GLUTAMINE SYNTHETASE ACTIVITY DURING GROWTH OF FREE-LIVING *RHIZOBIUM* SPECIES

BY

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A Thesis
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TO MY PARENTS

ABSTRACT

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Whole cells of Rhizobium spp. strains 122 DES, R309, 32H1 and B30 were grown in CS7 and LNB5 for four-ten days. Enzyme activity of glutamine synthetase (GS) (EC6.3.1.2.) was optimum in cultures of 122 DES grown for six days and in the other test strains at seven days. In cells of Rhizobium strains 122 DES and R309, higher levels of GS were observed in cells grown at 28°C than at 20°C whereas strains 32H1 and B30 showed optimum activity at 20°C. The relative states of adenylylation of GS were calculated by determining the ratio of forward activity:transferase activity. Strain R309 showed an average of 14% unadenylylated GS whereas strain B30 showed an average of 36% unadenylylated enzyme. Cells of strain B30 which were deprived of glutamine did not show any observable difference in the amount of unadenylylated enzyme. These preliminary findings indicate that although an increase in total GS activity was observed at six-seven days, the enzyme remained at least partially adenylylated. Due to technical problems it was difficult to determine nitrogenase activity except in a few cases. Nitrogenase activity in strains 122 DES and 32H1 showed a maximum activity at 7d. Ultrastructural studies of cells of strain R309 grown for 2 days on CS7 showed a bacteroid-type of cell with a wide subwall space and convolutions of the plasma membrane. Cells grown on media deprived of molybdenum showed a thick rigid rhizobium-type of cell wall without formation of membrane tubules.

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ABBREVIATIONS

ATase	adenyly transferase
CTAB	cetyltrimethylammoniumbromide
$E_{0}, E_{1} \cdots E_{12}$	average number of adenylyl groups per GS molecule
EDTA	(sodium) ethylenediaminetetraacetic acid
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
PBS	phosphate-buffered saline

INTRODUCTION

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The role of leguminous plants in maintaining the fertility of agricultural soils by enriching the soil with nitrogen was first established by Boussingault in 1838 (Mishustin and Shil'nikova). The association of rhizobia with legumes was first noted by Lachmann (1858) who observed that the root nodules of leguminous plants contain vibrio-like microorganisms. Beijerinck in 1888 isolated a pure culture of nodule bacteria and demonstrated that rhizobia were responsible for the formation of nodules in which atmospheric nitrogen was assimilated. The rhizobia-legume symbiosis is now being investigated with renewed interest as concern over the world protein resources and the ever-increasing cost of the energy-based fertilizer industry necessitate finding a less expensive method of providing nitrogen to increase crop yield.

Until recently, it has been mandatory to use an *in vivo* test system involving plant nodulation for the study of symbiotic nitrogen-fixation. However, in 1975 five papers were published documenting rhizobial cultures which carried out the fixation of nitrogen in the free-living state. It was this development that introduced a valuable tool for investigating the genetic and biochemical prerequisites of symbiotic nitrogen-fixation.

The nitrogen-fixing enzyme complex, nitrogenase (EC1.7.99.2.) has been the focus of biochemical studies concerned with the elucidation of factors involved in the reduction of N_2

to NH_4^+ and the nature of the enzyme complex. More recently attention has been directed to other problems such as the control of synthesis of nitrogenase and other enzymes. Glutamine synthetase (GS) (EC6.3.1.2.), the enzyme catalyzing the synthesis of glutamine from ammonia, is in a key position to regulate the flow of fixed nitrogen into the cell. Moreover, since ammonia is produced from atmospheric nitrogen, GS has been implicated as a control in nitrogen-fixation by regulating the transcription of nitrogenase. A plethora of information has accumulated on the regulatory aspect of GS in controlling the enzymes of ammonia assimilation, including itself. This regulatory scheme involves repression-derepression and an adenylylation-deadenylylation cascade system of the glutamine synthetase enzyme.

The mechanism of nitrogenase has been well investigated but the mechanisms which control its induction have not yet been elucidated. The emphasis of this study has been on the determination of glutamine synthetase activity levels during the period of maximum nitrogenase activity and includes data on nitrogenase and ultrastructural changes. The aim of the study was to acquire further insight into the relationship between glutamine synthetase and nitrogenase and a greater understanding of the nitrogen-fixing process in rhizobia.

HISTORICAL

HISTORICAL

I. Nitrogen fixation by free-living rhizobia

The study of nitrogen fixation in rhizobia had for many years been hampered by the complexity of symbiotic systems. However, through the development of an *in vitro* system, it is now possible to investigate and hopefully elucidate the many aspects of nitrogen fixation in these species previously at an experimental disadvantage.

Biological nitrogen fixation is an important process which contributes about two-thirds of all fixed nitrogen to the biosphere. This process has been the focus of much research over the years due to the ever-increasing cost of the energy-based fertilizer industry, increasing world population and over a ten-fold increase in the use of chemical nitrogen fertilizer since 1950 (Yates, 1976). It is obvious that new ways to supplement or improve the present methods of nitrogen supply are needed and increased understanding of the nitrogen fixation mechanism will eventually help to increase the world resources of protein.

In 1893, Winogradsky isolated the first free-living microorganism, able to fix atmospheric nitrogen. The anaerobic organism, *Clostridium pasteurianum* grew luxuriantly at the expense of nitrogen gas in a culture medium lacking any other nitrogen source. Beijerinck, in 1888, had succeeded in isolating root nodule bacteria from leguminous plants and demonstrated that the nodules were needed for healthy plant development. These classic experiments focused attention on

the crucial role played by microbes in the nitrogen cycle and initiated the study of biological nitrogen fixation (Mishustin and Shil'nikova, 1971).

Organisms known to fix nitrogen belong to the procaryotae and consist of certain bacteria and blue-green algae which harbor the nitrogen fixation (nif) genes. The nitrogen-fixing bacteria are divided into two main groups: those which are free-living and those which fix nitrogen in a symbiotic association with plants. The best known example of the latter association is the genus Rhizobium and the agriculturally important legumes.

Rhizobia are gram-negative aerobic, heterotrophic, non-spore forming organisms which can invade the roots of leguminous plants via an infection thread and form root nodules. Within the root nodules they change to a non-reproductive form called bacteroids and start fixing nitrogen. Both plants and bacteria show a host-strain specificity in this respect. The ability to form nodules is probably the most important characteristic distinguishing rhizobia from other bacterial genera. Until recently, this also meant experimental disadvantages. Progress in the study of the physiology and genetics of rhizobia has proceeded slowly since the studies have necessarily involved the inoculation of legume plants and extraction of the bacteroids from the modules. Until a few years ago it was thought that rhizobia could only fix nitrogen symbiotically i.e. only after transformation into the bacteroid form occurred. However, in 1975 three papers appeared in Nature which documented fixation in free-living rhizobia (Pagan et al., 1975;

Kurz and LaRue, 1975; McComb et al., 1975).

The dogma that rhizobia never fix nitrogen except within the nodule of a plant crumbled in two stages. Initially, the nitrogenase system in rhizobia was shown to occur in association with tissue cultures of leguminous plants (Holsten et al., 1971). It was then demonstrated that non-leguminous plants could induce nitrogenase activity in rhizobia as well (J.J. Child, 1975; Scowcroft and Gibson, 1975). Subsequent to this, conditions were discovered for fixation occurring in the absence of plant material (Pagan et al., 1975). The ability to fix nitrogen ex planta, as it is termed, is principally restricted to the slow-growing class of which the 'cowpea' group is typical. This property displayed by some rhizobia is proof that the genes for nitrogen fixation (nif) are contained in the genetic material of the bacteria and are not derived from the plant cells, as was once thought. Immunological evidence of the capability of free-living Rh. japonicum to synthesize a portion of nitrogenase was provided by Bishop et al. (1975).

Nitrogenase is the name given to the complex that catalyzes biological nitrogen fixation. Knowledge of nitrogenase stems from three advances made between 1960 and 1965. The first breakthrough occurred in 1960 when a cell-free extract of nitrogenase prepared from Clostridium pasteurianum (Carnahan et al., 1960) demonstrated a number of requirements of the nitrogenase complex. The enzyme was found to be oxygen sensitive and anaerobic conditions were required for activity. A source of reducing power and ATP were essential as well as the presence of Mg^{+2} ions and ferredoxin as an

electron carrier (Moustafa and Mortenson, 1967). In Clostridium the source of energy and reducing power was the pyruvate phosphoroclastic system which provided electrons to nitrogenase via ferredoxin (or flavodoxin) and supplied energy by producing acetyl phosphate and hence ATP. Later Bulen et al. (1965), working with Azotobacter and Rhodospirillum spp., substituted sodium dithionite for the reductant and introduced creatine phosphate, creatine kinase, and ADP as an ATP-generating system which provided as assay system for nitrogenase. The third break through came in 1965 when Dilworth in Australia and Schollhorn and Burris in Wisconsin simultaneously discovered that nitrogenase reduced acetylene to ethylene in Clostridium pasteurianum (Dilworth, 1966; Schollhorn and Burris, 1966). Because acetylene reduction takes place not only with enzyme preparations but also with living bacteria, plants or excised nodules, and soil or water samples, it received widespread acceptance as the standard assay for nitrogen fixation (Hardy et al., 1968). Prior to the finding that nitrogenase reduces acetylene to ethylene, the isotopic tracer method was widely accepted as the best criterion for N_2 fixation. Briefly, the method entailed exposing a culture of suspected nitrogen-fixing organisms to $^{15}N_2$ and after adequate incubation, subjecting the culture to Kjeldahl digestion followed by distillation and conversion of the ^{15}N enriched ammonia to $^{15}N_2$. Gas samples were then analyzed in a mass spectrometer (Burris and Wilson, 1957). The isotope technique was introduced in the early 1940s by Burris et al. (1943) and has been used since as a confirmatory test for nitrogen fixation. The tedium of the analysis and expense of $^{15}N_2$ has precluded its

widespread use as a screening method. The acetylene reduction method, on the other hand, is easy to perform, gives rapid results and is relatively inexpensive. The use of gas chromatography to detect the ethylene formed has provided an extremely sensitive method for monitoring nitrogenase activity (Bergersen, 1970).

