilibrating buffer. Fractions (10 ml each) containing glutamine synthetase activity were pooled.

Enough polyethylene glycol was added to the DEAE-cellulose eluate to obtain 15% saturation. The solution was stirred in an ice-bath for 10 minutes and centrifuged. The supernatant was discarded and the protein pellet was suspended in 8.5 ml of 10.0 mM imidazole-HCl, pH 7.3, 1.0 mM $MnCl_2$, 1.0 mM dithiothreitol and 0.2 N NaCl.

The concentrated enzyme was chromatographed on a column of Bio-Gel A-5m (figure 1) which previously had been washed with at least 4 bed volumes of the suspension buffer. Fractions (10 ml each) containing at least half of the activity peak were pooled. At this stage the enzyme was used to determine the optimum reaction parameters for the biosynthetic assay. Whenever necessary the enzyme was stored at -80 C after freezing in liquid N_2. This resulted in no significant loss in total activity.

A summary of the purification procedure is shown in table 4. The enzyme was purified to at least 90% homogeneity as determined by discontinuous gel electrophoresis (figure 2) (9). Further attempts to purify the enzyme usually resulted in loss of activity and loss in specific activity. The use of a glutamate-agarose affinity column which had been used to purify the nodule cytosol enzyme (29) did not

Figure 1. Chromatography of glutamine synthetase through Bio-Gel A-5m.



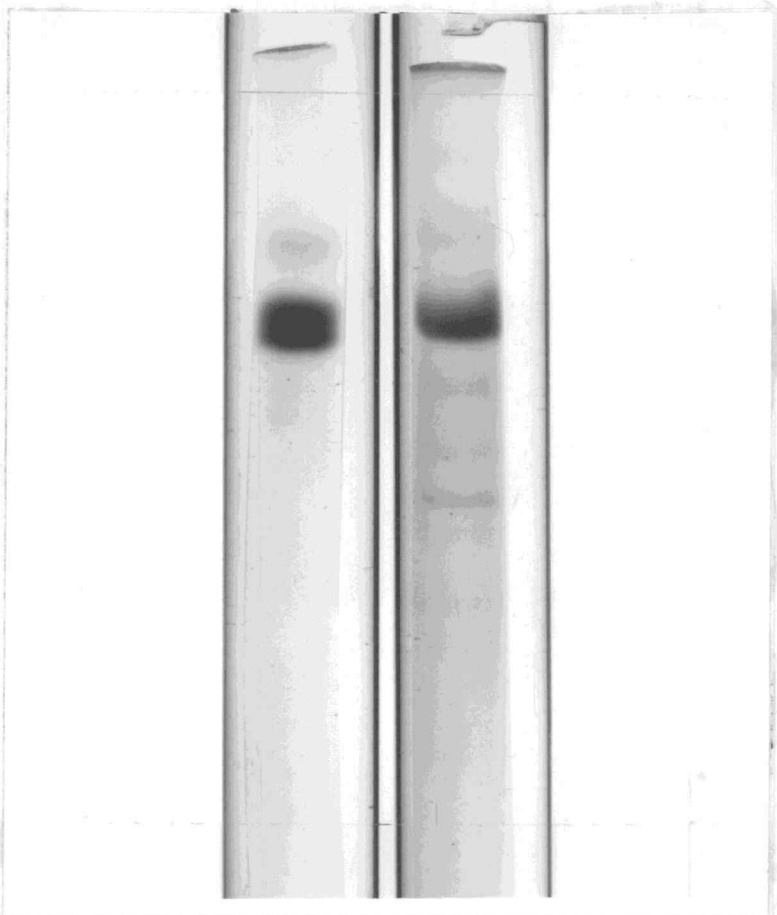
Enzyme fractions from the DEAE-cellulose were concentrated to 8.5 ml and chromatographed at 20 ml/h (10 ml per fraction) through a column (2.5 x 90 cm) of Bio-Gel A-5m previously equilibrated and eluted with 0.2 N NaCl containing 10.0 mM imidazole-HCl, pH 7.3, 1.0 mM $MnCl_2$ and 1.0 mM DTT. ●, A_{280}; o, glutamine synthetase ($E_{3.3}$) transferase activity.

Table 4. Purification of glutamine synthetase from soybean root nodule bacteroids. The enzyme was purified from 160 g of bacteroids extracted from 1 kg of soybean nodules which had been stored for 12 months at -80 C.

Fraction	Total protein (mg)	Specific activity (units*/mg)	Recovery (%)
Crude extract	7770	0.4	100
Polyethylene glycol (0-10%) ppt.	768	5.0	109
DEAE-cellulose eluate	515	5.4	83
Polyethylene glycol (0-15%) ppt.	184	14.0	78
Bio-Gel A-5m eluate	20	56.0	34

* A unit of activity is defined under methods.

Figure 2. Illustration of the results of polyacrylamide-gel electrophoresis of glutamine synthetase from nodule bacteroids and free-living Rhizobium japonicum 505.



Gel (a) contained 50 ug of glutamine synthetase ($E_{3.3}$) from nodule bacteroids and is representative of the purity of the Bio-Gel A-5m eluate. Gel (b) contained 30 ug of glutamine synthetase ($E_{8.5}$) from free-living Rhizobium japonicum 505 and is representative of the Sephadex G-100 eluate described in table 7. The enzyme samples in 5% sucrose, 10.0 mM imidazole-HCl, pH 7.3 and 1.0 mM $MnCl_2$ were layered on the surface of 5% acrylamide gels (0.5 x 12.5 cm) and subjected to electrophoresis as described in methods.

enhance purification of the glutamine synthetase from nodule bacteroids

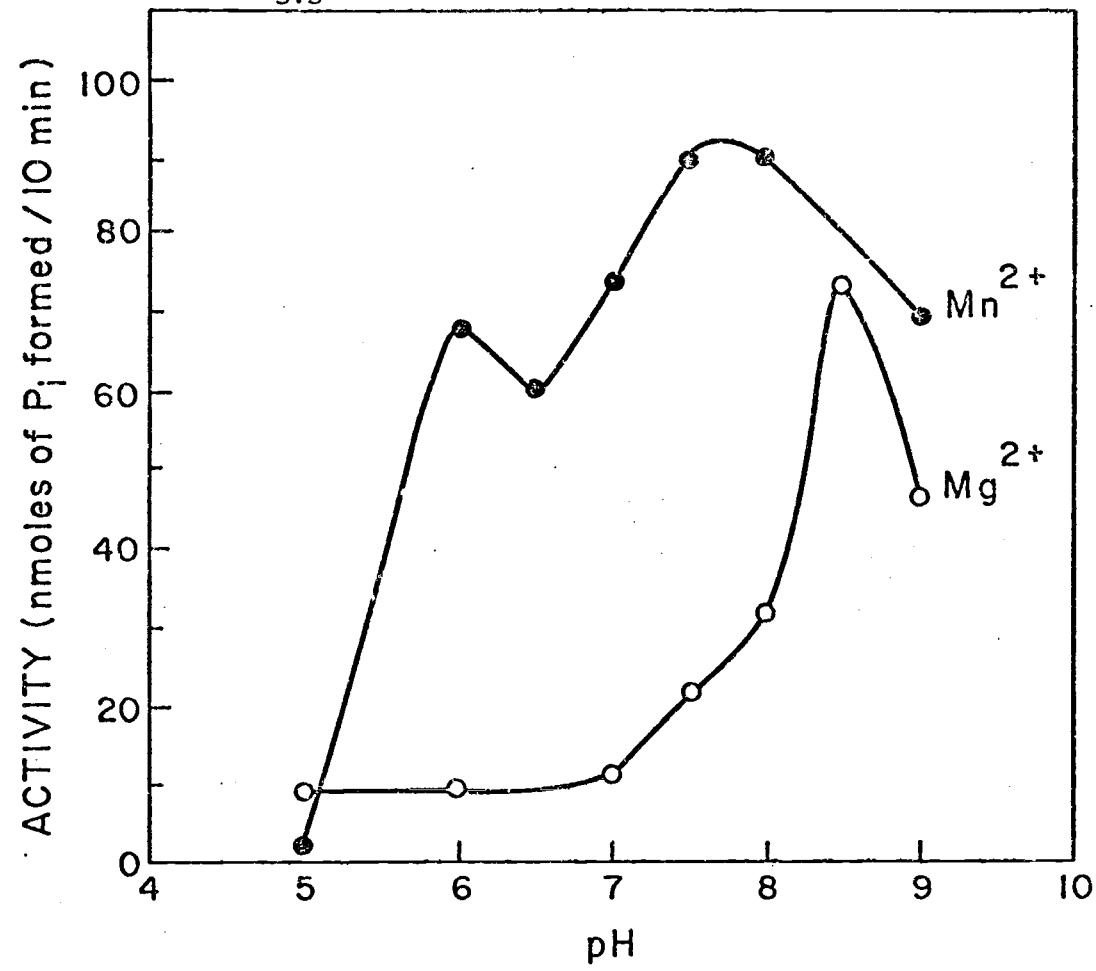
Properties. The purified enzyme with a specific activity of 55.0 units of transferase activity per mg protein was used to determine the properties of glutamine synthetase in the biosynthetic reaction.

Relative Adenylylation Value. The relative adenylylation of purified glutamine synthetase from the bacteroids was determined by the SVD method (62) and by the transferase reaction in the presence and absence of 60.0 mM Mg^{2+}. The values obtained for the enzyme were 1.63 and 1.38, respectively. The n value determined from absorbances of the transferase reaction in the presence and absence of 60.0 mM Mg^{2+} was 3.3. Glutamine synthetase from this particular batch of soybean nodule bacteroids is relatively unadenylylated.

pH Optima. The pH optimum of the glutamine synthetase biosynthetic activity was 8.5 in the presence of 5.0 mM $MgCl_2$ and 7.75 in an assay containing 5.0 mM $MnCl_2$. Although figure 3 shows that biosynthetic activity is less in the presence of $MgCl_2$ than in the presence of $MnCl_2$, subsequent experiments involving divalent cation specificity revealed that the $MgCl_2$ concentration was not optimal. Transferase activity of the relatively unadenylylated enzyme ($E_{3.3}$) has a pH optimum at 7.0 in the presence of 0.3 mM $MnCl_2$ (figure 4).

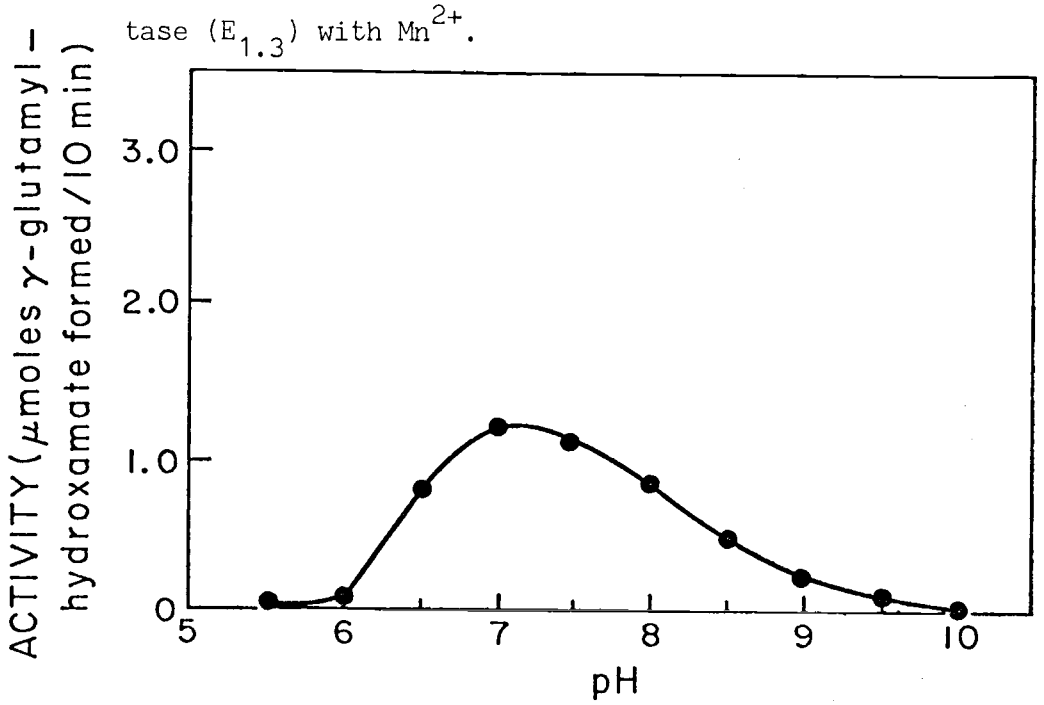
Metal Requirement. The biosynthetic activity of glutamine synthetase reached maximum velocity at pH 8.5 in the presence of 20.0 mM $MgCl_2$. At pH 7.75 maximum biosynthetic activity was observed with 5.0 mM $MnCl_2$. Results in figure 5 also show that $MnCl_2$ inhibited the reaction at

Figure 3. pH Optima of the biosynthetic activity of glutamine synthetase ($E_{3.3}$) with either Mg^{2+} (o-o) or Mn^{2+} (●-●).