The capacity of excised nodules from soybean plants to fix atmospheric nitrogen had been demonstrated convincingly in 1952 by use of the $^{15}N_2$ technique (Aprison and Burris, 1952). With the discovery of the acetylene reduction assay, investigating the nitrogen-fixing abilities of bacteroids in soybean root nodules became a less arduous task. About the same time, it was shown that host cell-free extracts of bacteroids could catalyze N_2 reduction and provided the first opportunity for the definition of biochemical events in N_2 fixation by legumes. Requirements for N_2 fixation appeared to be similar to those in Azotobacter vinelandii (Bulen et al., 1965), including the need for an ATP-generating system and sodium dithionite. Bergersen and Turner (1967) and Koch et al. (1967) determined some of the properties of the nitrogenase system in soybean root nodules. The nitrogenase complex appears to be similar in all organisms so far investigated.

Nitrogenase catalyzes the biological reduction of atmospheric nitrogen, which is very stable (valence state of zero) to ammonia (valence state of minus three):

$$N_2 + 6\,H^+ + 6\,e^- \longrightarrow 2\,NH_3$$

The enzyme has been isolated from approximately 20 prokaryotes and extensively purified from four bacteria permitting the components of the nitrogenase complex to be identified. The enzyme consists of two non-haem, iron-sulphur proteins (Bulen and LeComte, 1966; Mortenson et al., 1967). Neither protein alone has any demonstrable activity. The larger protein has a molecular weight of around 220,000 and is composed of four subunits of two types. It contains 20 to 30 iron atoms and acid-labile sulphur groups and two molybdenum atoms per molecule. This larger protein is referred to as the molybdoferredoxin protein (MoFe). The smaller protein has a molecular weight of about 60,000 with two subunits of equal size and four iron atoms and acid-labile sulphur groups per molecule. The smaller protein is referred to as azoferredoxin (Fe)(for reviews see Zumft and Mortenson, 1975; Dalton, 1974; Dalton and Mortenson, 1972). Both proteins are readily inactivated by oxygen but the second smaller protein is more sensitive (Eady et al., 1972; St. John et al., 1974). Careful anaerobic techniques must be used throughout the preparation, purification and assay of nitrogenase components (Burris, 1969). The MoFe and Fe proteins form an equimolar complex during the reaction which is necessary for nitrogenase activity. Hageman and Burris (1978) observed that the two proteins dissociate after each electron is transferred between them and suggest designating the MoFe as nitrogenase and the Fe protein as nitrogenase reductase.

Under physiological conditions the Fe protein is reduced by ferredoxin or a flavoprotein and specifically binds magnesium

adenosine 5'-triphosphate (MgATP) (Bui and Mortenson, 1968). Upon binding the MgATP the potential of the Fe protein becomes substantially more negative and it acquires the unique ability to reduce the MoFe protein (Smith et al., 1973). The reduced MoFe protein serves as an electron sink capable of reducing all of the substrates of nitrogenase.

Nitrogenase is a versatile enzyme, because in addition to dinitrogen and acetylene, it is now well established that nitrogenase will reduce a number of other triple-bonded substrates such as nitrous oxide, azide, cyanide, methyl isocyanide, protons and analogs of some of these compounds (Dalton and Mortenson, 1972). Reduction of these compounds showed the same requirements as did reduction of N_2 and was accompanied by a hydrolysis of ATP and evolution of H_2. Some of the substrates were found to be competitive with N_2, such as N_2O, while others were nonspecific (HCN, N_3). Acetylene is non-competitive with N_2 while N_2 is competitive with acetylene. It has been suggested that acetylene may be reduced by a less completely reduced electron sink than is required by nitrogenase (Rivera-Ortiz and Burris, 1975) because reduction of C_2H_2 is a two electron step, while N_2 reduction requires three two-electron steps. The rate of N_2-fixing activity is therefore about one-third of the acetylene-reducing activity.

Control over nitrogenase is exerted in two ways. The first is a coarse control in which synthesis of the enzyme is repressed by an excess of one of the products of its activity, namely ammonia (or ammonium ions). Ammonia represses the synthesis of nitrogenase

presumably by acting as a corepressor or precursor to a corepressor. The second control system is mediated specifically by the ATP:ADP ratio and affects the activity of existing nitrogenase (Moustafa and Mortenson, 1967; Kennedy, 1970). When the ATP:ADP ratio reaches 0.5, further ATP utilization by nitrogenase and hence nitrogen fixation is completely inhibited. Presumably this type of control is exerted so that when the ATP supply within the cell is low, the remaining ATP can be directed into more important cell functions. Recently, emphasis has been placed on the role of glutamine synthetase as a control of nitrogenase synthesis in a number of organisms including the enterics (Nagatani *et al.*, 1971; Tubb, 1974; Shanmugam *et al.*, 1975) and the rhizobia (Bishop *et al.*, 1976; Ludwig and Signer, 1977). This aspect of nitrogenase control will be dealt with in more detail in the last section.

Dinitrogen fixation by purified nitrogenase or by crude extracts from all N_2-fixing organisms is an anaerobic process. The aerobic N_2-fixers such as *Azotobacter spp.*, some blue-green algae, and the rhizobia-legume symbiosis can reconcile their ability to catalyze this O_2-sensitive process only if it occurs in an internal possibly localized anaerobic environment. In the N_2-fixing tissues of legume root nodules the presence of a myoglobin-like haemoprotein called leghaemoglobin is well known.

Kubo (1939) was the first to show that legume root nodules contained a soluble haemoglobin-type pigment which possessed the property of reversible oxygenation. This property was confirmed by Keilin and Wang (1945) and later by Virtanen and coworkers (1947)

who also established the relationship between leghaemoglobin and nodule effectiveness. Nash and Schulman (1976) confirmed the finding that leghaemoglobin is in a reduced state throughout the life cycle of the nodule.

A number of physiological roles have been proposed to explain the observed correlations between leghaemoglobin concentration and nitrogen fixation (Appleby et al., 1975; Bergersen, 1971). The role which seems most likely was proposed by Yocum in 1964 who suggested that leghaemoglobin may act by facilitating the diffusion of O_2 to the N_2-fixing bacteroids under the low pO_2 existing in the dense tissue. The increased respiration leads to an increase in ATP available for nitrogenase function. In this way leghaemoglobin acts as a sophisticated oxygen transport protein and damage to nitrogenase is avoided.

The slime characteristic of many aerobic nitrogen-fixing bacteria and blue-green algae may also have a protective function in inhibiting the diffusion of oxygen. Pankhurst and Craig (1978) found that cultures of Rhizobium sp. strain 32H1 developed two types of cells in a layer of soft agar. The pleiomorphic type 2 cells of strain 32H1 were present in the middle of the soft agar layer. The type 1 cells were located near the soft agar surface and secreted large amounts of extracellular slime which reduced the rate of O_2 diffusion and may be important for the development of the type 2 cells in the colony.

Nitrogenase, in the absence of reducible substrates, reduces H^+ to H_2 (Hoch et al., 1960). Apparently, H_2 evolution during N_2 fixation is an inherent property of the nitrogenase reaction. It

was suggested by Burns and Hardy (1975) that no net ATP-dependent production of hydrogen via nitrogenase occurs *in vitro*. However Schubert and Evans (1976) found that with most symbionts only 40-60% of the electron flow to nitrogenase was transferred to N_2; the remainder went to H_2 evolution. Energy loss through nitrogenase-dependent H_2 evolution is important because four or five ATP molecules are consumed per pair of electrons transferred regardless of electron acceptor (Hadfield and Bulen, 1969). It appears that some N_2-fixing symbionts possess an hydrogenase which recycles and utilizes H_2 evolved by the nitrogenase system. Dixon (1969) performed experiments with pea nodule bacteroids and determined that H_2 oxidation via the hydrogenase system was coupled to the synthesis of ATP, thus allowing the rhizobia to conserve the energy provided by the plant. Another explanation recently proposed was that oxidation of H_2 results in "respiratory protection" for the oxygen-labile nitrogenase proteins (Emerich *et al.*, 1979). Conditions have been described for hydrogenase expression in free-living cultures of rhizobia (Maier *et al.*, 1978) and this will hopefully be useful in evaluating the importance of the hydrogenase system in the nitrogen-fixing process of legumes.

The capacity for free-living rhizobia to fix nitrogen appears to be dependent upon two factors: the oxygen concentration and the available nutrients. Starting with early studies on nitrogen fixation in the active brei of soybean nodules it was shown that fixation was dependent on the oxygen concentration (Bergersen, 1966). When reports on nitrogen fixation in free-living rhizobia first

appeared (Keister, 1975; Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975; Tjepkema and Evans, 1975), the major determinant of nitrogenase synthesis and activity seemed to be the oxygen concentration. Rhizobia can grow in air without difficulty provided they have a source of fixed nitrogen. However, the rhizobia have a poor respiratory protection mechanism and because their nitrogenase system is extremely oxygen sensitive, they have an oxygen tolerance lower than most other aerobic nitrogen-fixing microorganisms. High specific rates of acetylene reduction can be obtained only by careful adjustment of the oxygen concentration in the gas phase when performing the assay. The acetylene reduction assay permits manipulation of the atmosphere provided the bacteria are grown in a container which can be sealed airtight. Oxygen, acetylene and argon can then be injected in the desired proportions and samples of the atmosphere assayed at a later time for ethylene production.