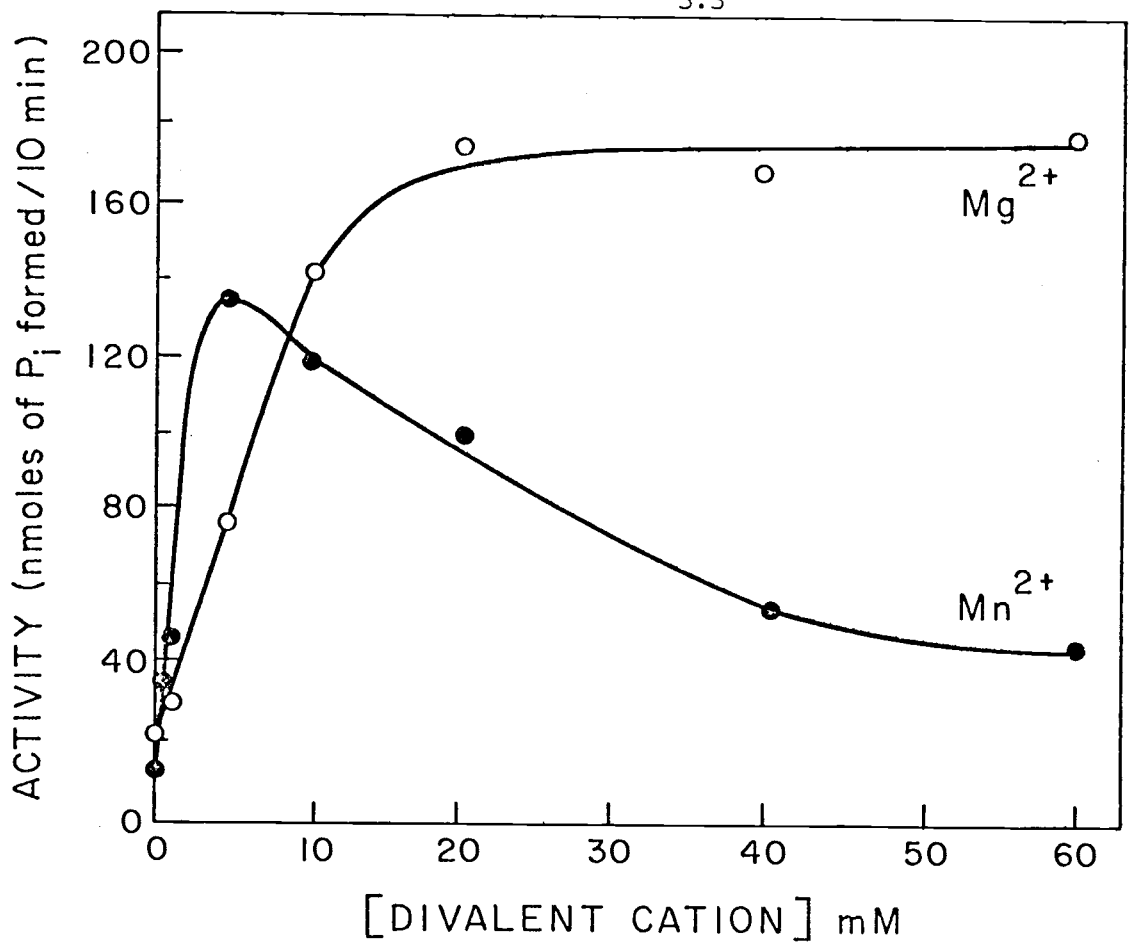
Assay mixtures contained 50.0 mM sodium glutamate, 50.0 mM Tes, 50.0 mM acetic acid, 50.0 mM NH_4Cl, 7.5 mM ATP, and 5.0 mM $MgCl_2$ (o-o) or 5.0 mM $MnCl_2$ (●-●). The reactions were initiated by the addition of 0.5 unit enzyme to a final reaction volume of 0.2 ml. The pH of the assay mixture was adjusted with NaOH. Reaction mixtures were incubated for 30 minutes at 37 C. Each data point represents the mean of triplicate determinations. Standard errors of means ranged from 1.0 to 12% of values reported.

Figure 4. pH Optimum of the transferase activity of glutamine synthetase ($E_{1.3}$) with Mn^{2+}.



Assay mixtures contained 50.0 mM Tes, 25.0 mM acetic acid, 30.0 mM hydroxylamine-HCl, 60.0 mM glutamine, 0.4 mM ADP, 20.0 mM arsenate and 0.3 mM $MnCl_2$. The reaction was initiated by the addition of 0.1 unit enzyme to a final reaction volume of 1.0 ml. Glutamine synthetase ($E_{1.3}$) from the bacteroids was prepared by treating with SVD for 90 minutes at 37 C. The pH of the assay mixture was adjusted with NaOH. The reaction mixtures were incubated for 10 minutes at 37 C and terminated by the addition of 2.0 ml of acidic $FeCl_3$ reagent (51).

Figure 5. The influence of divalent cations on the biosynthetic activity of glutamine synthetase ($E_{3.3}$) at respective pH optima.



Reaction mixtures contained 50.0 mM glutamate, 50.0 mM NH_4Cl, 7.5 mM ATP, 50.0 mM Tes-NaOH, 50.0 mM acetic acid and $MgCl_2$ (o-o) at pH 8.5 or $MnCl_2$ (●-●) at pH 7.75. Reactions were initiated by the addition of 0.5 unit of enzyme in a final reaction volume of 0.2 ml. Reaction mixtures were incubated for 30 minutes at 37 C. Each experimental point is the mean of triplicate determinations. S.E.M. ranged from less than 1 to 9% of values indicated.

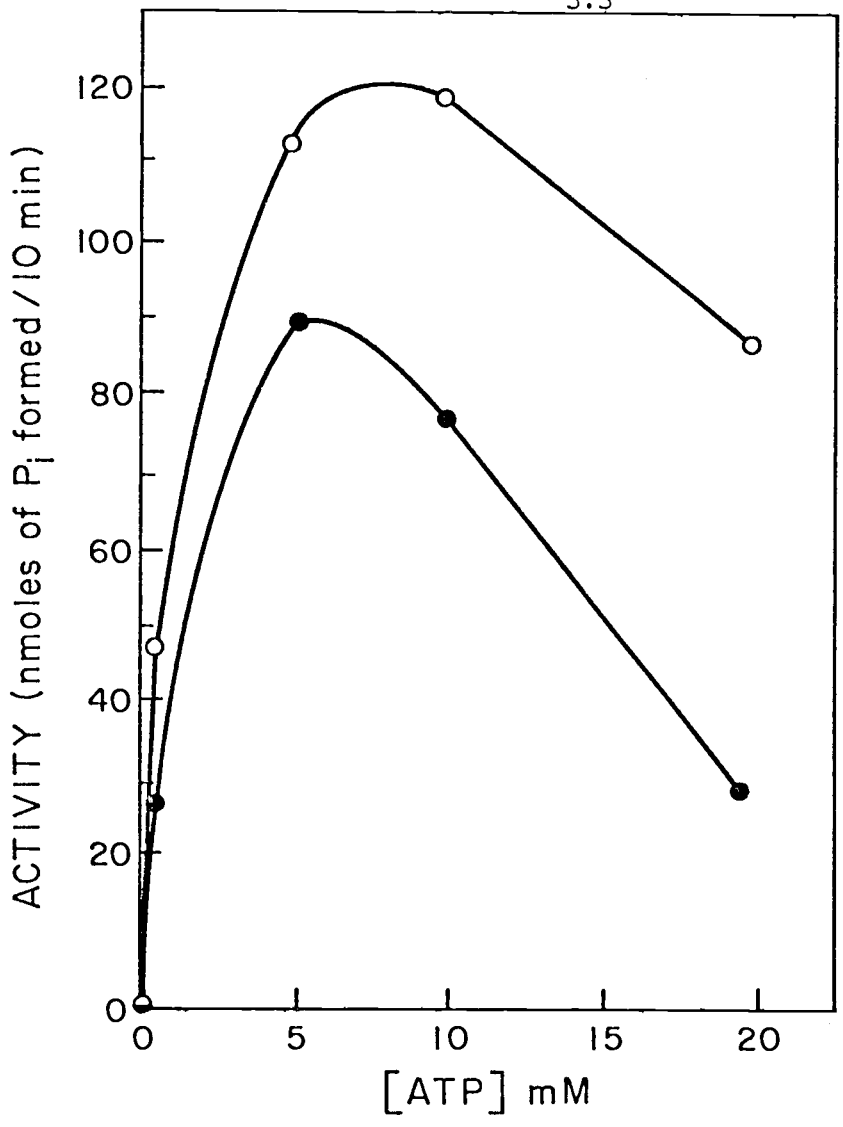
concentrations higher than 10.0 mM.

ATP Requirement. In an assay medium containing 20.0 mM $MgCl_2$, biosynthetic activity was highest with 10.0 mM ATP. In reaction mixtures with 5.0 mM $MnCl_2$, maximum activity was observed with 5.0 mM ATP (figure 6). Maximum velocity was attained at 20.0 mM ATP in the presence of both $MgCl_2$ and $MnCl_2$ as shown in figure 7. Experiments to demonstrate the effects of metal-ATP complex formation on the biosynthetic activity of the enzyme showed that free Mg^{2+} enhanced the reaction (figure 8a). Data shown in figure 8b indicated that biosynthetic activity was inhibited in the presence of excess Mn^{2+}.

Ammonium and L-Glutamate Saturation Curves. Experiments were conducted to determine saturation concentrations of ammonium and L-glutamate at optimum reaction conditions for the relatively unadenylylated enzyme from the bacteroids. Reactions were performed in the presence of either $MgCl_2$ or $MnCl_2$ at their respective pH optima. Inhibition of biosynthetic activity in the presence of $MgCl_2$ was observed at high concentrations of NH_4Cl. Data in Table 5 show that the NH_4Cl concentrations recommended for the biosynthetic assay by Shapiro and Stadtman (51) inhibited enzyme activity by almost 50%.

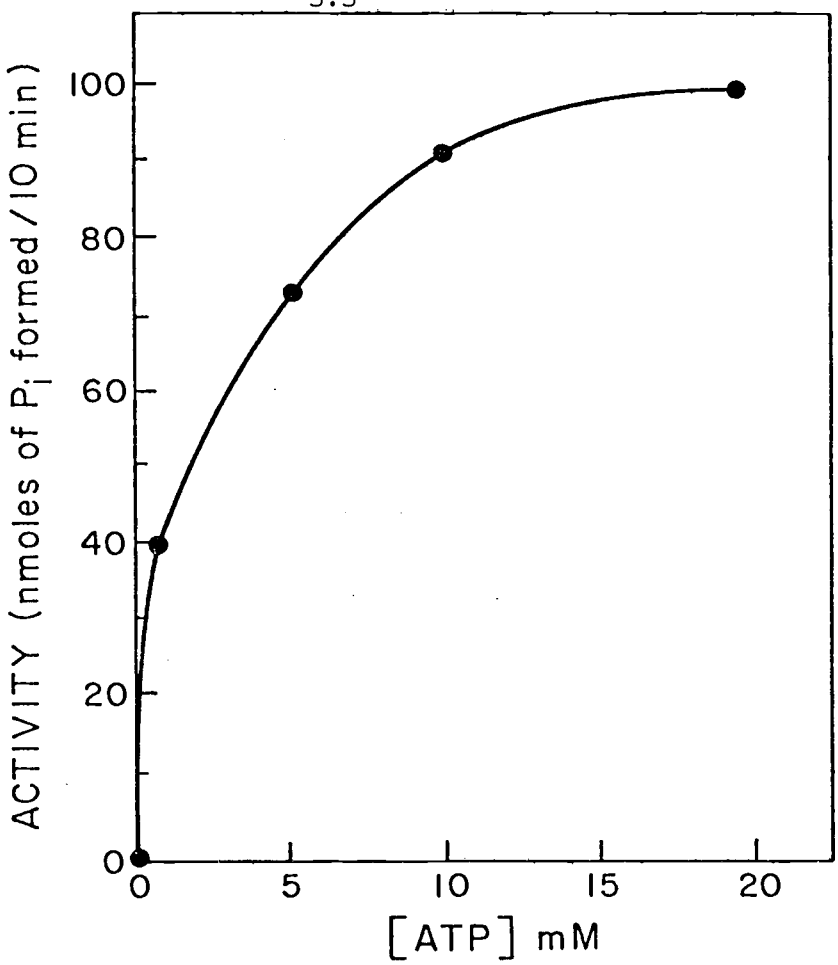
Results shown in figure 9 demonstrate that maximum activity occurred at 0.8 mM NH_4Cl with 0.5 unit of enzyme in the reaction medium. An apparent K_m value of 1.04 mM for NH_4Cl was determined by the method of Eisenthal and Cornish-Bowden (13). No inhibition of the reaction occurred in the presence of 5.0 mM $MnCl_2$ when the NH_4Cl concentration was increased to 15.0 mM. The enzyme has an apparent K_m of 1.4 mM for

Figure 6. The influence of ATP and divalent cations on the biosynthetic activity of glutamine synthetase ($E_{3.3}$).



Assay mixtures contained 50.0 mM glutamate, 50.0 mM NH_4Cl, 50.0 mM Tes, 50.0 mM acetic acid and 20.0 mM $MgCl_2$ (o-o) or 5.0 mM $MnCl_2$ (●-●). Reactions were initiated by the addition of 0.5 unit of enzyme to a final reaction volume of 0.2 ml. Reactions were incubated for 30 minutes at 37 C at pH 8.25. Each data point represents the mean of triplicate determinations. S.E.M. ranged from 0.6 to 12.0% of values reported.

Figure 7. The influence of ATP on the biosynthetic activity of glutamine synthetase ($E_{3.3}$) in the presence of Mg^{2+} and Mn^{2+}.



Assay mixtures contained 50.0 mM glutamate, 50.0 mM NH_4Cl, 50.0 mM Tes, 50.0 mM acetic acid, 20.0 mM $MgCl_2$ and 5.0 mM $MnCl_2$. Reactions were initiated by the addition of 0.5 unit of enzyme to a final reaction volume of 0.2 ml. Reactions were incubated for 30 minutes at 37 C at pH 8.25. Each data point reflects the mean of triplicate determinations. S.E.M. were less than 2% of values indicated.

Figure 8(a). Influence of MgATP on biosynthetic activity of glutamine synthetase ($E_{3.3}$).

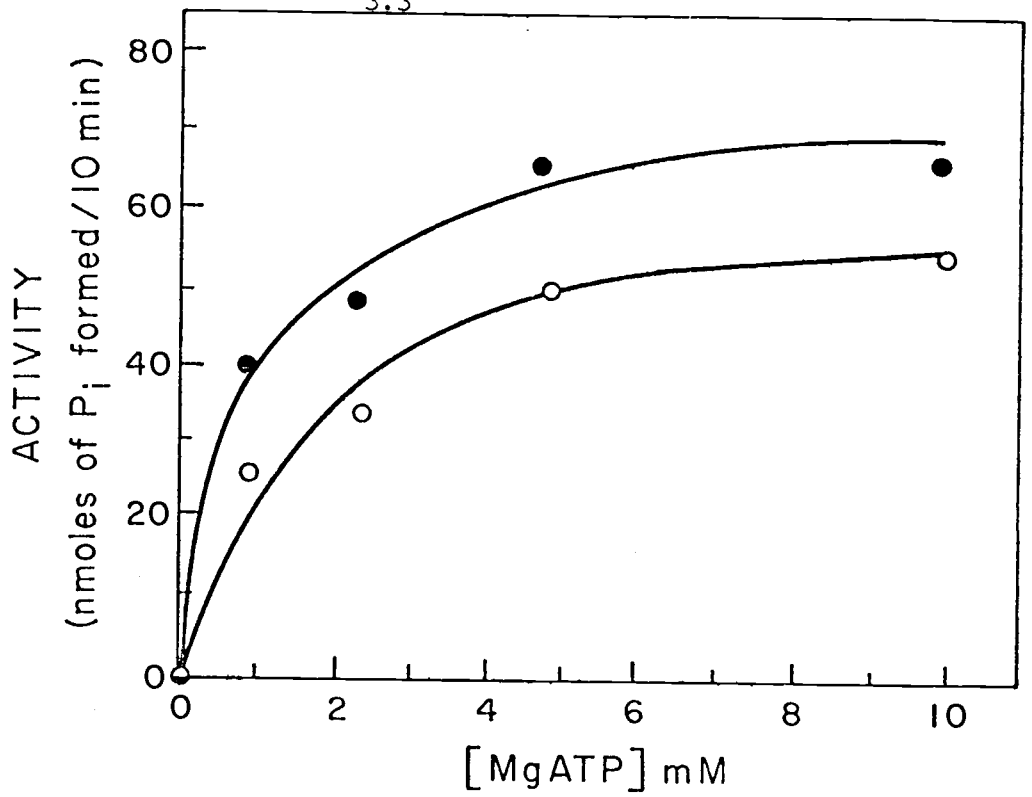


Figure 8(b). Influence of MnATP on biosynthetic activity of glutamine synthetase ($E_{3.3}$).