Pagan *et al.* (1975) were successful in demonstrating detectable levels of nitrogenase activity in *Rhizobium sp.* strain 32H1 grown on solid media (CS7) when the concentration of oxygen in the atmosphere was 20%. Presumably the amount of O_2 within the cells was sufficiently low in order to support nitrogen-fixing activity. Kurz and LaRue (1975) found that cultures of *R. japonicum* 61A76 fixed nitrogen in an atmosphere of 20% O_2 as well. Similar results were reported by Gibson et al. (1976) for strain 32H1. Pankhurst and Craig (1978) examined sections of a soft agar layer

and found that rhizobia growing only in the middle of the soft agar layer contained nitrogenase suggesting that the activity was restricted to zones within which the oxygen concentration was favourable. Moreover the rhizobia, present as a distinct band of cells at a constant depth in the agar, were similar in morphology to the nitrogen-fixing bacteroids formed by strain 32H1 in cowpea root nodules.

Pagan *et al.* (1975) were unable to demonstrate nitrogen-fixing activity in cultures of 32H1 grown in liquid CS7 media , which was attributed to an unfavourable oxygen tension in the liquid culture. Later workers determined the oxygen requirements for optimum nitrogen fixation by free-living rhizobia, and stressed the importance of reduced levels of O_2. Nitrogenase activity developed when cultures of 32H1 were grown at a dissolved oxygen concentration $< 1\mu M$ in either an agitated liquid medium (Keister and Evans, 1976) or in a chemostat (Bergersen *et al.*, 1976). When strain 32H1 was grown in a stationary liquid medium (Evans and Keister, 1976), the optimum oxygen concentration in the atmosphere was $\sim 20\%$. The nitrogenase activity in the stationary cultures was probably restricted to zones where the dissolved oxygen concentration was favourable for the maintenance of continued activity. However, when the stationary cultures were shaken, nitrogen fixation was inhibited by 1% or more oxygen. It is evident that the dissolved O_2 concentration for N_2-fixation of cultures in a liquid media needs to be carefully controlled. Keister and Evans (1976) determined that a concentration of oxygen $< 0.1\%$ was best for the initiation of acetylene

reduction, but the highest specific rate was found at ~0.13% oxygen in the gas phase of liquid cultures. The cell density of liquid cultures was also associated with the oxygen tolerance (Keister, 1975). Keister showed that high specific rates of acetylene reduction could be attained only by carefully balancing a low cell density with a low oxygen concentration. Nitrogenase activity was found to decline rapidly with higher cell densities in agitated liquid cultures, whereas stationary liquid cultures required relatively high cell densities to establish a tolerable oxygen level before nitrogenase activity could be expressed. Reduced levels of oxygen appear to be essential for the development of the nitrogenase system in free-living rhizobia but the exclusion of oxygen inhibits both growth and enzymic activity.

In a number of studies where direct contact between plant cells and rhizobia was not essential for nitrogenase induction, it appeared that the plant cells were providing unknown factors for the induction of N_2-fixation. This prompted researchers to examine several plant metabolites, including selected sugars and citric acid cycle intermediates as possible inducers of nitrogenase activity. The type and concentration of various sugars, sugar alcohols, carboxylic acids and combined nitrogen were shown to have marked effects on the presence, level and duration of nitrogenase activity as did the physical conditions such as oxygen tension and temperature.

A number of defined media were formulated containing a source of fixed nitrogen, other than nitrate, and more than one carbon source in addition to the usual inorganic compounds. Pagan

et al. (1975) found that when arabinose, succinate or glutamine was omitted from their CS7 medium, nitrogenase activity was low or undetectable. They found that fumarate could replace the succinate, and galactose the arabinose in the basic medium. Kurz and LaRue (1975) screened carbohydrates which promoted the callus-Rhizobium association and found xylose, arabinose and galactose preferred sugars. Any of these sugars plus sucrose would induce nitrogenase activity in the LNB5 medium but not if only one carbon source was available.

Bergersen and Turner (1967) reported that in washed suspensions of bacteroids, N_2-fixation was stimulated by oxidizable substrates including succinate, fumarate, and pyruvate. Gibson et al. (1976) examined the effects of a number of citric acid cycle intermediates and found that without carboxylic acids in the CS7 medium, nitrogenase activity was low or undetectable unless a high level of arabinose was provided.

Kurz and LaRue (1975) found that neither growth nor acetylene reduction was observed when the Rhizobia were subcultured on nitrogen-free media. The LNB5 medium contained fixed nitrogen in the form of potassium nitrate and ammonium sulphate (1mM). The levels of fixation were not significantly changed in the presence of L-methionine sulphone, which can interfere with NH_3 repression of nitrogenase. Evans and Keister (1976) found that a combined nitrogen source, such as yeast extract or casamino acids, was required for effective nitrogenase activity in Rhizobium sp. 32H1 but could be replaced by glutamate and glutamine. Pagan et al. (1975) found

that if glutamine was replaced by ammonium sulphate (1mM) in the CS7 medium nitrogenase activity was still evident but the level of activity was reduced. Higher concentrations of fixed nitrogen, such as 10mM ammonium sulphate (Kurz and LaRue, 1975) and 10mM ammonium chloride (Evans and Keister, 1976), did however inhibit N_2-fixation.

The requirement for fixed nitrogen has not been established yet although it is possible that small amounts of fixed nitrogen are needed for the induction of nitrogenase synthesis. Fixed nitrogen, which inhibits nitrogenase in many organisms, indicates that the controls on nitrogenase in rhizobia are not the same as those in other nitrogen-fixing bacteria. Part of the symbiotic condition may require the fixed nitrogen.

Diversity in colonial morphology of slime-producing nitrogen-fixing microorganisms has been recognized for some time. Jensen *et al.* (1960) first described the tendency of *Derxia gummosa* to form either massive or small colonies on nitrogen-free agar. Hill (1971) determined that only the massive colonies contained active nitrogenase which appeared to be associated with the oxygen concentration. On nitrogen-free media the small colony types of *D. gummosa* were observed when grown in air. However, only massive colony types were present when grown in less than 0.20 atm O_2. Variations in the colony morphology of *Rhizobium spp.* have also been recognized for some time and in an early review, Allen and Allen (1950) suggested a correlation between the colony type and the ability to fix nitrogen. Herridge and Roughley (1975) described isolates of a *Rhizobium sp.* which formed large mucoid colonies and were inefficient while others gave

rise to small dry efficient colonies in the host plant. More recently several workers have reported the isolation of colonial variants of rhizobia and investigated a number of differences between these variants (Kuykendall and Elkan, 1976; Mulongoy and Elkan, 1977; Upchurch and Elkan, 1977; Upchurch and Elkan, 1978).

Kuykendall and Elkan (1976) used a nonionic detergent to isolate four derivatives from a pure strain of R. japonicum 110 on the basis of colony morphology. These derivatives are not detectable with standard pure culture techniques due to cell adherence thus genetically mixed colonies often result. The nitrogen-fixing efficiency of the two small non-slimy clones was 5- to 10- fold more efficient than the larger clones. Characterization of these derivatives also disclosed a clear difference in the ability to utilize carbohydrates (Kuykendall and Elkan, 1976; Mulongoy and Elkan, 1977). Upchurch and Elkan (1977) investigated the same clones and found that the small non-slimy derivatives were sensitive to salt with depressed growth rates and were significantly more effective in both symbiotic and free-living nitrogen-fixation. The large slimy derivatives were relatively unaffected by salt. Upchurch and Elkan (1978) investigated the fate of fixed nitrogen in small and large derivatives of two R. japonicum strains. The small efficient nitrogen-fixing derivatives assimilated less fixed nitrogen by excreting more ammonia under nitrogen-fixing conditions. These findings support the idea that rhizobia can simultaneously derepress nitrogenase while repressing the ammonia assimilatory

enzymes. In symbiotic nitrogen-fixation a mechanism such as this would function to benefit the plant.

As the single major supplier of biologically fixed nitrogen, the legume-Rhizobium symbiosis is of paramount importance in designing strategies for the achievement of higher and more efficient rates of nitrogen fixation. The recent introduction of methodology for the fixation of nitrogen by rhizobia in the free-living state has paved the way towards intensive investigations now underway on the nitrogenase system unhampered by the complex association of the host plant.

II. Cellular Regulation of Glutamine Synthetase Activity

The role of glutamine is of central importance in the nitrogen metabolism of microorganisms because of its involvement in the synthesis of compounds vital to the cell. The amide group of glutamine is the preferred source of nitrogen for the biosynthesis of at least six different compounds; tryptophan, adenylic acid, cytidylic acid, glucosamine-6-P, carbamyl phosphate and histidine (Woolfolk et al, 1966; Stadtman et al, 1967). In addition, the α-amino group of glutamine can serve as a source of nitrogen for the synthesis of various amino acids via specific transaminases some microorganisms produce. The transaminase catalyzes transfer of the α-amino group of glutamine to various α-ketoacids. Thus, the synthesis of glutamine may be regarded as the first step in a highly branched pathway leading ultimately to the biosynthesis of a large number of various compounds.