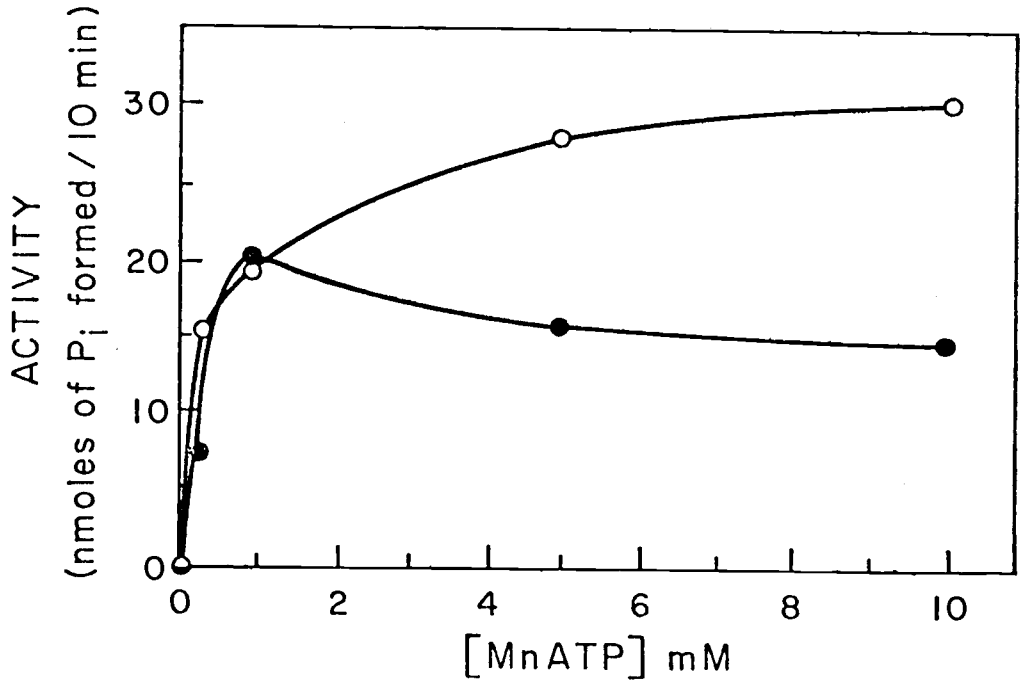


Figure 8(a). Influence of MgATP on biosynthetic activity of glutamine synthetase ($E_{3.3}$).

Assay mixtures contained 50.0 mM glutamate, 50.0 mM NH_4Cl, 50.0 mM Tes-NaOH and 50.0 mM acetic acid at pH 8.5. Equimolar concentrations of $MgCl_2$ and ATP were added to each reaction to attain concentrations of MgATP indicated in the figure. The reactions were initiated by the addition of 0.5 unit enzyme (●-●) or 0.5 unit enzyme pre-incubated with 20.0 mM $MgCl_2$ (o-o) in a final reaction volume of 0.2 ml. The reaction mixtures were incubated for 30 minutes at 37 C. Each experimental point represents the mean of triplicate determinations. S.E.M. ranged from 2 to 12% of values reported.

Figure 8(b). Influence of MnATP on biosynthetic activity of glutamine synthetase ($E_{3.3}$).

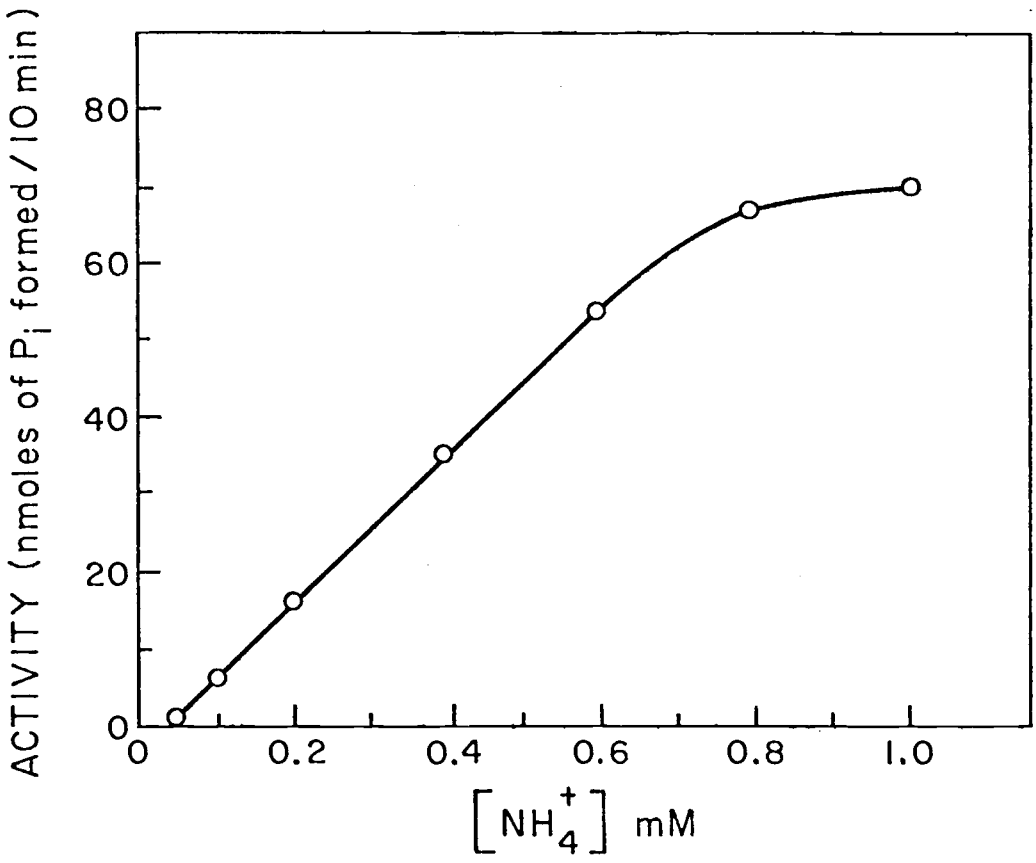
Reaction mixtures contained 50.0 mM glutamate, 5.0 mM NH_4Cl, 50.0 mM Tes-NaOH and 50.0 mM acetic acid at pH 7.75. Equimolar concentrations of $MnCl_2$ and ATP were added to each reaction to attain concentrations of MnATP indicated in the figure. The reactions were initiated by the addition of 0.5 unit of enzyme (o-o) or 0.5 unit enzyme pre-incubated with 5.0 mM $MnCl_2$ (●-●) to a final reaction volume of 0.2 ml. The reaction mixtures were incubated for 30 minutes at 37 C. Each data point represents the mean of triplicate determinations. S.E.M. ranged from 1 to 7% of values reported.

Table 5. Inhibition of glutamine synthetase ($E_{3.3}$) biosynthetic activity by NH_4Cl. Reaction mixtures contained 50.0 mM glutamate, 50.0 mM Tes, 50.0 mM acetic acid, 20.0 mM $MgCl_2$, 7.5 mM ATP and NH_4Cl at concentrations indicated. The reaction medium was adjusted to pH 8.5 with NaOH. Reactions were initiated by the addition of 0.5 unit enzyme ($E_{3.3}$) in a total reaction volume of 0.2 ml. Reaction mixtures were incubated for 30 minutes at 37 C. Each value represents the mean $\pm$ S.E.M. of triplicate determinations.

Concentration of NH_4Cl (mM)	*Inhibition(%)
0.9	---
4.3	2.5 $\pm$ 2.50
8.5	25.6 $\pm$ 1.81
17.0	31.2 $\pm$ 2.32
42.5	48.5 $\pm$ 2.34

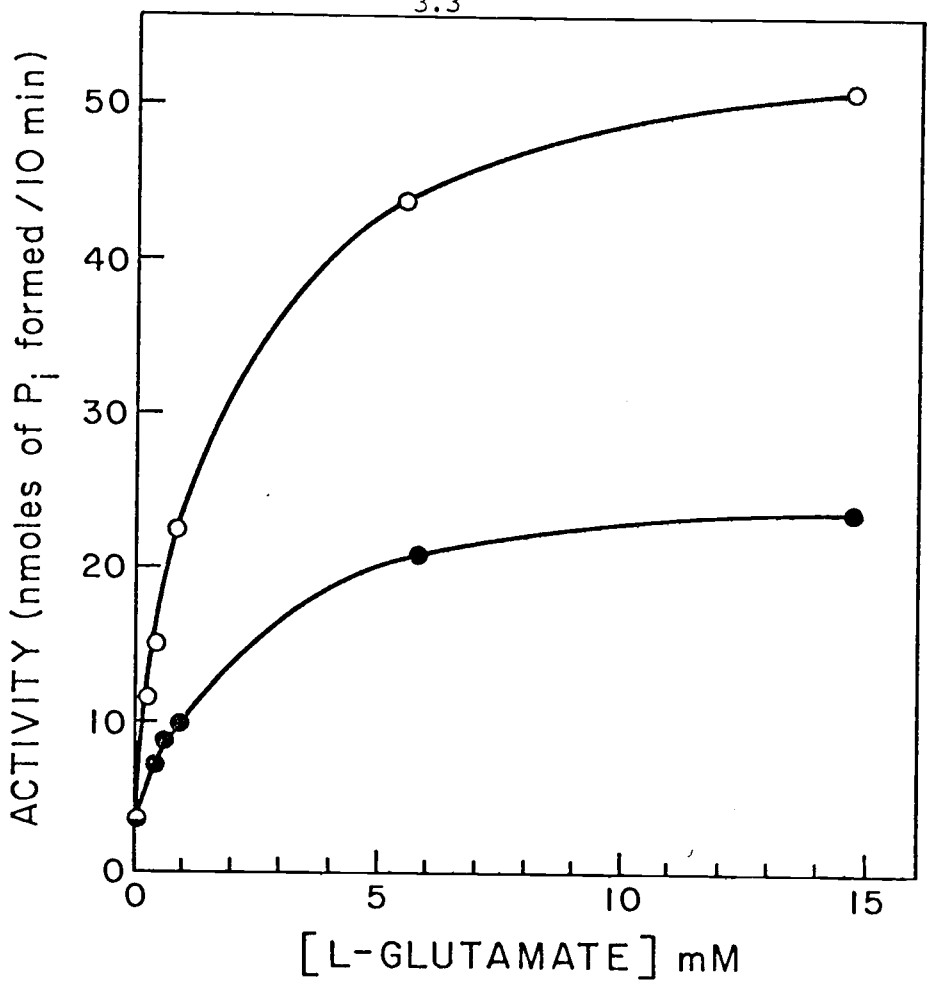
* %Inhibition based on highest biosynthetic activity observed in reaction mixtures containing 0.9 mM NH_4Cl.

Figure 9. Influence of NH_4Cl on biosynthetic activity of glutamine synthetase ($E_{3.3}$) in the presence of $MgCl_2$.



Reactions mixtures contained 50.0 mM glutamate, 50.0 mM Tes-NaOH, 50.0 mM acetic acid, 20.0 mM $MgCl_2$ and 7.5 mM ATP at pH 8.5. Reactions were initiated by the addition of 0.5 units of enzyme in a final reaction volume of 0.2 ml. Reaction mixtures were incubated for 30 minutes at 37 C. Each experimental point represents the mean of six replicate determinations. S.E.M. values were 1.0% or less of values reported.

Figure 10. Influence of L-glutamate on biosynthetic activity of glutamine synthetase ($E_{3.3}$).



Assay mixtures contained 50.0 mM Tes, 50.0 mM acetic acid, and 5.0 mM NH_4Cl. Reactions with 20.0 mM $MgCl_2$ (o-o) also contained 7.5 mM ATP and were adjusted to pH 8.5 with NaOH. Reactions with 5.0 mM $MnCl_2$ (●-●) contained 5.0 mM ATP and were adjusted to pH 7.75 with NaOH. The reactions were initiated by the addition of 0.5 unit of enzyme in a final reaction volume of 0.2 ml and incubated for 30 minutes at 37 C. Each experimental point represents the mean of duplicate determinations. Standard errors of means were less than 2% of reported values.

L-glutamate in an assay medium containing 20.0 mM $MgCl_2$ and pH 8.5. Results plotted in figure 10 show that activity was highest in an assay medium containing $MgCl_2$. This activity at saturating concentrations of L-glutamate was over 2-fold greater than the activity of reactions in the presence of $MnCl_2$.

Purification and Properties of Glutamine Synthetase from Free-living Rhizobium japonicum 505

Purification of the Enzyme (Procedure I). The bacteria were suspended in twice their weight of 0.1 M imidazole-HCl, pH 7.3 and disrupted in a French pressure cell at 3.5×10^7 newtons per meter^2 (5000 psi). The disrupted cells were centrifuged at 40,000g for 60 minutes. All steps were conducted between 0 and 4 C unless otherwise stated.

The supernatant was used as the crude preparation and the pellet was discarded. Sufficient streptomycin sulfate was added to obtain 3% (w/v). The solution was stirred for 10 minutes and centrifuged. All centrifugations were at 15,000g for 15 minutes unless otherwise stated. The pellet was discarded.

Sufficient polypropylene glycol was added to the supernatant to obtain 5% (v/v). The mixture was stirred for 10 minutes in an ice-bath and then centrifuged. The supernatant contained almost 95% of total activity and therefore the pellet was discarded. Enough polypropylene glycol was added to the supernatant to obtain 20% (v/v). The mixture was stirred as before and centrifuged. The supernatant lacked glutamine synthetase activity and therefore was discarded.

The pellet was suspended in 10.0 mM imidazole-HCl, pH 7.3, 1.0 mM $MnCl_2$ and 0.5 N NaCl. The suspension was centrifuged to clarify. The supernatant containing the enzyme activity was dialyzed against 150 volumes of a buffer containing 10.0 mM imidazole-HCl, pH 7.3, and 1.0 mM $MnCl_2$.

The dialyzed supernatant was added to a column of DEAE-cellulose

which had been equilibrated with 10.0 mM imidazole-HCl, pH 7.3, and 1.0 mM $MnCl_2$. The enzyme was eluted from the column by the addition of 50 ml of equilibrating buffer containing 0.2 N NaCl. The fractions containing glutamine synthetase were pooled and concentrated to 5.0 ml in an Amicon Diaflo cell equipped with a PM-30 membrane. The concentrate was chromatographed on a column of Bio-Gel A-5m (2.5 x 90 cm) with 10.0 mM imidazole-HCl, pH 7.3, containing 1.0 mM $MnCl_2$. A summary of the purification procedure is described in table 6 .

Purification of the Enzyme (Procedure II). Rhizobium japonicum 505 cells were suspended as in procedure I and glutamine synthetase was purified in the same manner as described for the bacteroid enzyme. A column of PVP and XAD-4 (1:1) was washed with eight volumes of 10.0 mM imidazole-HCl, pH 7.3, containing 1.0 mM $MnCl_2$. The crude extract was passed through the column to remove possible contaminating phenolics. A summary of the purification procedure is described in table 7.

Glutamine synthetase enzymes with E_n values of 10.5 and 8.5 were purified by procedure II. Attempts to purify these enzymes further resulted in loss of total activity and decrease in specific activity. Electrophoretic gels shown in figure 2 demonstrate the results of additional purification steps.

Properties. Enzyme preparations with specific activities of 20.0 and 44.0 units of transferase activity per mg protein with E_n values of 10.5 and 8.5, respectively, were used to determine the properties of adenylylated glutamine synthetase in the biosynthetic reaction.

Table 6. Purification of glutamine synthetase from free-living Rhizobium japonicum 505. Procedure I. The enzyme was purified from 58.0 g of Rhizobium japonicum 505 which had been cultured in the defined medium shown in table 1 with glutamate as the nitrogen source.

Fraction	Total protein (mg)	Specific activity (units*/mg)	Recovery (%)
Crude extract	2325	1.0	100
Streptomycin sulfate supernatant (3%)	2608	0.8	95
Polypropylene glycol (5-20%) ppt.	115	12.9	65
DEAE-cellulose eluate	37	45.4	74
Bio-Gel A-5m eluate	11	88.1	43

* A unit of activity is defined under methods.

Table 7. Purification of glutamine synthetase from free-living Rhizobium japonicum 505. Procedure II. The enzyme was purified from 43.0 g of Rhizobium japonicum 505 which had been cultured in 9.0 liters of the defined medium in table 1 with glutamine as the nitrogen source. Additional steps in purification included concentration of the Bio-Gel A-5m eluate with polyethylene glycol and chromatography on a column of Sephadex G-100 to remove residual PEG.

Fraction	Total protein (mg)	Specific activity (units*/mg)	Recovery (%)
Crude extract	2625	1.3	100
XAD-4:PVP eluate	2250	1.3	86
Polyethylene glycol (0-10%) ppt.	672	3.7	77
DEAE-cellulose eluate	360	7.0	77
Polyethylene glycol (0-15%) ppt.	161	16.1	79
Bio-Gel A-5m eluate	25	44.0	33
Polyethylene glycol (5-15%) ppt.	23	23.0	15
Sephadex G-100 eluate	13	35.0	13

* A unit of activity is defined under methods.

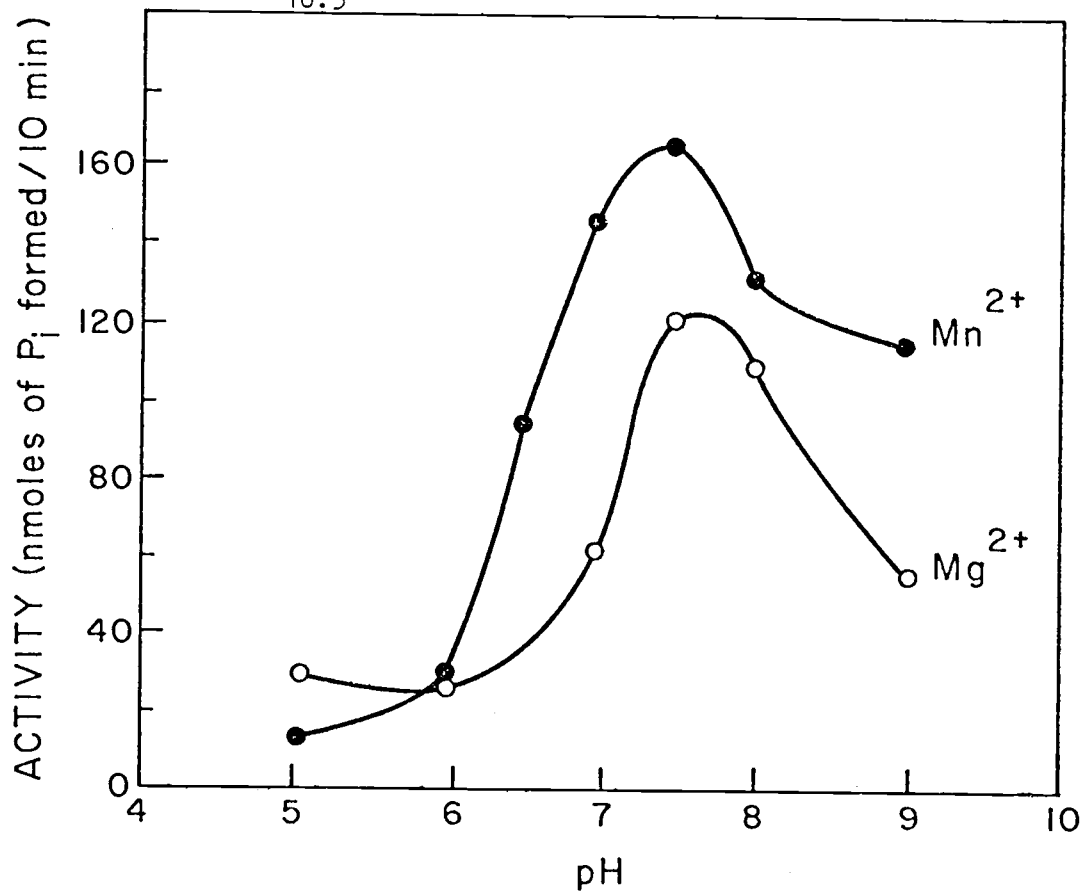
Relative Adenylylation Values. The relative adenylylation of the enzymes from the free-living Rhizobium japonicum was determined by the SVD method (62) and by the transferase reaction in the presence and absence of 60.0 mM Mg^{2+} as previously described. The E_n values obtained for the enzymes were 10.5 and 8.5.

pH Optima. Glutamine synthetase ($E_{10.5}$) displayed biosynthetic activity with pH optima at 7.5 in the presence of either 20.0 mM $MgCl_2$ or 5.0 mM $MnCl_2$. Higher activity was observed in assay media containing $MnCl_2$. Results are described in figure 11. The pH optimum for the transferase reaction in the presence of Mn^{2+} was about 7.3 (figure 12).

Ammonium and L-Glutamate Saturation Curves. Experiments to determine saturating concentrations of ammonium and L-glutamate were conducted with the highly adenylylated glutamine synthetase ($E_{10.5}$). Biosynthetic reactions were conducted in the presence of either $MgCl_2$ or $MnCl_2$ at pH 7.5. Data plotted in figures 13 and 14 show typical Michaelis-Menten equation curves for corresponding substrates, L-glutamate and ammonium in the presence of $MnCl_2$. The Michaelis-Menten constants were determined by the method of Eisenthal and Cornish-Bowden (13). In the presence of Mn^{2+} the adenylylated glutamine synthetase ($E_{10.5}$) had apparent K_m values of 2.31 and 1.38 mM for glutamate and ammonium, respectively. The apparent K_m for glutamate in the presence of Mg^{2+} was 10.07 mM. Inhibition of the reaction in the presence of $MgCl_2$ was not observed in 15.0 mM NH_4Cl (table 8).

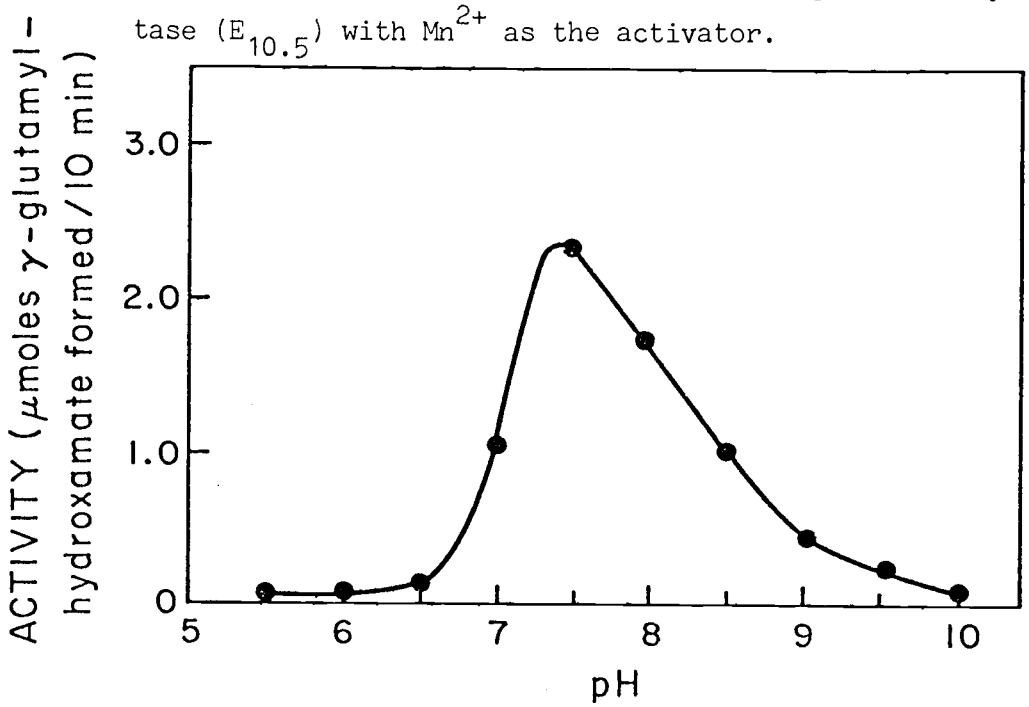
Metabolic Effectors of the Biosynthetic Reaction. Nucleotides,

Figure 11. pH Optima of the biosynthetic activity of glutamine synthetase ($E_{10.5}$) with either Mg^{2+} or Mn^{2+}.



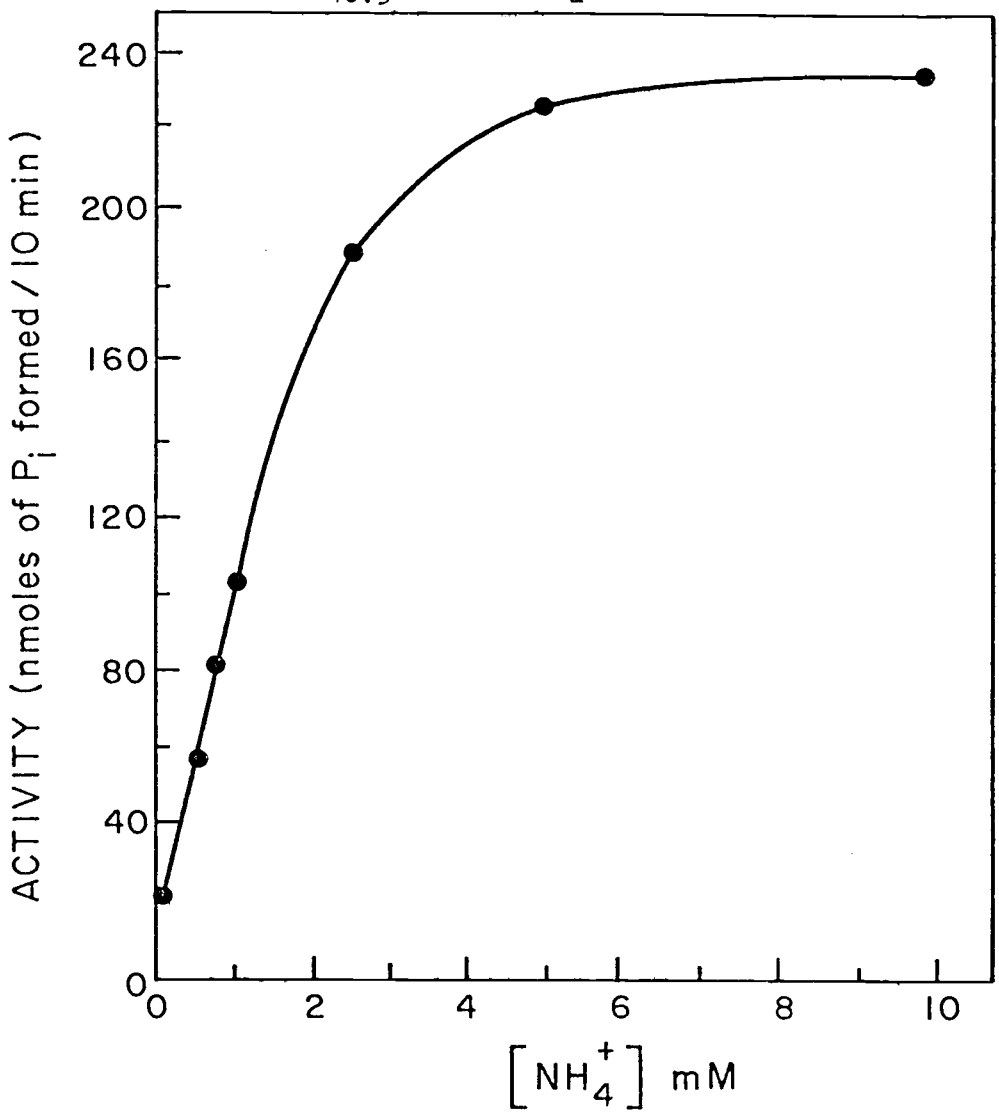
Assay mixtures contained 50.0 mM glutamate; 50.0 mM Tes, 50.0 mM acetic acid; 5.0 mM NH_4Cl; 7.5 mM ATP and 20.0 mM $MgCl_2$ (o); or 5.0 mM ATP and 5.0 mM $MnCl_2$ (•). The pH was adjusted with NaOH. The reactions were initiated by the addition of 1.0 unit of enzyme in a final volume of 0.2 ml. Reaction mixtures were incubated for 30 minutes at 37 C. Each experimental point represents the mean of triplicate determinations. S.E.M. ranged from 1 to 13% of values reported.