Glutamine is synthesized in a reaction catalyzed by the enzyme glutamine synthetase (GS) (Speck, 1949; Elliott, 1948; Elliott, 1951).

$$\text{L-Glutamate} + \text{ATP} + NH_4^+ \xrightarrow{Mg^{2+}\ \ Mn^{2+}} \text{L-Glutamine} + \text{ADP} + \text{Pi}$$

Glutamine(amide): α-ketoglutarate amino transferase oxido-reductase (NADP)(GOGAT)(glutamate synthase) completes the pathway by

converting α-ketoglutarate to glutamate (Wulff et al, 1967).

$$\text{L-Glutamine} + {}^{\alpha}\text{-Ketoglutarate} + \text{NADPH} + H^{+} \longrightarrow 2\text{L-Glutamate} + NADP^{+}$$

Ammonia evolved through dinitrogen fixation becomes assimilated into cellular nitrogen via the combined action of glutamine synthetase and glutamate synthase. Another enzyme, glutamate dehydrogenase (GDH) is also responsible for the assimilation of ammonia into cellular material. GDH, however, has a poor affinity for ammonia i.e. a high Km, and is of no use at low ammonia concentrations (Brenchley and Magasanik, 1974). At high ammonia concentrations, GDH catalyzes the formation of glutamate (Meers et al, 1970):

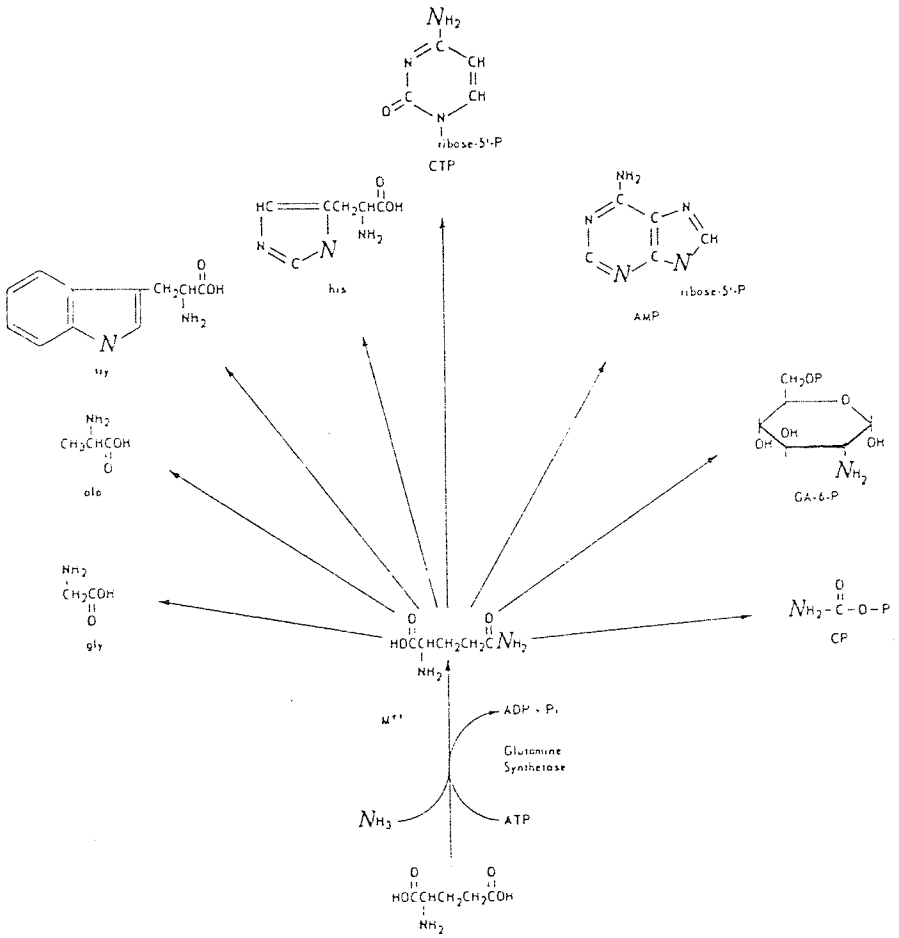
$$NH_3 + \alpha\text{-Ketoglutarate} + \text{NADPH} + H^{+} \longrightarrow$$
$$\longrightarrow \text{L-glutamate} + NADP^{+} + H_2O$$

Below 1mM NH_4^{+} , GS plays an essential role in ammonia assimilation. Due to the unfavorable equilibrium for glutamate formation, it seems evident that GS should occupy a strategic role in nitrogen metabolism and, from the standpoint of cellular regulation, be an effective target for metabolite control.

Bacterial glutamine synthetase is subject to rigorous control by at least four mechanisms: (1) repression and derepression of enzyme synthesis (2) cumulative feedback inhibition by multiple end-products of glutamine metabolism (3) enzyme catalyzed attachment of 5′-AMP groups to specific tyrosine residues, effecting modulation of GS activity and alterations in divalent cation specificity and in feedback inhibitor responses, and (4) modulation of

GS activity by variations in the ratios of ATP, Mn^{24} and other nucleotide di-and tri-phosphates (Stadtman et al, 1967; Shapiro and Stadtman, 1970).

Figure 1.



Biosynthesis and metabolic fates of glutamine. M^{++} is a divalent cation, especially Mg^{++} or Mn^{++}; CP, carbamyl phosphate; GA-6-P, glucosamine-6-phosphate.

(from Stadtman et al.,1967)

It appears that the adenylylating-deadenylylating system as well as

the transcriptional regulating mechanism are the main mechanisms controlling the function of glutamine synthetase.

It has been established that the synthesis of GS is dependent upon the nitrogen concentration of the culture medium. Nitrogen-rich media, especially with high concentrations of ammonium salts, decrease the level of GS activity in enteric bacteria (Woolfolk et al., 1966; Mecke and Holzer, 1966). This is not surprising in view of the fact that, at high concentrations, ammonium ion will replace glutamine as the nitrogen donor in a great many biosynthetic reactions (Meister, 1962). The composition of the medium determines not only the activity but also the intracellular level of glutamine synthetase. Cells growing in a medium containing a carbon source and a growth-rate limiting concentration of ammonia produce the highest levels of the enzyme. Cells growing in a medium with an excess of ammonia show a 6-10-fold reduction in amount of enzyme synthesized (Prival et al, 1973). This fluctuation in the levels of GS activity is the result of regulation at the point of transcription of GS synthesis which reflects the intracellular concentration of messenger RNA (m-RNA) complementary to DNA for the structural gene (gln-A) of GS (Streicher et al., 1975; Magasanik et al., 1974; Tyler et al., 1974). In organisms devoid of GS, the gln A m-RNA is undetectable suggesting that transcription of gln A is not regulated simply by repression via the GS protein. Rather, GS itself may regulate the transcription of its structural gene (Weglenski and Tyler, 1977). There is strong evidence indicating that GS also regulates transcription of a number of other genes, all involved in

nitrogen assimilation (Streicher et al., 1974; Magasanik et al., 1974).

Autoregulation by GS was first proposed by Magasanik and coworkers (1974) as a result of investigations of glutamine-requiring mutants of Klebsiella aerogenes. Such mutants contain no or very little GS and have mutations at three unlinked sites on the chromosome gln A, gln B, and gln D. Mutations in gln A and in gln E, a site linked to gln B restore the ability to produce GS to a mutant in gln B (Prival et al., 1973; Streicher et al., 1975; Foor et al., 1975). On the other hand, organisms with a mutation mapping within the structural gene for GS (gln A) can also have a high constitutive level of GS (Deleo and Magasanik, 1975; Streicher et al., 1975). It would appear that the mutation in the chromosomal gln A gene has altered the GS so that it fails to repress GS while retaining enzymatic activity. Normal GS which can be replaced by the episomal gln A^+ gene will restore repression.

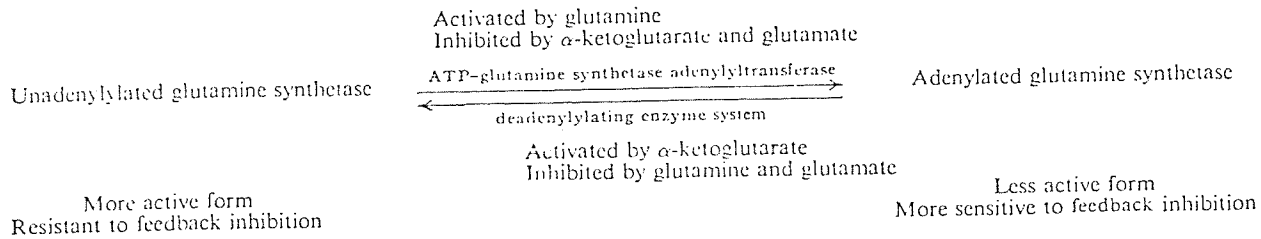
It is also possible that the synthesis of GS is activated by unadenylylated GS and repressed by adenylylated GS. Lesions in the structural genes for proteins involved in the GS adenylylation system can alter the rate of production of GS in an inverse manner to their effects on the state of adenylylation of the enzyme (Foor et al., 1975; Magasanik et al., 1974).

As previously mentioned, GS is subject to rigorous control by a number of mechanisms. In addition to transcriptional control, a control involving the adenylylation or deadenylylation of selected tyrosine residues on the enzyme exists. This leads to

the adenylylated inactive form or the deadenylylated active form of GS. Intermediary forms of partially adenylylated GS also exist (Shapiro and Stadtman, 1970).

Shapiro investigated the requirements of the deadenylylating reaction (Shapiro, 1969). Glutamine had previously been shown to stimulate the adenylylating process (Kingdon et al., 1967).

Figure 2.



(Shapiro, 1969)

The adenylylated form predominates when the bacteria are grown under conditions of ammonia excess and conversely the deadenylylated form predominates when the supply of nitrogen is limited.

GS catalyzes the biosynthetic (forward) reaction involving L-glutamate and ammonia to form L-glutamine (equation 1) but it can also catalyze a transferase reaction involving L-glutamine and hydroxylamine to form γ-glutamylhydroxamate (equation 2)(Shapiro and Stadtman, 1970b).

(1) L-glutamate + ATP + NH_4^+ $\xrightarrow{M^{2+}}$ L-glutamine + ADP + Pi

$$(2)\quad \text{L-glutamine} + NH_2OH \xrightarrow[\text{ADP}]{M^{2+}} \gamma\text{-glutamylhydroxamate} + NH_4^+$$
arsenate

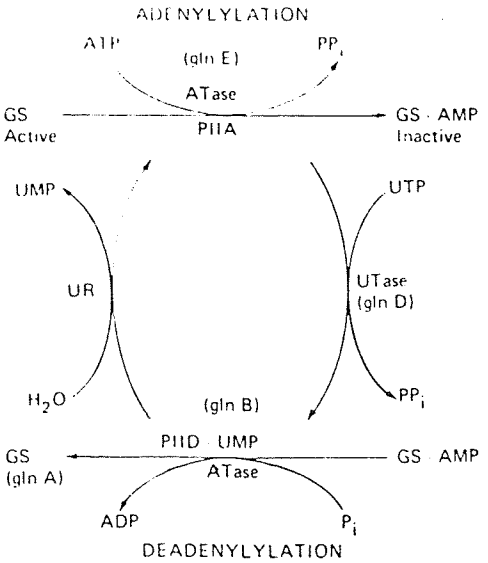
The adenylylated form of GS can only catalyze the forward reaction in the presence of Mn^{2+}. The deadenylylated form requires either Mg^{2+}, Co^{2+}, or CA^{2+}, but cannot utilize Mn^{2+}. The transferase activities of both forms of GS are activated by Mn^{2+}, whereas Mg^{2+} is able to support the transferase activity of only the deadenylylated enzyme. This provides a tool for assaying the two forms of glutamine synthetase in that the transferase assay in the presence of Mn^{2+} can be used to assay total GS activity, whereas the forward assay in the presence of Mg^{2+} measures GS activity due to the deadenylylated form.

Woolfolk and coworkers purified glutamine synthetase from Escherichia coli in 1966. It was in the deadenylylated form and at that time it was not known that two forms existed (Woolfolk et al., 1966). The structure of GS from E.coli was unambiguously disclosed in electronmicrographs (Valentine et al., 1968) which showed that the enzyme consisted of 12 discrete subunits arranged in two hexagonal rings superimposed on one another. The subunit molecular weight was calculated to be 46,000 while the enzyme had a total mol wt of 592,000 as determined by sedimentation equilibrium (Shapiro and Ginsburg, 1968). When purified the enzyme had variable amounts of Mn^{2+} associated with it which stabilized the native dodecameric structure, protecting it from reacting with sulfhydryl reagents and other mild denaturants (Shapiro and Stadtman, 1967). As well,

preparations of purified GS differed from one another in the amount of covalently bound AMP residues they contained. The content of AMP varied from 0 to 12 equivalents per mole i.e. a maximum of 1 ATP to a tyrosine residue on each of the subunits (Shapiro and Stadtman, 1970).

The inactivation of GS by adenylylation requires ATP and two proteins called adenylyltransferase (ATase) and P_{II}. These proteins are also required for the removal of the adenylyl groups which then restores biosynthetic function to the glutamine synthetase. The decision to adenylylate or deadenylylate depends on the form of P_{II}. Uridylylated P_{II} (P_{IID}) activates deadenylylation while removal of uridylyl groups from P_{IID} (P_{IIA}) activates adenylylation. Uridylyltransferase (UTase) catalyzes the uridylylation of P_{II} while a different enzyme is responsible for removal of the uridylyl groups (Kingdon et al., 1967; Shapiro, 1969).

Figure 3.



Adenylylation and deadenylylation of glutamine synthetase, adapted from Ginsburg and Stadtman, GS, glutamine synthetase (EC 6.3.1.2); ATase, adenylylation enzyme; UTase, uridylyl-removing enzyme; gln, gene controlling production of glutamine synthetase.

(Magasanik, 1977)

Shapiro et al. (1967) determined that the growth conditions played a decisive role in determining the catalytic properties of GS. The adenylyltransferase was found to be activated by glutamine and inhibited by α-ketoglutarate, UTP, CTP, and other nucleotide derivatives, whereas these compounds had opposite effects on the deadenylylating enzyme. Consequently, the state of adenylylation in vivo is determined by the intracellular levels of these effectors. High concentrations of ammonium salts in the growth medium will favor the conversion of α-ketoglutarate to glutamate resulting in an increase in the glutamine level. Subsequently, stimulation of adenylyltransferase .P_{IIA} occurs and results in the conversion of unadenylylated GS to the adenylylated form. However, low concentrations of ammonium salts favor the accumulation of α-ketoglutarate and depletion of glutamine resulting in activation of the deadenylylating enzyme and conversion to the active form of GS (Shapiro and Stadtman, 1970b).

The activity of glutamine synthetase in gram-negative bacteria is inversely proportional to the state of adenylylation (Kingdon et al., 1967) which can be estimated by a number of methods. As previously mentioned, it is possible to measure the total catalytic activity of adenylylated and unadenylylated GS using the transferase assay. The unadenylylated enzyme itself is measured using the forward assay. This permits the number of adenylylated subunits per mole, n, to be calculated from the ratio of activity in the transferase assay: forward assay.

$$n = \frac{\text{forward activity}}{\text{transferase activity}} \times 12$$

This method of determining the E_n value (state of adenylylation) has the advantage that only catalytic amounts of enzyme are required and the enzyme need not be pure, so long as interfering activities are absent (Shapiro and Stadtman, 1970b). Since GS will bind up to 12 equivalents of adenyly groups per mole, it can exist in at least 12 different forms. Reference to enzyme preparations having different average states of adenylylation are designated E (enzyme) and followed by a subscript $\bar{0}$, $\bar{1}$, $\bar{2}$ ..... $\overline{12}$, to denote the average number of adenylyl groups per 600,000 mol wt. This designation does not imply that all molecules of GS present have the same number of AMP groups attached to them but rather, it pertains to the average number of adenylyl groups per mole. The term, E_0, E_1,.....E_{12} (without bar over subscript) is reserved for the designation of apparently homogeneous preparations in which each enzyme molecule has the subscript number of adenylyl groups attached to it.

The state of adenylylation of GS can also be estimated using a spectrophotometric assay as well, but this is only possible for a purified preparation. For the unadenylylated enzyme (E_0) the ratio of absorbancy at 260 nm to that at 290 nm is 0.89. The binding of AMP to the enzyme subunits increases the absorbancy at 260 nm but has no significant effect at 290 nm. Since the molar absorbancy indices of bound and free AMP are the same, the increase in absorbancy of the enzyme at 260 nm is directly proportional to the number of

equivalents of AMP bound. The theoretical relationship between the average number of equivalents of AMP bound per mole of enzyme and the ratio of absorbancies is as follows (Shapiro and Stadtman, 1970b):

$$\text{Moles AMP/ mole enzyme} = (15.3)\ \frac{A_{260nm}}{A_{290nm}} - 13.6$$

Recently Stadtman *et al.* (1979) examined six different enzymic procedures for determining the average state of adenylylation. All methods depended on measurements of the γ-glutamyltransferase activity in assay mixtures containing Mn^{2+} at a pH where adenylylated subunits are equally active and under conditions where only unadenylylated subunits are active. An evaluation of the reliability of the methods resulted in reproducability of the $\bar{n}$ values to within ± 1 unit ($\pm$ 8%). The validity of all methods, however, depends upon strict adherence to the prescribed conditions and the determination of the unique isoactivity pH for the assay system. Activity measurements at the unique cross-over point (isoactivity point) is a measure of the total enzyme concentration, independent of the state of adenylylation.

III. Regulation by Glutamine Synthetase

Glutamine synthetase (GS) is in a key position to modulate the overall flow of fixed nitrogen (ammonium ion) to organic nitrogen in the N_2-fixing cell. Since both the amount and form of glutamine synthetase change in response to the ammonia concentration of the medium, it has been suggested that GS is responsible not only for glutamine biosynthesis but for the assimilation of nitrogen into glutamate through the GDH and GS-GOGAT pathways. This regulation is effected by GS interaction with DNA which activates the transcription of certain genes (Tyler *et al.*, 1974). A number of workers have also suggested GS to be an activator of the histidase and nitrogenase operons which are involved in the utilization of certain nitrogenous compounds (Prival *et al.*, 1973; Magasanik *et al.*, 1974; Streicher *et al.*, 1974; Tubb, 1974). Most of the evidence supporting this idea has accumulated from work on the enterics and has indicated that in *E. coli* and *Klebsiella*, glutamine synthetase regulates nitrogen assimilation through a cascade effect identical to the one responsible for the regulation of the enzymatic activity of GS already described in the previous section. An essential step in the utilization of ammonia by bacteria is the incorporation of ammonia into glutamic acid. Glutamic acid not only provides a portion of the carbon skeleton for the amino acids of the "glutamic acid family", but also the amino groups of several amino acids via transamination as well as some of the nitrogen atoms of purine and pyrimidine nucleotides through reactions with glutamine.

The assimilation of ammonia into glutamate by bacteria proceeds via either glutamate dehydrogenase (GDH) or the glutamine synthetase/glutamate synthase (GS-GOGAT) pathway, depending upon the organism and ammonia concentration of the environment (Tempest et al., 1973).