Figure 12. pH Optimum of the transferase activity of glutamine synthetase ($E_{10.5}$) with Mn^{2+} as the activator.



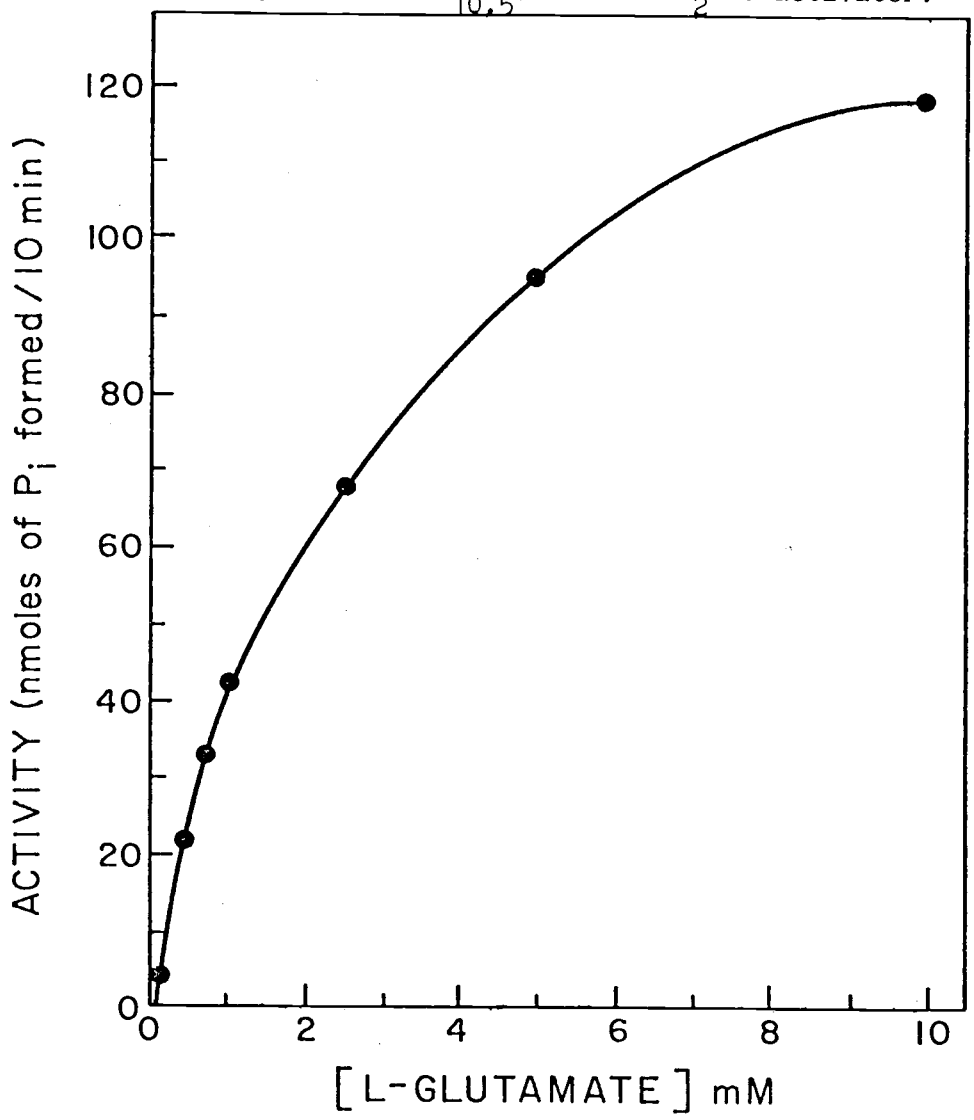
Assay mixtures contained 50.0 mM Tes, 25.0 mM acetic acid, 30.0 mM hydroxylamine-HCl, 60.0 mM glutamine, 0.4 mM ADP, 20.0 mM arsenate and 0.3 mM $MnCl_2$. The reaction was initiated by the addition of 0.2 unit of enzyme in a final reaction volume of 1.0 ml. The pH was adjusted with NaOH. The reaction mixtures were incubated for 10 minutes at 37 C and terminated by the addition of 2.0 ml of $FeCl_3$ reagent (51). Each data point represent the average of duplicate determinations.

Figure 13. Influence of NH_4Cl on biosynthetic activity of glutamine synthetase ($E_{10.5}$) with $MnCl_2$ as activator.



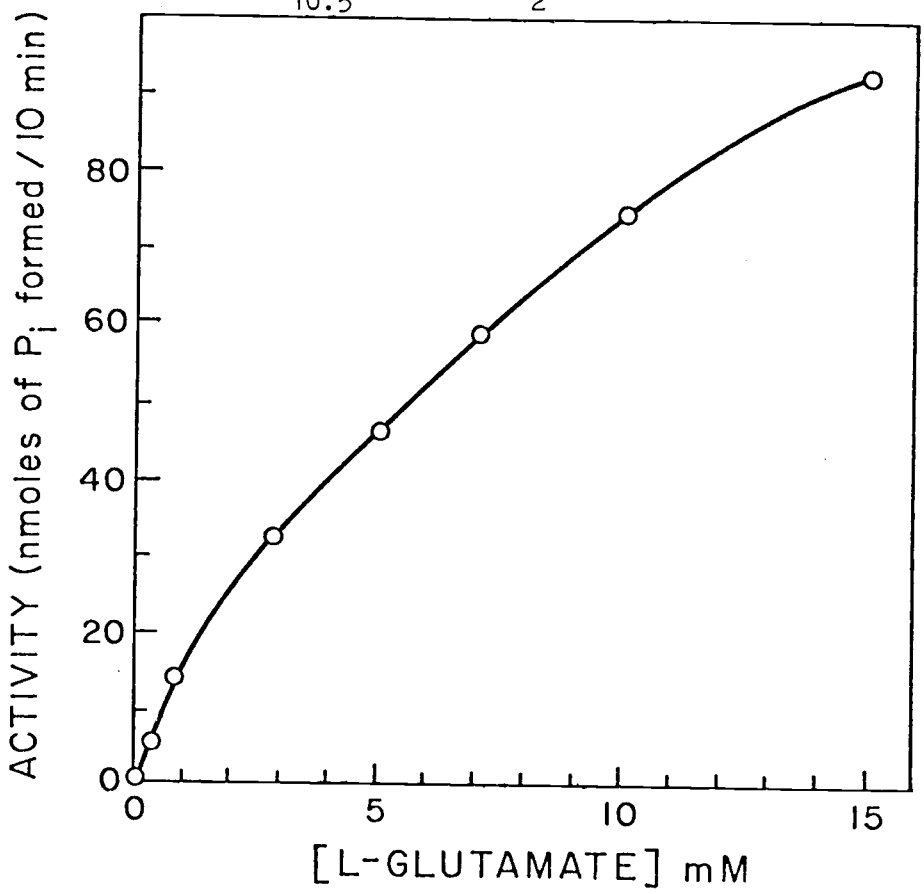
Assay mixtures contained 50.0 mM Tes-NaOH, 50.0 mM acetic acid, 50.0 mM glutamate, 5.0 mM ATP, 5.0 mM $MnCl_2$ and NH_4Cl at concentrations indicated. Reactions in a final volume of 0.2 ml were initiated by the addition of 0.6 unit of enzyme. The mixtures were at pH 7.5 and were incubated for 30 minutes at 37 C. Each data point represents the mean of triplicate determinations. Standard errors of means were less than 2.0% of values reported.

Figure 14. Influence of L-glutamate on the biosynthetic activity of glutamine synthetase ($E_{10.5}$) with $MnCl_2$ as activator.



Assay mixtures contained 50.0 mM Tes-NaOH, 5.0 mM NH_4Cl, and 50.0 mM acetic acid. Reactions with 5.0 mM $MnCl_2$ contained 5.0 mM ATP and were conducted at pH 7.5. Reactions were initiated by the addition of 0.6 unit of enzyme to a final reaction volume of 0.2 ml. The mixtures were incubated for 30 minutes at 37 C. Each experimental point represents the mean of triplicate determinations. S.E.M. ranged from less than 1.0% to 10.0% of values reported.

Figure 15. Influence of glutamate on biosynthetic activity of glutamine synthetase ($E_{10.5}$) with $MgCl_2$ as the activator.



Assay mixtures contained 50.0 mM Tes-NaOH, 5.0 mM NH_4Cl, and 50.0 mM acetic acid at pH 7.5. Reactions also contained 20.0 mM $MgCl_2$ and 7.5 mM ATP. Reactions were initiated by the addition of 0.6 unit of enzyme to a total of 0.2 ml. The mixtures were incubated for 30 minutes at 37 C. Each data point represents the mean of triplicate determinations. S.E.M. values were 1.0% or less of values reported.

Table 8. Influence of NH_4Cl on biosynthetic activity of the glutamine synthetase ($E_{10.5}$) from free-living Rhizobium japonicum 505. Reaction mixtures contained 50.0 mM glutamate, 50.0 mM Tes, 50.0 mM acetic acid, 20.0 mM $MgCl_2$, 7.5 mM ATP and NH_4Cl at concentrations indicated. The reaction medium was adjusted to pH 7.5 with NaOH. Reactions were initiated by the addition of the enzyme to a final reaction volume 0.2 ml. Reaction mixtures were incubated for 30 minutes at 37 C. Each value shown represents the mean $\pm$ S.E.M. of triplicate determinations.

Concentration (mM)	*Activity
0.0	0.0
1.0	43.0 $\pm$ 1.0
5.0	67.5 $\pm$ 2.0
10.0	84.5 $\pm$ 1.0
15.0	87.5 $\pm$ 2.2

* Activity expressed as nmoles of P_i formed per 10 minutes.

organic acids and amino acids involved in the assimilation of ammonia were included in the biosynthetic reaction. Biosynthetic activity of highly adenylylated enzymes in the presence of each individual effector was compared to the control reactions without effectors. Results in table 9 show that the biosynthetic reaction was inhibited 100% by 5 mM 2-oxoglutaric acid and oxalacetic acid. Aspartic acid and adenosine 5'-monophosphate inhibited the reaction catalyzed by 0.6 unit of $E_{10.5}$ enzyme 67% and 52%, respectively. Adenosine 5'-diphosphate caused about 7% inhibition. Glutamine and asparagine had no effect.

The effects of 2-oxoglutaric acid and oxalacetic acid at 0.5 mM and 1.0 mM are shown in table 10. Neither organic acid inhibited the reaction at 0.5 mM. The reaction was inhibited about 14% by 1.0 mM 2-oxoglutaric acid and 8% by 1.0 mM oxalacetic acid.

The results of similar effector experiments involving 1.0 unit of enzyme from free-living Rhizobium japonicum 505 with an E_n value of 8.5 are shown in tables 11 and 12. Inhibition of the biosynthetic reaction was increased to 57% by 1.0 mM 2-oxoglutaric acid and to 44% by 1.0 mM oxalacetic acid.

Table 9. Inhibition of glutamine synthetase ($E_{10.5}$) by selected nucleotides, organic acids and amino acids. Assay mixtures contained 50.0 mM Tes, 50.0 mM acetic acid, 50.0 mM sodium glutamate, 10.0 mM NH_4Cl, 20.0 mM $MgCl_2$, 7.5 mM ATP and 5.0 mM inhibitor. The pH of the mixture was adjusted to 7.5 with NaOH. The reaction tubes were incubated for 30 minutes at 37 C. Each value represents the mean ± S.E.M. of triplicate determinations.

Inhibitor (at 5 mM)	*Inhibition(%)
AMP (monosodium)	52 ± 1.5
ADP (disodium)	7 ± 2.0
2-oxoglutaric acid (monopotassium)	100
oxalacetic acid (free acid)	92 ± 2.5
aspartic acid (monosodium)	67 ± 1.8
asparagine (monohydrate)	2 ± 2.0
glutamine	5 ± 1.4

* %Inhibition compared to control assay without added inhibitor.

Table 10. Inhibition of glutamine synthetase ($E_{10.5}$) by 2-oxoglutaric acid and oxalacetic acid. Assay mixtures contained 50.0 mM Tes, 50.0 mM acetic acid, 50.0 mM glutamate, 10.0 mM NH_4Cl, 20.0 mM $MgCl_2$, 7.5 mM ATP and inhibitor at concentrations indicated in table. The pH was adjusted to 7.5 with NaOH. The reactions, initiated by the addition of 0.6 unit of enzyme in a final volume of 0.2 ml, were incubated for 30 minutes at 37 C. Each value represents the mean $\pm$ S.E.M. of triplicate determinations.

Inhibitor	Concentration (mM)	*Inhibition(%)
2-oxoglutaric acid (monopotassium)	0.5	0
	1.0	14.0 $\pm$ 4.0
oxalacetic acid (free acid)	0.5	0
	1.0	8.0 $\pm$ 1.8

* %Inhibition compared to control assay without added inhibitor.

Table 11. Inhibition of glutamine synthetase ($E_{8.5}$) by selected nucleotides, organic acids and amino acids. Assay mixtures contained 50.0 mM Tes, 50.0 mM acetic acid, 50.0 mM glutamate, 10.0 mM NH_4Cl, 20.0 mM $MgCl_2$, 7.5 mM ATP and 5.0 mM inhibitor. The pH was adjusted to 7.5 with NaOH. The reactions in a final volume of 0.2 ml were initiated by the addition of 1.0 unit of enzyme and incubated for 30 minutes at 37 C. Each value represents the mean ± S.E.M. of triplicate determinations.

Inhibitor (5.0 mM)	*Inhibition(%)
AMP (monosodium)	72.0 ± 0.5
ADP (disodium)	41.0 ± 5.2
2-oxoglutaric acid (monopotassium)	100 ± 0.0
oxalacetic acid (free acid)	100 ± 0.0
aspartic acid (monosodium)	90.0 ± 0.9
asparagine (monohydrate)	0 ± 1.2
glutamine	2.0 ± 1.2

* %Inhibition compared to control assay without added inhibitor.

Table 12. Inhibition of glutamine synthetase ($E_{8.5}$) by 2-oxoglutaric acid and oxalacetic acid. Assay mixtures contained 50.0 mM Tes, 50.0 mM acetic acid, 50.0 mM glutamate, 10.0 mM NH_4Cl, 20.0 mM $MgCl_2$, 7.5 mM ATP and inhibitor at concentrations indicated in table. The pH was adjusted to 7.5 with NaOH. The reactions in a final volume of 0.2 ml were initiated by the addition of 0.6 unit enzyme and incubated for 30 minutes at 37 C. Each value represents the mean $\pm$ S.E.M. of triplicate determinations.