The study of mutants in Klebsiella aerogenes has revealed the role of glutamine synthetase as a regulator of the synthesis of enzymes responsible for the formation of glutamate (Brenchley et al., 1973). Previous work on E. coli strains unable to catabolize glutamate suggested that GDH was not involved in the major pathway of glutamate metabolism (Marcus and Halpern, 1969). Thus it appears that most of the nitrogen is assimilated via the GS-GOGAT route. In addition, unlike GDH the GS-GOGAT pathway can function under conditions of ammonia limitation. It was found that strains lacking GDH activity grow as well as wild-type strains on media containing both high and low concentrations of ammonia or other nitrogen sources (Brenchley and Magasanik, 1974; Pahel et al., 1978). Brenchley et al. (1973) demonstrated that loss of the GS-GOGAT pathway resulted in a requirement of glutamate for growth. Apparently the role of GDH in ammonia assimilation can be completely replaced by the GS-GOGAT pathway (for review see Tyler, 1978).

In nitrogen-fixation of detached soybean nodules Bergersen (1965) demonstrated that ammonia was the primary product and it was later shown that nearly all of the fixed ammonia was found outside the bacteroid (Bergersen and Turner, 1967). Subsequently the

enzymes of ammonia assimilation, glutamate synthase (Nagatani et al., 1971; Robertson et al., 1975), glutamate dehydrogenase (Grimes and Fottrell, 1966) and glutamine synthetase (Kennedy, 1973; Dunn and Klucus, 1973) were found in both plant and bacteroid fractions. Extensive studies have been made on the levels of glutamine synthetase, glutamate synthase and glutamate dehydrogenase in nitrogen-fixing bacteroids. The enzyme levels varied with the strains tested and the cultural conditions.

The finding that the major part of glutamine synthetase activity was present in the plant fraction of the nodule (Dunn and Klucus, 1973) whereas the GS activities were low in bacteroids examined by Brown and Dilworth (1975) indicates that bacteroids are not nitrogen limited. The high level of nitrogen fixation by the acetylene reduction rates of the intact nodule was insufficient to account for the GS present in the bacteroid. Brown and Dilworth (1975) suggested that ammonia produced in the nodule was more likely to be assimilated by plant enzymes since no evidence existed for the GS-GOGAT system in root nodules. Similarly according to McParland et al. (1976) the major portion of GS activity was associated with the cytosol rather than with the bacteroids which would support the idea that assimilation occurs outside the bacteroid. Purified glutamine synthetase from the cytosol of soya-bean root nodules confirmed that the enzyme was of plant origin (McParland et al., 1976). The function of the ammonia-assimilatory enzymes of the bacteroids may be to maintain an adequate supply of amino

acids for bacteroid proteins, or they may be constitutively synthesized. Scott et al. (1976) suggested that the excreted ammonia could be converted to asparagine by a co-operative system comprising glutamine synthetase, glutamate synthase and asparagine synthetase.

In bacteroids from pea (Pisum sativum) and bean (Vicia faba) a similar pattern was observed with the glutamate dehydrogenase and glutamate synthase enzymes. The activity of these enzymes was much lower than the glutamine synthetase and nitrogenase indicating that neither pathway incorporated ammonia (Kurz et al., 1975). Low activities of GS, GOGAT and GDH were also shown in sonicated bacteroid preparations although a high activity of GS was found in the nodule supernatants indicating that the GS-GOGAT pathway was inoperative in root nodules and ammonia is probably assimilated through enzymes of the plant system (Brown and Dilworth, 1975). In a study on ammonia assimilation in R. japonicum, Fottrell and Mooney (1969) found that GDH was present in cultures grown on glutamate or aspartate but that the GS activity was a 100- fold greater when grown on glutamate. In bacteroids of R. japonicum Bishop et al. (1976) showed that the GS was not influenced by ammonia even under conditions where nitrogenase activity was inhibited. Kondorosi et al. (1977) developed a GOGAT-deficient mutant which was unable to assimilate ammonia but was fully effective. A number of workers demonstrated low levels of the enzymes of ammonia assimilation in bacteroids (Brown and Dilworth, 1975; Robertson et al., 1975; Kurz et al., 1975). This evidence supports the view that the bacterial

GS-GOGAT system is not important in the transportation of fixed nitrogen in the nodule.

From the accumulated evidence plant enzymes appear to be a major factor in symbiotic nitrogen fixation. The assimilatory enzymes in bacteroids may promote the excretion of fixed nitrogen which can then be assimilated and utilized by the plant.

In free-living rhizobia, the enzymes involved in ammonia assimilation have been investigated to determine their role in the regulation of nitrogen metabolism free from factors associated with the plant-bacteria symbiosis. The enzyme levels of GS, GOGAT and GDH in free-living rhizobia did not correspond to the enzyme activities obtained in bacteroids (Brown and Dilworth, 1975; Planqué *et al.*, 1977) indicating an altered response to varying ammonia concentrations. This may have important implications since GS has both an assimilatory and regulatory role in nitrogen metabolism (Streicher *et al.*, 1974). Brown and Dilworth (1975) also found that the levels of GS, GOGAT and GDH appeared to be controlled by the NH_4^+ concentration of the culture medium. However, this observation was made without testing for *in vitro* nitrogen fixation. O'Gara and Shanmugam (1976) found that the majority of fixed nitrogen in free-living nitrogen-fixing cultures of rhizobia was exported into the cell supernatant, analogous to the situation in bacteroids. They presented evidence that the export of NH_4^+ may be closely linked to the control of the ammonia assimilatory enzymes. L-Glutamate repressed glutamate synthase whereas NH_4^+ was found to repress

glutamine synthetase. O'Gara and Shanmugam (1976) hypothesized that the export of excess nitrogen fixed by free-living rhizobia was accomplished by simultaneously derepressing their Nif genes while repressing genes for NH_4^+ assimilation.

In free-living nitrogen-fixing R. meliloti Kondorosi et al. (1977) showed that ammonia was assimilated via the GS-GOGAT pathway and no GDH could be detected. The level of GOGAT activity was approximately the same on various nitrogen sources tested. However the level of glutamine synthetase in the cells varied with the type of nitrogen supplied and even at high ammonia concentrations GS was still able to provide the bacteria with assimilated ammonia. These results contrasted those of Magasanik et al. (1974) who found that under similar conditions with Klebsiella, the GS-GOGAT pathway was repressed and ammonia assimilation proceeded via GDH. Kondorosi et al. (1977) suggested GS to be a controlling element in the nitrogen metabolism of R. meliloti even though they did not find evidence for an adenylylation-deadenylylation system of GS in these bacteria. Ammonium assimilation in Rhizobium sp. 32H1 was investigated and the GDH pathway was found to be inoperative (Ludwig, 1978) but the GS-GOGAT pathway operated at a slow rate under strict negative control by ammonium itself. The glutamine synthetase activity was modulated by both repression-derepression and reversible adenylylation indicating that strain 32H1 is an efficient nitrogen-fixing strain which restricts its own ammonium assimilation in order to maximize the output of fixed nitrogen to the host plant during symbiosis.

Ammonium assimilation in rhizobia appears to differ

significantly from that of enteric bacteria in a number of respects. GDH does not have a role in assimilating ammonia; it is a strictly catabolic enzyme. Rhizobial GS and GOGAT exhibit low catalytic activities under all growth conditions, however, GS total activity is present at high levels suggesting that rhizobial GS has a low turnover number relative to that of enteric bacteria. Higher ammonium concentrations are required to repress and adenylylate enteric bacterial GS as compared to rhizobial GS (Ludwig, 1978).

Wild-type cultures of K. pneumoniae excrete only traces of NH_4^+ into their environment during growth in contrast to the nodule bacteria. Genetic manipulation of K. pneumoniae can cause a loss of control and excretion of NH_4^+ into the medium by interrupting the flow of NH_4^+ via glutamate into cell material, without altering NH_4^+ production (nitrogenase activity) (Shanmugam and Valentine, 1975). Shanmugam and Valentine produced a GOGAT-deficient mutant of K. pneumoniae which after induction of nitrogenase initially produced a small amount of ammonium ion. Because GOGAT was missing and GDH repressed at low NH_4^+ concentrations, the conversion of NH_3 to glutamate was blocked and the NH_4^+ produced was transported into the medium. As the level of NH_4^+ reached approx. 0.1 mM, GDH activity was expressed providing a second pathway for reutilization of NH_4^+ abolishing glutamate auxotrophy. The accumulation of NH_4^+ in the medium, resulted in the synthesis of nitrogenase being repressed. Such a scheme is plausible in bacteroids as previous results demonstrate the GDH pathway is inoperative.

In enterics NH_4^+ is assimilated via two pathways tightly

regulated by the NH_4^+ concentration of the medium (Tempest et al., 1970; Nagatani et al., 1971). Glutamine synthetase, the key enzyme in the GS-GOGAT pathway was found to be repressed by a high NH_4^+ concentration. A correlation between the levels of histidase and GS in K. aerogenes under NH_4^+ limitation was reported by Prival and Magasanik (1971) and Prival et al. (1973). Mutant strains deficient in GS were unable to derepress histidase activity. A similar relationship between nitrogenase activity and GS in K. pneumoniae was shown by Tubb (1974). Further investigations (Streicher et al., 1974; Shanmugam et al., 1975) led to the proposal that the non-adenylylated form of glutamine synthetase could induce nitrogenase synthesis and repression of GS or its conversion into the adenylylated form caused repression of nitrogenase synthesis. Shanmugam and Valentine (1975) also implicated GS as a control in nitrogenase synthesis in K. pneumoniae.