Inhibitor	Concentration (mM)	*Inhibition(%)
2-oxoglutaric acid (monopotassium)	0.5	18.0 $\pm$ 1.6
	1.0	57.0 $\pm$ 3.0
oxalacetic acid (free acid)	0.5	16.0 $\pm$ 1.6
	1.0	44.0 $\pm$ 1.9

* %Inhibition compared to control assay without added inhibitor.

Influence of NH_4^+ on Glutamine Synthetases from Nodules and Free-living Rhizobium japonicum

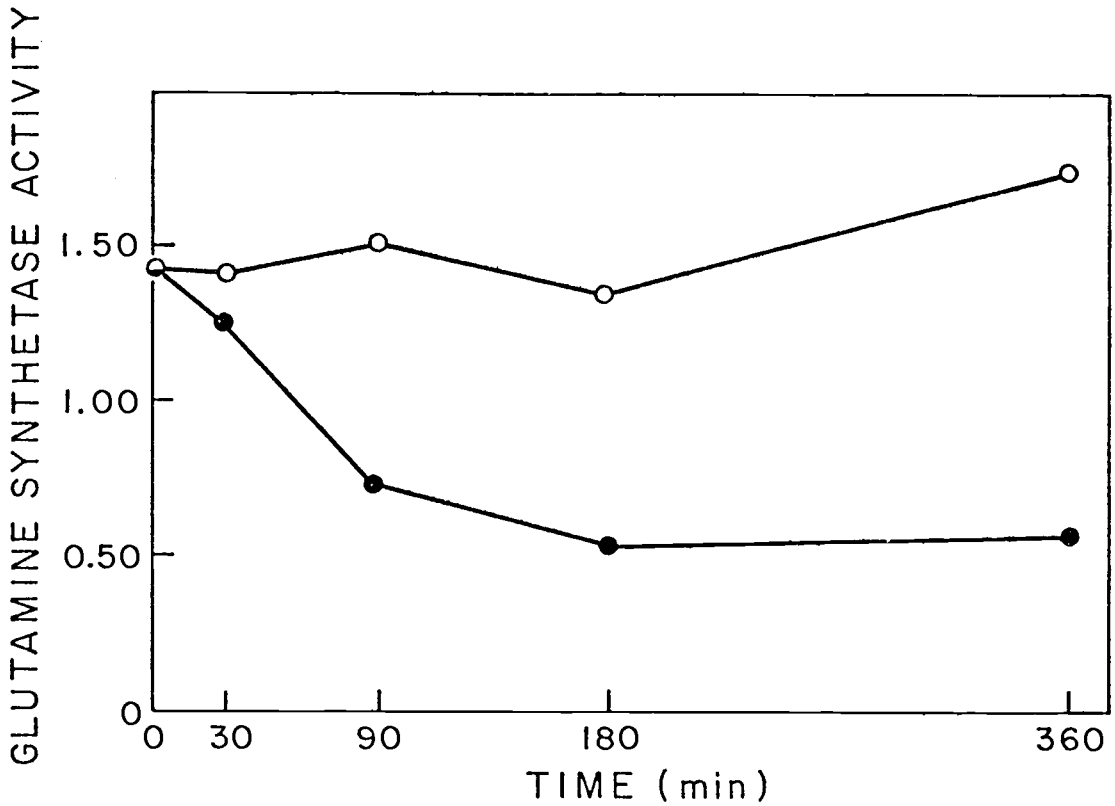
Effects of NH_4^+ on Glutamine Synthetase from Free-living Rhizobium japonicum. The response of glutamine synthetase to ammonium was of interest because of the postulated role that the enzyme has in the biosynthesis of itself and other enzymes (28). Rhizobium japonicum was cultured in the medium described in table 1. The cultures were incubated on a rotary shaker at 30 C. After 36 hours, the cells were collected by centrifugation and suspended in the same medium without glutamate. The cells were incubated as before under nitrogen-limiting conditions for four hours. Nitrogen-starved cultures of Rhizobium japonicum were incubated in the presence of 15.0 mM $(NH_4)_2SO_4$. The control cultures were incubated in the presence of Na_2SO_4. After 180 minutes a 58% reduction in glutamine synthetase activity was noted (figure 16). The relative adenylylation reached a maximum after 90 minutes of incubation (figure 17) in both the ammonium-treated and the control cultures. At 90 minutes the E_n value of glutamine synthetase for the ammonium-treated cells was approximately 8.0. The E_n value of glutamine synthetase from the control cells was about 2.4. This difference is significant because of the different properties displayed by the adenylylated ($E_{8.0}$) and unadenylylated ($E_{2.4}$) enzymes.

Effects of Nitrogen Deprivation on Adenylylation of Glutamine Synthetase from Bacteroids in Nodulated Roots. Efforts to influence the adenylylation state of glutamine synthetase from the bacteroid in situ or in suspensions by the addition of excess ammonium were unsuccessful

(5). Nodulated soybean roots were deprived of nitrogen in an attempt to induce deadenylylation of glutamine synthetase in the bacteroids. The nodulated soybean roots were incubated for 12 hours in a gas mixture of 80% Ar and 20% O_2. A second sample of nodulated roots was incubated in a similar gas mixture with the addition of 10% C_2H_2 (high concentrations of C_2H_2 non-competitively inhibit N_2 reduction by nitrogenase (5)). There was no appreciable influence on the relative adenylylation of glutamine synthetase in the treated samples as compared to the control samples of nodules.

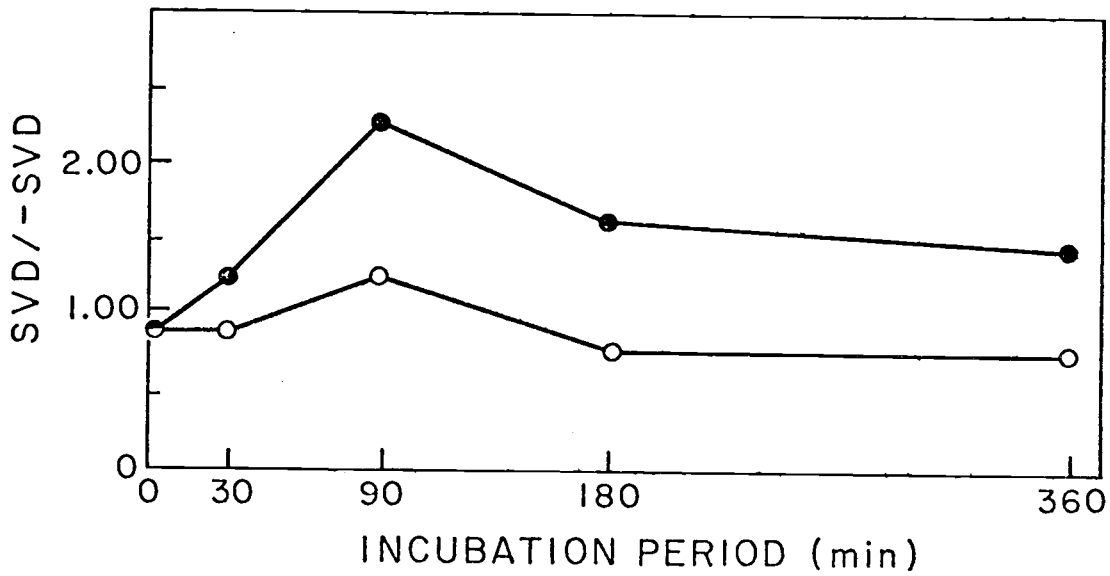
Effects of NH_4^+ on Adenylylation of Bacteroid and Cytosol Glutamine Synthetases in Sliced Nodules. Uptake of free ammonium by intact soybean nodules was reported to be from 8.4 to 14.9 ueq per g fresh nodule when the nodules were exposed to nutrient solutions containing $(NH_4)_2SO_4$ concentrations from 5.0 to 30.0 mM (5). In an effort to further increase the uptake of ammonium, experiments were conducted in which one sample of soybean nodules was sliced then incubated in the presence of 15.0 mM NH_4Cl for 2 hours while vigorously shaking. For comparison, a second sample was treated in a similar fashion but was incubated in the presence of 15.0 mM KCl. Determination of the relative adenylylation values of the glutamine synthetase from the bacteroids from the two samples showed no significant difference. Both samples displayed adenylylation values of about 1.35. Glutamine synthetase from the cytosol had relative adenylylation values of 0.95 and 1.03, respectively. These values are within experimental error and suggest no adenylylation at all.

Figure 16. Effect of NH_4^+ on glutamine synthetase activity in free-living Rhizobium japonicum.



All conditions were as described in the legend for figure 17 (5). Each experimental point represents the mean of triplicate determinations of specific activity (umoles of ɣ-glutamylhydroxamate produced per mg protein at 25 C. o-o, 15.0 mM Na_2SO_4 (control); ●-●, 15.0 mM $(NH_4)_2SO_4$.

Figure 17. Effect of NH_4^+ on adenylylation of glutamine synthetase in free-living Rhizobium japonicum.



Cells were cultured as described in text. All procedures until the final harvesting of the cells were conducted aseptically. The crude extracts were prepared as described by Bishop, et al. (5). The relative adenylylation of glutamine synthetase was determined by the SVD procedure previously described under methods. Five hundred ml of cells in 1.0 liter flasks were harvested at each time period. Relative adenylylation values are means of duplicate determinations. o-o, 15.0 mM Na_2SO_4 (control); ●-●, 15.0 mM $(NH_4)_2SO_4$.

Comparison by Relative Adenylylation and Gel Electrophoresis of Glutamine Synthetases from the Cytosol and Bacteroids from Soybean Nodules.

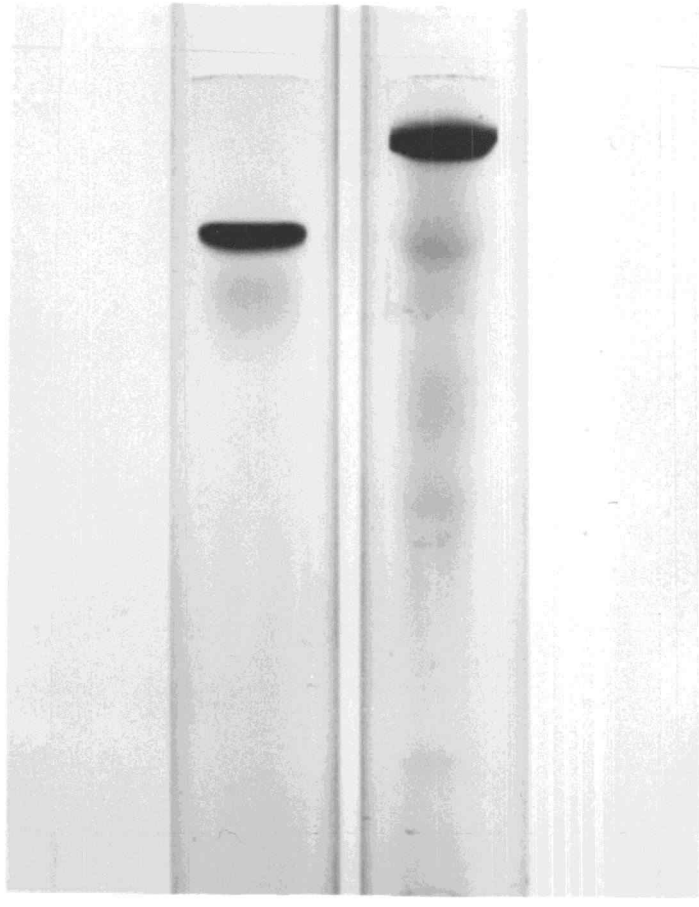
The Relative Adenylylation of Glutamine Synthetases in Cytosol and Bacteroids from Soybean Nodules. Experiments were conducted to determine the relative adenylylation state of the glutamine synthetases from the soybean nodule cytosol and bacteroids. Table 13 shows that the relative adenylylation values for the cytosol enzyme varied around 1.00 consistently when treated with SVD. The adenylylation values for glutamine synthetase from the bacteroids varied from 1.27 to 2.44 when determined by SVD technique. These values changed slightly when the enzyme was in the presence of Mn^{2+} or Mg^{2+} plus Mn^{2+}.

Comparison of Glutamine Synthetases from Cytosol and Bacteroid Fractions from Soybean Nodules by Electrophoretic Mobility. Purified glutamine synthetase from the cytosol fraction of the soybean nodule was obtained from Dr. R. McParland and partially purified glutamine synthetase from the bacteroid fraction were layered on electrophoretic gels. Migration of the enzymes into the 5% gel is shown in figure 18. Electrophoretic mobility of the glutamine synthetase from the cytosol fraction had an R_f value of 0.19 and the enzyme from the bacteroids had an R_f value of 0.07.

Table 13. The relative adenylylation of glutamine synthetase in the cytosol and bacteroids from soybean nodules. Relative adenylylation values were determined as previously described under methods. Bacteroid crude extracts were prepared by the procedure described by Bishop, et al. (5). The cytosol crude extract was prepared by Dr. R. H. McParland and has been reported in a collaborative investigation (29). Values shown represent the mean of duplicate determinations.

Experiment	Source of enzyme	Relative adenylylation SVD/-SVD	$Mn^{++}/Mg^{++}+ Mn^{++}$
1	Bacteroid	1.27	1.45
	Cytosol	1.01	----
2	Bacteroid	1.35	1.50
	Cytosol	0.99	----
3	Bacteroid	2.44	2.56
	Cytosol	----	----
4	Bacteroid	2.22	2.00
	Cytosol	0.96	----

Figure 18. Comparison of glutamine synthetases from soybean nodule cytosol and from R. japonicum bacteroids by use of gel electrophoresis.



(a) (b)

Gel (a) contained 20 ug of purified glutamine synthetase from the cytosol fraction of the soybean nodule (29). Gel (b) contained 40 ug of glutamine synthetase from the nodule bacteroid. The enzyme samples in 5% sucrose, 10.0 mM imidazole-HCl, pH 7.3 and 1.0 mM $MnCl_2$ were layered on the surface of 5% polyacrylamide gels (0.5 x 8 cm) and subjected to electrophoresis as described in methods.

DISCUSSION

In the legume nodule, nitrogenase is synthesized in the bacteroids where it catalyzes the reduction of N_2 to ammonia. Soybean nodule exudates have been reported to contain asparagine, asparatic acid, glutmine, and glutamic acid at concentrations of 125, 29, 20 and 5 umoles per ml, respectively (70). Wolk, et al., (69) established that the glutamine synthetase and glutamate synthase are the primary enzymes involved in the incorporation of ammonium into nitrogenous metabolites in blue-green algae. The research of Robertson (43) and O'Gara, et al., support the view that glutamine synthetase plays an analogous role in legumes. Glutamine synthetase has been proposed to have a positive role in the transcription of the nif operon (59). Studies by Dunn and Klucas (12), Brown and Dilworth (7), and McParland, et al., (29), have shown that glutamine synthetase activity is greater in the cytosol (non-bacteroid) fraction than in the bacteroid fraction of the legume nodule. Information concerning the properties of the glutamine synthetases from the soybean nodule was needed to better understand the roles of glutamine synthetase in the assimilation of ammonium and the control of nitrogenase synthesis in legume nodules. Also, it was of interest to study the changes that occur in the properties of glutamine synthetase of Rhizobium japonicum as a result of symbiosis with the host legume.

Glutamine Synthetase from Nodule Cytosol. Collaborative research with Dr. R. H. McParland (29), which is referred to only briefly in this thesis, led to a fairly thorough characterization of the glutamine synthetase from the soybean nodule cytosol. The enzyme constitutes

approximately 2% of the total soluble protein in the cytosol. Activity of glutamine synthetase in the cytosol is over 10-fold greater than that in the nodule bacteroids. For example, the total glutamine synthetase activity in 100 g of soybean nodules was 4000 units while the bacteroids extracted from 100 g nodules usually contained about 300 units of glutamine synthetase activity.

The enzyme from the cytosol has been purified 50-fold over the crude extract. Purity was established by sedimentation equilibrium analysis and by gel electrophoresis. It was concluded that glutamine synthetase from the nodule cytosol was similar to the enzymes from the pea leaf (36) and sheep brain (46). The molecular weight of the enzyme from the cytosol is 376,000 and is composed of 8 subunits arranged in a cubical structure. Each subunit has a molecular weight of about 47,300. Evidence obtained from SVD treatment of the glutamine synthetase from the cytosol suggests an absence of an adenylylation system. This conclusion is consistent with research on glutamine synthetases from eucaryotic systems and also gram-positive bacteria (28). Robertson and Warburton (43) have concluded that glutamine synthetase in the cytosol fraction of the lupin nodule plays a major role in the assimilation of the product of N_2 reduction.

Glutamine Synthetase from Nodule Bacteroids. The glutamine synthetase from soybean nodule bacteroids was purified extensively (about 100-fold); however, about 90% of the final product was estimated to be homogeneous. Although the enzyme retained transferase activity when frozen in liquid nitrogen and stored at -80 C, almost total loss of

activity occurred after 8 hours at 4 C. Fifty percent loss of total activity was routinely experienced during batch purification steps with either polyethylene glycol or polypropylene glycol. All transferase activity was lost when ammonium sulfate was used in the purification procedure. This behavior is unlike that for the glutamine synthetase from Escherichia coli (51). Attempts to purify glutamine synthetase from bacteroids by $ZnSO_4$ precipitation as reported for Escherichia coli (33) were unsuccessful.

Although difficulties were encountered in purification of glutamine synthetase from nodule bacteroids, the information derived from the partially purified preparations indicates some similarities with the glutamine synthetase from E. coli. The enzyme from the nodule bacteroids was partially adenylylated with values ranging between 1.0 and 3.0 (E_0 and E_8). This evidence indicated that the activity of glutamine synthetase in the bacteroid may be regulated by an adenylylation system in a manner similar to the control systems described in other gram-negative bacteria (62). Efforts to alter the relative adenylylation of the enzyme from the bacteroids in situ were unsuccessful. It was not possible to influence the adenylylation of the glutamine synthetase by the addition of excess ammonium to the soybean nutrient medium in situ or to sliced nodules in vitro. Nitrogen-deprivation of nodules in vitro did not result in deadenylylation of the glutamine synthetase in the bacteroids. The major effect that was observed by the addition of excess ammonium to the nodules was a decrease in nitrogenase activity (5). O'Gara and Shanmugam (35) have shown that as much as 94% of $^{15}N_2$ fixed by free-living Rhizobium japonicum was excreted as NH_4^+ into the medium.

Miflin and Lea (32) suggest that the bacteroid membrane may function exclusively for the elimination of NH_4^+ and, therefore, uptake of excess ammonium by the bacteroid may not occur. The decrease in nitrogenase activity resulting from the addition of NH_4^+ may not be caused by a direct influence of ammonium but may be influenced by a product of NH_4^+ assimilation. This influence may be only on the synthesis of nitrogenase and not on the adenylylation system of glutamine synthetase.

Bacteroids contain about 8.0 mM magnesium and a concentration of manganese less than 1 ppm, the latter of which is too low to activate the glutamine synthetase reaction. It may be concluded, therefore, that Mg^{2+} is the activator of the physiologically active, unadenylylated glutamine synthetase in the biosynthetic reaction in bacteroids.

The apparent K_m values as determined by the direct linear plot method of Eisenthal and Cornish-Bowden (13) were 1.04 mM and 1.4 mM for ammonium and L-glutamate, respectively. It was observed that concentrations of NH_4Cl higher than 1.0 mM caused an inhibition of the biosynthetic reaction in the presence of Mg^{2+} but not in the presence of Mn^{2+}. Maximum biosynthetic activity was shown at 20.0 mM Mg^{2+}. Concentrations of Mn^{2+} higher than 5.0 mM caused an inhibition of the biosynthetic reaction. In the presence of 20.0 mM Mg^{2+} maximum biosynthetic activity was attained with 7.5 mM ATP. Greater activity was observed when equimolar concentrations of $MgCl_2$ and ATP were added to reactions containing 20.0 mM Mg^{2+}. This suggests that uncomplexed Mg^{2+} is required for enzyme activity.

The biosynthetic activity of the glutamine synthetase ($E_{3.3}$) from the bacteroid had pH optima at 8.5 and 7.8 in the presence of either

Mg^{2+} or Mn^{2+}, respectively. The unadenylylated enzyme from E. coli was reported to have a pH optimum at 8.0 in the biosynthetic assay containing Mg^{2+} (20). Glutamine synthetases from the pea leaf (36) and soybean nodule cytosol (29) have pH optima at 8.2 and 8.0, respectively, in the biosynthetic assay containing Mg^{2+}. Maximum biosynthetic activity for glutamine synthetase from both eucaryotic tissues and bacteria has been reported within a pH range of 7.6 to 8.3 when the assay mixture contained Mg^{2+}. In this respect glutamine synthetase from nodule bacteroids is similar to other glutamine synthetases.

Glutamine Synthetase from Free-living Rhizobium japonicum. The failure of attempts to culture rhizobia in a defined medium that would result in the synthesis of either a completely adenylylated or unadenylylated glutamine synthetase has made purification and characterization of the enzyme difficult. Many of the external conditions which influence the adenylylation state of glutamine synthetase in either free-living Rhizobium japonicum or soybean nodule bacteroids remain to be defined. Rhizobium japonicum cultured in the defined medium as described under "Experimental and Results" resulted in a yield of 5.0 g of cells per liter when the cells were harvested after 80 hours of incubation. The substitution of 5.0 mM NH_4Cl for glutamate in the defined medium (a nitrogen-limiting condition) resulted in slow growth, low yield (0.5 g cells per liter) and a low adenylylation state glutamine synthetase. Although the methods employed to show the effect of excess ammonium and nitrogen-deprivation on the adenylylation state of glutamine synthetase on small volume cultures of Rhizobium japonicum were

successful, the necessary aseptic methods were not practical for large volume cultures. Routinely the cells were cultured in the defined medium containing glutamate as the nitrogen source. Typically, these cells contained an adenylylated glutamine synthetase (e.g, $E_{10.5}$ and $E_{8.5}$).

Glutamine synthetase from free-living Rhizobium japonicum was purified 80-fold over the crude extract. The crude extract from the free-living rhizobia had a specific activity which was three-fold greater than that in crude extract from the bacteroids. Like the enzyme from the bacteroids, glutamine synthetase from free-living Rhizobium japonicum remained stable for up to 2 months when frozen in liquid nitrogen and stored at -80 C. Yields in total activity during the purification procedures were analogous to the yields obtained with the enzyme extracted from the bacteroids. Glutamine synthetases from nodule bacteroids and free-living Rhizobium japonicum showed similar electrophoretic mobilities. Use of the transferase assay system to detect activity on gels showed the enzymes had similar R_f values. Gels stained with Coomassie Brilliant Blue also showed that the major protein bands corresponded with the bands that exhibited activity on the gels.

The relatively adenylylated glutamine synthetase ($E_{10.5}$) from free-living Rhizobium japonicum had a pH optima at 7.5 for biosynthetic activity in the presence of either Mg^{2+} or Mn^{2+}. Optimal pH conditions for biosynthetic activity of the enzyme from Escherichia coli have been reported to be 7.2 and 7.5 in the presence of Mg^{2+} (20). The unadenylylated glutamine synthetase ($E_{3.3}$) from rhizobia bacteroids showed the shift in pH optima as does the enzyme from Escherichia coli (20). The

higher activity is observed for the adenylylated enzyme from rhizobia in the presence of Mn^{2+}. The change in divalent cation response by the adenylylated enzyme from free-living rhizobia is analogous to that seen in Escherichia coli (20).

The apparent K_m value for glutamate of the adenylylated enzyme from rhizobia in the biosynthetic assay in the presence of Mg^{2+} was ten-fold greater than the affinity observed for the unadenylylated glutamine synthetase from rhizobia bacteroids. The K_m values reported for the unadenylylated and adenylylated enzymes from Escherichia coli for glutamate were 5.0 mM and 2.0 mM, respectively, in the biosynthetic assay containing Mg^{2+}. The affinity constants for glutamate of glutamine synthetase from other organisms ranged from 0.8 mM to 13.0 mM. Properties shown in table 14 indicate that K_m values for ammonium were lower than for glutamate in both eukaryotic tissues and the prokaryotic organisms listed.

Tronick, et al. (62), reported that glutamine synthetases from gram-negative bacteria were antigenically related. Glutamine synthetase from rhizobia is regulated by adenylylation as are the enzymes of other gram-negative bacteria (62). Therefore, it is reasonable to assume that the glutamine synthetase from rhizobia is similar to the enzyme from other gram-negative bacteria. In this study no evidence was obtained indicating that glutamine synthetase in rhizobia plays a role in the control of the synthesis of nitrogenase. The possibility of a role, however, was not excluded.

Table 14. Comparison of some properties of glutamine synthetases.

Source of enzyme	Molecular weight	Number of subunits	K_m for substrates	
			NH_4^+	glutamate
Soybean nodule cytosol	376,000	8	ND	ND
bacteroid	ND[1]	ND	1.04 mM	1.4 mM
R. japonicum free-living	ND	ND	1.4 mM	10.0 mM
E. coli	592,000	12	NR[2]	5.0 mM
			NR	2.0 mM
B. subtilis	600,000	12	NR	0.8 mM
Pea leaf	370,000	8	1.5-2.2 x 10^{-2}mM	11.4-13 mM
Sheep brain	392,000	8	0.18 mM	2.5 mM
Rat liver	352,000	8	NR	5.0 mM

[1] ND (not determined)

[2] NR (not reported)

Table 14. Comparison of some properties of glutamine synthetases.

Source of enzyme	Functional adenylylation system	pH Optimum	
		transferase	biosynthetic
Soybean nodule cytosol	none	6.5	8.0
bacteroid	yes	$7.0_{E_{1.3}}$	$8.5_{E_{3.3}}$
R. japonicum free-living	yes	$7.3_{E_{10.5}}$	$7.5_{E_{10.5}}$
E. coli	yes	$7.8_{E_{2.3}}$	$8.0_{E_{2.3}}$ $7.8_{E_{2.3}}$
		$6.8_{E_{11.8}}$	$7.2_{E_{11.8}}$ $7.5_{E_{9.0}}$
B. subtilis	none	NR	7.2
Pea leaf	none	NR	8.2
Sheep brain	none	NR	7.0-7.4
Rat liver	none	NR	NR

Table 14. Comparison of some properties of glutamine synthetases.

Properties of the glutamine synthetases from the nodule bacteroids ($E_{3.3}$) and free-living Rhizobium japonicum ($E_{10.5}$) were compared to the properties of glutamine synthetases from soybean nodule cytosol (29), Escherichia coli (55), Bacillus subtilis (11), pea leaf (36, 37, 38), sheep brain (31) and rat liver (31).

SUMMARY

The objectives of this investigation included the development of a purification procedure for the glutamine synthetases from the soybean nodule bacteroids and free-living Rhizobium japonicum, and the determination of properties of the enzymes. Also, the research was designed to determine the influence that glutamine synthetase has on the assimilation of ammonium and the control of nitrogenase synthesis in legume nodules. The results of this investigation may be summarized as follows:

1. The procedure for the purification of glutamine synthetase included streptomycin sulfate treatment to remove nucleic acids, polypropylene glycol or polyethylene glycol fractionation, step-wise elution from a DEAE-cellulose column and chromatography on Bio-Gel A-5m. The enzymes were purified to an extent of over 90 percent homogeneity as determined by disc gel electrophoresis.

2. The apparent K_m values for the substrates ammonium and glutamate were 1.45 mM and 1.4 mM, respectively, for the physiologically active glutamine synthetase. Apparent K_m values were determined by the direct linear plot method described by Eisenthal and Cornish-Bowden (13). Concentrations of ammonium greater than 1.0 mM caused inhibition of the biosynthetic reaction.

3. Higher biosynthetic activity was observed in the presence of Mg^{2+} than in the presence of Mn^{2+} for the physiologically active, unadenylylated glutamine synthetase. Biosynthetic activity has a pH optimum at 8.5 in the presence of Mg^{2+}. This value is near the pH optima reported for glutamine synthetases of other organisms.

Maximum biosynthetic activity was observed when the ratio of Mg^{2+} to ATP was greater than 4. The activity in the biosynthetic assay was inhibited when the ratio of Mn^{2+} to ATP was greater than 1.0.

4. The glutamine synthetases from soybean nodule cytosol and bacteroids are different as shown by electrophoretic mobilities and activities after SVD treatment. Also, they behave differently during purification. Glutamine synthetases from free-living Rhizobium japonicum and Rhizobium japonicum bacteroids appear to be regulated by the adenylylation system. They have similar electrophoretic mobilities. No evidence was obtained of fundamental difference in the glutamine synthetases from bacteroids and free-living rhizobia.

5. The adenylylation state of glutamine synthetase from bacteroids was not affected by the addition of excess ammonium in situ to intact nodulated plants. The addition of excess ammonium to nitrogen-deprived (fixed nitrogen) cultures of Rhizobium japonicum caused a decrease in glutamine synthetase activity and an increase in the state of adenylylation as determined by SVD treatment and divalent cation specificity.