An understanding of the control of nitrogenase synthesis in rhizobia is also under study. The experimental approach has been to study NH_4^+ repression of nitrogenase and to try and ascertain the relationship between nitrogenase and glutamine synthetase (Tubb, 1976; Scowcroft et al., 1976; Bishop et al., 1976).

A source of combined nitrogen which normally inhibits nitrogen-fixation in free-living bacteria is essential for effective nitrogenase synthesis of rhizobia grown in culture (Gibson et al., 1976). Repression of the nitrogenase in active culture was studied by inducing the enzyme and then transferring the culture to a medium containing various nitrogen compounds or inhibitors of the

assimilatory enzymes (Scowcroft et al., 1976). Ammonia, glutamine and nitrate all inhibited nitrogenase activity and the effect of the ammonia was immediate and dependent on the ammonium ion concentration. Methionine sulphoximine and methionine sulphone, inhibitors of the GS and GOGAT enzymes which will allow derepression of nitrogenase synthesis in K. pneumoniae and A. vinelandii in the presence of NH_4^+ (Gordon and Brill, 1974), did not prevent NH_4^+ inhibition of nitrogenase activity in nitrogen-fixing cultures of strain 32H1 (Scowcroft et al., 1976).

Glutamine synthetase activity in cultures of 32H1 showing inhibition of nitrogenase activity by NH_4^+ showed no change in total GS activity in either the presence or absence of Mg^{2+}. Glutamate dehydrogenase was also not detected in the cultures. The failure to observe a change in the state of adenylylation of GS, the absence of GDH, the nature of the inhibition by NH_4^+ and the inability to alleviate the inhibition with methionine sulphoximine and methionine sulphone led Scowcroft et al. (1976) to conclude that control of nitrogenase synthesis and activity in strain 32H1 was not effected through the regulation of glutamine synthetase in contrast to that observed in the wild-type K. pneumoniae.

The role of adenylylated glutamine synthetase in the regulation of nitrogenase activity was investigated in the cowpea Rhizobium sp. CB756 (Bergersen and Turner, 1976). Oxygen-limited continuous cultures of this strain showed high levels of nitrogenase activity which were not significantly affected by excess ammonium ions or glutamine. However, when the O_2- limitation was

relieved, the apparent repression of nitrogenase synthesis occurred when NH_4^+ was in excess. The steady state concentration of dissolved O_2 remained low and an increase in adenylylation of GS was observed. Bergersen and Turner (1976) suggested that the restricted supply of ATP during O_2-limited growth interferes with adenylylation of GS thus preventing repression of nitrogenase in the presence of excess ammonium ions.

On the basis that nitrogenase activity in free-living rhizobia is dependent on a favourable oxygen supply, Scowcroft et al. (1976) proposed that NH_4^+ and glutamine may alter the O_2 demand of the entire culture so that the supply is not adequate for nitrogenase activity. The results showed that nitrogenase activity which was inhibited by NH_4^+ in strain 32H1 could be relieved by increasing the O_2 tension.

The activity and extent of adenylylation of glutamine synthetase in both free-living and bacteroid forms of R. japonicum were examined in the presence of excess ammonia (Bishop et al., 1976). The addition of excess NH_4^+ to the medium resulted in repression and adenylylation of GS in the free-living bacteria while in bacteroid preparations GS was not consistently influenced by the addition of NH_4^+. This information as well as the observation that normal levels of GS in legume bacteroids were found to be relatively low (5-20%) compared to levels in nitrogen-starved free-living R. japonicum (Brown and Dilworth, 1975; Dunn and Klucus, 1973), indicates that bacteroids may have lost their capability to regulate GS via the adenylylation-deadenylylation cascade control system.

A number of reports began to appear which supported the involvement of GS in the regulation of nitrogenase activity in cultured rhizobia and opened up a controversy in this area. Streicher and coworkers (1974) first speculated that the regulatory system for nitrogen-fixation by the Rhizobium-legume symbiosis may be similar to that in Klebsiella. Tubb (1974) reported an "ammonium effect" on nitrogenase activity in Rhizobium sp. 32H1 due to growth on NH_4^+ as sole nitrogen source but not complete repression of the enzyme. Glutamate-initiated growth was observed to result in an excretion of NH_4^+ and it was suggested that the GS-GOGAT pathway may be responsible for establishing a symbiotic infection. However, not until 1977 was more convincing evidence offered in support of glutamine synthetase as a controlling element in the nitrogen metabolism of Rhizobium spp. Ludwig and Signer (1977) isolated GS auxotrophs of strain 32H1 and showed that mutants deficient in GS activity were deficient in nitrogenase activity as well. A glutamine-requiring derivative of the 'fast-growing' species R. meliloti was isolated by other workers which yielded ineffective nodules on Medicago sativa (Kondorosi et al., 1977). Their results indicate that GS plays a role in the regulation of nitrogenase activity in both free-living Rhizobium and bacteroids, although the mechanism is still unclear.

Two forms of GS, differing in their isoelectric points, were isolated from cell-free extracts of R. japonicum (Darrow and Knotts, 1977). It was suggested that the possession of two forms of GS may confer a physiological advantage to rhizobia when entering

a symbiotic relationship. Ludwig (1978) found that strain 32H1 possessed no alternative to the GS-GOGAT pathway and restricted its own ammonium assimilation in order to maximize the contribution of fixed nitrogen to the host plant. A closely balanced system between NH_4^+ production and utilization is obviously not operational in agronomically important nitrogen-fixing organisms such as the symbiotic root-nodule bacteria since these microorganisms are obligated to produce NH_4^+ for plant growth.

Thus the controls of ammonium assimilation in cultures of rhizobia appear to be more stringent, indicating a reduced ability to incorporate fixed nitrogen into cellular material. This occurs at a great expense to the bacteria suggesting that rhizobia are committed to the evolutionary benefits of symbiosis.

MATERIALS AND METHODS

MATERIALS AND METHODS

Bacterial Strains

The following Rhizobium spp. were used in the course of this investigation:

The 'cowpea' Rhizobium strain 32H1 was obtained from Dr. T. LaRue (Prairie Regional Laboratory, Saskatoon, Canada).

The 'cowpea' Rhizobium strain CB756 was obtained from Dr. F.J. Bergersen (Division of Plant Industry, CSIRO, Canberra, Australia) subsequently designated as R_{309} (Univ. of Hawaii).

The 'cowpea' Rhizobium strain CB756 was obtained from Dr. Bohlool (University of Hawaii, Honolulu, Hawaii) subsequently designated as B_{30}.

The R. japonicum strain USDA 122 (DES), a small colony derivative of USDA strain 122 which was isolated by David Emerich, was obtained from Dr. N.E.R. Campbell (Dept. of Microbiology, University of Manitoba, Winnipeg, Canada).

Maintenance of Cultures

Yeast extract-mannitol agar (YEM) (yeast extract (Difco), 1.0g; mannitol, 10.0 g; $CaCl_2$, 1.0 g; NaCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.20 g; $K_2HPO_4 \cdot 3H_2O$, 0.65 g; agar, 15.0 g; and distilled water to 1 liter) was used for the maintenance of stock culture slants. The pH of the medium was adjusted to 7.2 with 1 N NaOH and the medium was sterilized at 120°C for 20 min.

Growth Media

CS7

A modification of the defined medium of Pagan et al. (1975) was used for strains R309, 32H1 and B30. The media contained (g/l): glutamine, 0.145; L-glutamic acid, 1.0; Na-succinate, 6.8; myo-inositol, 1.0; L-arabinose, 3.75; KH_2PO_4, 0.27; $MgSO_4 \cdot 7H_2O$, 0.015; $MnSO_4 \cdot 4H_2O$, 0.01; H_3BO_3, 0.005; KI, 0.001; $CuSO_4 \cdot 5H_2O$, 0.00015; $ZnSO_4 \cdot 7H_2O$, 0.001; $CoCl_2 \cdot 6H_2O$, 0.0001; $CaCl_2 \cdot 2H_2O$, 0.11; $Na_2MoO_4 \cdot 2H_2O$, 0.0025; 0.0134; Fe citrate, 0.01; agar (BBL), 10.0; and deionized distilled water. The salts were prepared as concentrated stock solutions and added to the dissolved organic compounds and the pH was adjusted to 6.5 with 1 N NaOH. The media was made up to volume and sterilized by filtration with a Nalgene disposable filter unit (.45μm) and dispensed in 10 ml vol. Sterile cooled molten agar was added to an equal volume (2 x conc) of the liquid medium and was dispensed in 8 ml vol into 24 ml serum bottles or 150 ml into Roux bottles.

LNB5

A modified LNB5 medium described by Maier and Brill (1976) was used for R. japonicum strain 122 DES. The medium contained (g/l): D-gluconate, 5.0; Na-glutamate, 1.0; yeast extract (Difco), 1.0; inositol, 0.1; sucrose, 10.0; L-arabinose, 10.0; $NaH_2PO_4H_2O$, 0.15; $CaCl_2 \cdot 2H_2O$, 0.15; $MgSO_4 \cdot 7H_2O$, 0.25; $MnSO_4 \cdot H_2O$, 0.01; H_3BO_3, 0.003; $ZnSO_4 \cdot 7H_2O$, 0.002; FeEDTA, 0.028; $NaMoO_4 \cdot 2H_2O$, 0.25 mg; $CuSO_4 \cdot 5H_2O$, 0.04 mg; $CoCl_2 \cdot 6H_2O$, 0.025 mg; KI, 0.78 mg;

thiamine HCl, 0.01; pyridoxal HCl, 0.001; agar (BBL), 12.0 and deionized distilled water. The vitamins and salts were added to the other ingredients and the pH adjusted to 5.5 with HCl. The medium was then made up to volume and sterilized by filtration. The solid media was prepared as for CS7.