BIBLIOGRAPHY

1. Adler, S. P., D. Purich and E. R. Stadtman. 1975. Cascade control of E. coli glutamine synthetase. Properties of P-2 regulatory protein and uridylyl transferase-uridydyl-removing enzyme. Journal of Biological Chemistry 250: 6264-6272.

2. Atkinson, D. E. 1968. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifers. Biochemistry 7: 4030-4034.

3. Beale, S. I., S. P. Gough and S. Granick. 1975. Biosynthesis of δ-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. Proceedings of the National Academy of Science, U.S.A. 72: 2719-2723.

4. Bergersen, F. J. and G. L. Turner. 1976. The role of O_2-limitation in control of nitrogenase in continuous cultures of Rhizobium sp. Biochemical Biophysical Research Communications 73: 524-531.

5. Bishop, P. E., J. G. Guevara, J. A. Engelke and H. J. Evans. 1976. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between Rhizobium japonicum and Glycine max. Plant Physiology 57: 542-546.

6. Brill, W. J. 1975. Regulation and genetics of bacterial nitrogen fixation. In: Annual Review of Microbiology (M. Starr, ed.), Vol. 29, pp. 109-129. Annual Review, Inc. Palo Alto, California.

7. Brown, C. M. and M. J. Dilworth. 1975. Ammonia assimilation by Rhizobium cultures and bacteroids. Journal of General Microbiology 86: 39-48.

8. Burns, R. C. and R. W. F. Hardy. 1975. Nitrogen Fixation In Bacteria And Higher Plants. Springer-Verlag, New York.

9. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. In: Annals of the New York Academy of Sciences, Vol. 121, pp. 404-427.

10. Denton, M. D. and A. Ginsberg. 1970. Some characteristics of the binding of substrates to glutamine synthetase from Escherichia coli. Biochemistry 9: 617-632.

11. Deuel, T. F., A. Ginsberg, J. Yeh, E. Shelton and E. R. Stadtman. 1970. Bacillus subtilis glutamine synthetase. Purification and physical characterization. Journal of Biological Chemistry 245: 5195-5205.

12. Dunn, S. D. and R. V. Klucas. 1973. Studies on possible routes of ammonium assimilation in soybean root nodule bacteroids. Canadian Journal of Microbiology 19: 1493-1499.

13. Eisenthal, R. and A. Cornish-Bowden. 1974. The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochemical Journal 139: 715-720.

14. Evans, H. J., B. Koch and R. Klucas. 1972. Preparation of nitrogenase from nodules and separation into components. In: Methods In Enzymology (A. San Pietro, ed.). Vol. 24B, pp. 470-476. Academic Press, New York.

15. Ginsberg, A. 1972. Glutamine synthetase of Escherichia coli: some physical and chemical properties. In: Advances in Protein Chemistry (C. B. Anfinsen, J. T. Edsale and F. M. Richards, eds.), Vol. 26, pp. 1-79. Academic Press, New York.

16. Gornall, A. G., C. J. Bardawill and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. Journal of Biological Chemistry 177: 751-766.

17. Hartman, S. C. 1973. Relationships between glutamine amidotransferases and glutaminases. In: The Enzymes of Glutamine Metabolism (S. Prusiner and E. R. Stadtman, eds.), pp. 319-330. Academic Press, New York.

18. Hartman, S. C. and S. Prusiner. 1973. Guanosine 5'-phosphate synthetase. In: The Enzymes of Glutamine Metabolism (S. Prusiner and E. R. Stadtman, eds.), pp. 409-420. Academic Press, New York.

19. Kennedy, I. R. 1966. Primary products of symbiotic nitrogen fixation. I. Short-term exposures of Serradella nodules to $^{15}N_2$. Biochimica et Biophysica Acta 130: 285-294.

20. Kingdon, H. S. and E. R. Stadtman. 1967. Regulation of glutamine synthetase. X. Effect of growth conditions on the susceptibility of E. coli glutamine synthetase to feedback inhibition. Journal of Bacteriology 94: 949-957.

21. Krebs, H. A. 1935. Metabolism of amino acids. IV. The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. Biochemical Journal 29: 1951-1969.

22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

23. Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods In Enzymology (S. P. Colowick and N. O. Kaplan, eds.), Vol. 3, pp. 447-454. Academic Press, New York.

24. Levintow, L. and A. Meister. 1954. Reversibility of the enzymatic synthesis of glutamine. Journal of Biological Chemistry 209: 265-280.

25. Loomis, W. D. 1959. Amide metabolism is higher plants. III. Distribution of glutamyl transferase and glutamine synthetase activity. Plant Physiology 34: 541-546.

26. Loomis, W. D. 1974. Overcoming problems of phenolics and quinones in the isolations of plant enzymes and organelles. In: Methods In Enzymology (S. Fleischer and L. Packer, eds.), Vol. 31, pp. 528-544. Academic Press, New York.

27. Ludwig, R. A. and E. R. Signer. 1977. Glutamine synthetase and control of nitrogen fixation in Rhizobium. Nature 267: 246-248.

28. Magasanik, B., M. J. Prival, J. E. Brenchley, B. M. Tyler, A. B. DeLeo, S. L. Streicher, R. A. Beuder and C. G. Paris. 1974 In: Current Topics in Cellular Regulation (B. L. Horecher and E. R. Stadtman, eds.), Vol. 8, pp. 119-138. Academic Press, New York.

29. McParland, R. H., J. G. Guevara, R. R. Becker and H. J. Evans. 1976. The purification and properties of the glutamine synthetase from the cytosol of soya-bean root nodules. Biochemical Journal 153: 597-606.

30. Meers, J. L., D. W. Tempest and C. M. Brown. 1970. 'Glutamine (amide): 2-oxoglutarate amino transferase oxido-reductase (NADP)', an enzyme involved in the synthesis of glutamate by some bacteria. Journal of General Microbiology 64: 187-194.

31. Meister, A. Glutamine synthetase of mammals. 1974. In: The Enzymes (P. D. Boyer, ed.), 3rd Edition, Vol. 10, pp. 699-754. Academic Press, New York.

32. Miflin, B. J. and P. J. Lea. 1976. Review: The pathway of nitrogen assimilation in plants. Phytochemistry 15: 873-885.

33. Miller, R. E., E. Shelton and E. R. Stadtman. 1974. Zinc-induced paracrystalline aggregation of glutamine synthetase. Archives of Biochemistry and Biophysics 163: 155-171.

34. Mitchell, C. A. and C. R. Stocking. 1975. Kinetics and energetics of light-driven chloroplast glutamine synthetase. Plant Physiology 55: 59-63.

35. O'Gara, F. and K. T. Shanmugam. 1976. Regulation of nitrogen fixation by rhizobia export of fixed N_2 as NH_4^+. Biochemica et Biophysica Acta 437: 313-321.

36. O'Neal, D. and K. W. Joy. 1973. Glutamine synthetase of pea leaves. I. Purification, stabilization and pH optima. Archives of Biochemistry and Biophysics 159: 113-122.

37. O'Neal, T. D. and K. W. Joy. 1974. Glutamine synthetase of pea leaves. Divalent cation effects, substrate specificity, and other properties. Plant Physiology 54: 773-779.

38. O'Neal, T. D. and K. W. Joy. 1975. Pea leaf glutamine synthetase. Regulatory properties. Plant Physiology 55: 968-974.

39. Postgate, J. R. 1968. How microbes fix nitrogen. Science Journal 4: 69-74.

40. Powers, S. G. and J. F. Riordan. 1975. Functional arginyl residues as ATP binding site of glutamine synthetase and carbamyl phosphate synthetase. Proceedings of the National Academy of Science, U.S.A. 72: 2616-2620.

41. Quispel, A. 1974. Chapter 1. General introduction. In: The Biology of Nitrogen Fixation (A. Quispel, ed.), pp. 1-8. North-Holland Publishing Company, Amsterdam. American Elsevier Publishing Company, Inc., New York.

42. Rhee, S. G., P. B. Chock and E. R. Stadtman. 1976. Mechanistic studies of glutamine synthetase from E. coli: An integrated mechanism for biosynthesis, transferase and ATPase reactions. Biochimie 58: 35-49.

43. Robertson, J. G. and M. P. Warburton. 1975. Induction of glutamate synthase during nodule development in lupin. FEBS Letters 55: 33-37.

44. Robertson, J. G., K. J. F. Farnden, M. P. Warburton and J. A. M. Banks. 1975. Induction of glutamine synthetase during nodule development in lupin. Australian Journal of Plant Physiology 2: 265-272.

45. Rognes, S. E. 1975. Glutamine-dependent asparagine synthetase from Lupinus luteus. Phytochemistry 14: 1975-1982.

46. Rowe, W. B., R. A. Ronzio, V. P. Wellner and A. Meister. Glutamine synthetase (sheep brain). In: Methods In Enzymology (H. Tabor and C. W. Tabor, eds.), Vol. 17. Academic Press, New York.

47. Segal, A., M. S. Brown and E. R. Stadtman. 1974. Metabolite regulation of the state of adenylylation of glutamine synthetase. Archives of Biochemistry and Biophysics 161: 319-327.

48. Shanmugam, K. T. and C. Morandi. 1976. Amino acids as repressors of nitrogenase biosynthesis in Klebsiella pneumoniae. Biochemica et Biophysica Acta 437: 322-332.

49. Shanmugam, K. T., I. Chan and C. Morandi. 1975. Regulation of nitrogen fixation-derepressed mutants of K. pneumoniae. Biochemica et Biophysica Acta 408: 101-111.

50. Shapiro, B. M. and A. Ginsberg. 1968. Effects of specific divalent cations on some physical and chemical properties of glutamine synthetase from Escherichia coli. Taut and relaxed enzyme forms. Biochemistry 7: 2153-2167.

51. Shapiro, B. M. and E. R. Stadtman. 1970. Glutamine synthetase (Escherichia coli). In: Methods In Enzymology (H. Tabor and C. W. Tabor, eds.), Vol. 17, pp. 900-922. Academic Press, New York.

52. Snell, F. D. and C. T. Snell. 1949. Colorimetric Methods of Analysis. 3rd Edition, pp. 656-657. D. Van Nostrand Company, Inc., Toronto.

53. Speck, J. F. 1949. The enzmatic synthesis of glutamine, a reaction utilizing adenosine triphosphate. Journal of Biological Chemistry 179: 1405-1426.

54. Stadtman, E. R., A. Ginsberg, W. B. Anderson, A. Segal, M. S. Brown and J. E. Ciardi. 1971. Regulation of glutamine metabolism in E. coli by enzyme catalyzed adenylylation and deadenylylation of glutamine synthetase. In: Molecular Basis of Biological Activity (K. Gaede, B. L. Horecher, and W. J. Whelan, eds.). The Pan-American Association of Biochemical Societies (PAABS) Symposium, Vol. 1, p. 127. Academic Press, New York.

55. Stadtman, E. R. and A. Ginsberg. 1974. The glutamine synthetase of Escherichia coli: Structure and control. In: The Enzymes (P. D. Boyer, ed.), 3rd Edition, Vol. 10, pp. 755-807. Academic Press, New York.

56. Stahl, J. and L. Jaenicke. 1972. Investigations of the structure of glutamine synthetase from pig brain. European Journal of Biochemistry 29: 401-407.

57. Stewart, G. R. and D. Rhodes. 1976. Evidence for the assimilation of ammonia via the glutamine pathway in nitrate-grown Lemna minor L. FEBS Letters 64: 296-299.

58. Streeter, J. G. 1972. Nitrogen nutrition of field-grown soybean plants. I. Seasonal variations in soil nitrogen and nitrogen composition of stem exudates. Agronomy Journal 64: 311-314.

59. Streicher, S. L., K. T. Shanmugam, F. Ausubel, C. Morandi, and R. B. Goldberg. 1974. Regulation of nitrogen fixation in Klebsiella pneumoniae: Evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis. Journal of Bacteriology 120: 815-821.

60. Stumpf, P. K., and W. D. Loomis. 1950. Observations on a plant amide enzyme system requiring manganese and phosphate. Archives of Biochemistry 25: 451-453.

61. Tempest, D. W., J. L. Meers and C. M. Brown. 1970. Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route. Biochemical Journal 117: 405-407.

62. Tronick, S. R., J. E. Ciardi and E. R. Stadtman. 1973. Comparative biochemical and immological studies of bacterial glutamine synthetases. Journal of Bacteriology 115: 858-868.

63. Trotta, P. O., L. M. Pinkus, V. P. Wellner, L. Estis, R. H. Haschemeyer and A. Meister. 1973. Structure-function relationships in glutamine-dependent carbamyl phosphate synthetase. In: The Enzymes of Glutamine Metabolism (S.Prusiner and E. R. Stadtman, eds.), pp. 431-482. Academic Press, New York.

64. Tubb, R. S. 1974. Glutamine synthetase and ammonium regulation of nitrogenase synthesis in Klebsiella sp. Nature 251: 481-485.

65. Tyler, B., A. B. DeLeo and B. Magasanik. 1974. Activation of transcription of hut DNA by glutamine synthetase (histidine utilization (hut) operons/Salmonella typhimurium/control of glutamate-forming enzymes/cyclic AMP). Proceedings of the National Academy of Science, U.S.A. 71: 225-229.

66. Wedler, F. C. and F. M. Hoffmann. 1974. Glutamine synthetase of Bacillus stearothermophilus. I. Purification and basic properties. Biochemistry 13: 3207-3214.

67. Wedler, F. C. and P. D. Boyer. 1972. Substrate binding and reaction intermediates of glutamine synthetase (Escherichia coli W) as studied by isotope exchanges. Journal of Biological Chemistry 247: 984-992.

68. Withalt, B. 1973. NAD synthetase. In: The Enzymes of Glutamine Metabolism (S. Prusiner and E. R. Stadtman, eds.), pp. 421-430. Academic Press, New York.

69. Wolk, C. P., J. Thomas, P. W. Shaffer, S. M. Austin and A. Galonsky. 1976. Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium, Anabaena cylindrica. Journal of Biological Chemistry 251: 5027-5034.

70. Wong, P. P. and H. J. Evans. 1971. Poly-β-hydroxybutyrate utilization by soybean (Glycine max. Men.) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiology 47: 750-755.

71. Woolfolk, C. A., B. M. Shapiro and E. R. Stadtman. 1966. Regulation of glutamine synthetase. I. Purification and properties of glutamine synthetase from Escherichia coli. Archives of Biochemistry and Biophysics 116: 177-192.

72. Wyngaarden, J. B. 1973. Glutamine phosphoribosylpyrophosphate amidotransferase. In: The Enzymes of Glutamine Metabolism (S. Prusiner and E. R. Stadtman, eds.), pp. 365-386. Academic Press, New York.