Measurement of Growth

Stationary phase cells of strain R309 grown on YEM agar slants were washed with distilled water and harvested by centrifugation at 10,000 x g for 10 min in a Sorvall Superspeed RC-5 automatic refrigerated centrifuge at 4°C. Cells were washed once with liquid CS7 and then resuspended to an Absorbance of 0.30 at 680 nm as measured on a Coleman Junior IIA spectrophotometer. Samples of these cell suspensions (0.1 ml) were added to 9.9 ml of liquid media in screw cap test tubes (120 nm x 15 nm). The tubes were incubated at 28°C on a rotary shaker for 5-6 days. Absorbance was determined at 12h intervals at 680 nm.

Nitrogenase Assay

The inocula were prepared from a 4-5d culture on YEM agar. One drop of a heavy cell suspension in distilled water was added to an agar slant (8 ml) contained in serum bottles (24 ml) and was spread over the surface of the medium. The serum bottles, which were loosely cotton plugged, were incubated on their sides at 28°C. All samples were done in triplicate.

Procedure: Nitrogenase activity was assayed by acetylene

reduction (Hardy et al., 1968). After incubation for 4-8 days, the cotton plugs from the serum bottles were replaced with sterile Suba seals and the bottles were flushed for 60 s with argon. Two ml of the argon was withdrawn with a syringe and replaced with 0.5 ml oxygen and 1.5 ml acetylene. The bottles were then incubated at 28°C. Gas chromatography was performed using a Pye series 104 gas chromatograph equipped with a flame-ionization detector, with a 2 m phenyl isocyanate/porasil C column at an oven temp of 60°C. The GC 'Durapak' column packing was obtained from Waters Associates Inc., Milford, Mass. Gases were supplied by 'Linde' Specialty Gas. The limits of impurities in the O_2 were: 1ppm H_2, 3ppm H_2O, 3ppm N_2, 7ppm Ar, 1ppm CO, 1ppm CO_2. The Argon had been pre-purified and the acetylene was a purified grade. Uninoculated bottles served as controls. Gas samples (0.2 ml) were removed at 5h, 24h and 48h intervals (unless otherwise specified) and analyzed for their acetylene and ethylene content. The concentration of ethylene was calculated utilizing the value obtained from the ethylene standard and the calibrated gas volume of the flask.

Glutamine Synthetase Assay

The inoculum (3 ml) of a heavy suspension from the surface growth of a YEM slant was spread over the surface of medium (150 ml) contained in a Roux bottle and placed in a standard incubator at 28°C, or at 20°C or 37°C in a water bath incubator. The cells were harvested 4-10 days after incubation. For R309, 32H1 and B30 the following method was used. The surface growth was washed with 25 ml of a 0.1 mg/ml solution of CTAB (cetyltrimethyl

ammonium bromide - Sigma) in phosphate-buffered saline (PBS) at room temp. The cells were spun down at 12,000 x g for 10 min in a Sorvall RC-5 Superspeed refrigerated centrifuge at 4°C, washed once in 1% KCl and resuspended in 1.0 ml of 1% KCl. The suspension was strained through cheese cloth to remove agar particles and stored at 4°C. Cultures of R. japonicum 122 DES were pre-washed with 50 ml of a 0.1 mg/ ml solution of CTAB in PBS. The cells were spun for 3 min at full-speed in a Sorvall omin-mixer at 4°C to remove the slime and were collected by centrifuging at 30,000 x g for 20 min. The pellet was washed once in 1% KCl and resuspended in 1.0 ml of 1% KCl. The suspension was strained through cheese cloth and stored at 4°C.

Transferase Assay Procedure: The transferase activity of glutamine synthetase was determined by the γ-glutamyltransferase assay procedure of Shapiro and Stadtman (1970b)as modified by Bender et al. (1977). A concentrated reaction mixture was prepared fresh by mixing the following stock solutions in the order and proportions listed: distilled water, 7.53 ml; 1.0 M imidazole-HCl (pH 7.15), 2.25ml; 0.80 M hydroxylamine-HCl, 0.37 ml; 0.10 M $MnCl_2$, 45 µl; 0.28 M K-arsenate (pH 7.15), 1.5 ml; 40 mM Na-ADP (pH 7.0), 0.15 ml; CTAB (1 mg/ml), 1.5 ml. A blank was prepared in the same manner but the K-arsenate and ADP solutions were replaced with water. The assay mixture was adjusted to pH 7.15 at room temp with 2 M KOH and was used immediately or cooled to 4°C. The cell suspension in 1% KCl, prepared as described in the

procedure for glutamine synthetase, was added to 0.40 ml of the concentrated assay mixture to give a volume of 0.45 ml and equilibrated at 28°C for 5 min. The reaction was initiated by the addition of 0.05 ml of .20 M L-glutamine and allowed to proceed for 20 min. The reaction was stopped by the addition of 1.0 ml of the 'stop mix' which contained 10% $FeCl_3 \cdot 3H_2O$ in .02 N HCl, 6 N HCl, and 25% TCA in a 1:1:1 ratio. The samples were then mixed, centrifuged to remove any precipitate and the Absorbance was measured at 540 nm on a Gilford 2400 spectrophotometer. A standard curve was prepared with commercially obtained γ-glutamylhydroxamate. One unit of enzyme was defined as that amount required to catalyze the synthesis of 1 mole of γ-glutamylhydroxamate per minute. The Na-ADP (from equine muscle, Grade IX) and L-glutamic acid γ-monohydroxamate were obtained from Sigma.

Forward Assay Procedure: The ability of glutamine synthetase to form glutamine was determined by the forward reaction assay system of Bender et al. (1977) adapted from Ebner et al. (1970). The concentrated assay mixture was prepared daily by mixing the stock solutions in the order and proportions listed: distilled water, 7.2 ml; 1.0 M imidazole-HCl (pH 7.15), 2.0 ml; 0.80 M hydroxylamine-HCl, 1.25 ml; 3.0 M $MgCl_2$, 0.40 ml; 0.85 M monosodium L-glutamate, 4.2 ml; CTAB (1 mg/ml), 2.0 ml. The assay mixture was adjusted to pH 7.7 at room temp with 10 M KOH and was used immediately or cooled to 4°C. The cell suspension in 1% KCl was added to 0.40 ml of the concentrated assay mixture to give a vol of 0.44 ml. After 5 min of equilibration at 28°C, the reaction

was initiated by adding 0.06 ml of 0.20 M ATP and allowed to proceed for 20 min. Blanks were prepared without ATP. The reaction was terminated by the addition of 1.0 ml of the stop mix described in the transferase assay procedure. The mixture was immediately placed in a Vortex mixer to dissolve the precipitate that formed upon the addition of the stop mix. The samples were then centrifuged and the absorbancy was measured at 540 nm as for the GT assay. The adenosine 5'-triphosphate (from equine muscle, disodium salt) was obtained from Sigma Chemical Co. A standard curve was prepared with L-glutamic acid γ-mono-hydroxamate (Sigma) for calibration of the activity.

Determination of Protein

The bacterial suspension was digested in 1.0 M NaOH for 10 min at 100°C and the protein content assayed by the colorimetric method of Lowry et al. (1951). Standard protein solutions in the range of 50 to 250 μg/ml, were made with crystalline bovine serum albumin (Sigma) dissolved in distilled water. The samples were measured at 750 nm on a Coleman Junior II A spectrophotometer.

Bacterial Dry Weight

Bacterial cells from a 12d agar slant culture in a serum bottle were washed twice with distilled water and resuspended in 10 ml distilled water. The suspension was dried in an aluminum pan at 110°C in a drying oven overnight and the dry wt of the cells was determined.

Electron Microscopy

Cultures of R309 grown on solid CS7 medium with and without sodium molybdate incorporated into the medium were collected at 2d and 6d. The cells were fixed by the method of Kellenberger *et al.* (1958). The pellet of cells was dehydrated in graded concentrations of ethanol (10%, 30%, 40%, 50%, 70%, 90% and absolute) and infiltrated with epon. Sections were mounted on 400-mesh uncoated grids, stained with uranyl acetate (25%, w/v) and lead citrate (0.33%) and examined with an Hitachi HU-II electron microscope.

RESULTS

RESULTS

Colony Morphology

The colony morphologies of strains R309, 32H1 and B30 were observed on CS7 agar. Colonies of R309 and 32H1 were pinpoint, translucent, convex and glistening and colonial variants were observed in the cultures when viewed with a Zoom stereomicroscope showing larger, slime-producing clones as well as the small, dry clones. Colonies of B30 were creamy and characterized by some slime production. Colonies of 122 DES cultured on LNB5 medium were larger (~1mm) and often camouflaged by an excessive amount of extracellular polysaccharide slime.

Purity checks of the cultures were periodically carried out by subculturing on yeast extract-mannitol agar plates containing 10 ml/L of a 1:400 congo red solution (Hahn, 1966). Strains R309 and 32H1 failed to absorb congo red from this medium within a week of incubation while other genera exhibited marked absorption. Periodic examinations of cell suspensions of all strains by phase-contrast microscopy seemed to indicate pure cultures.

Growth of Rhizobium sp. R309

Rhizobium sp. R309, which showed high levels of nitrogenase activity *in vitro*, was chosen for the study of characteristic growth rates in liquid CS7 media at 28°C. Growth curves (Fig. 4) were obtained with inocula of 0.30, 0.12, and 0.065

Fig. 4. Growth curve of Rhizobium sp. R309 in liquid CS7 medium.

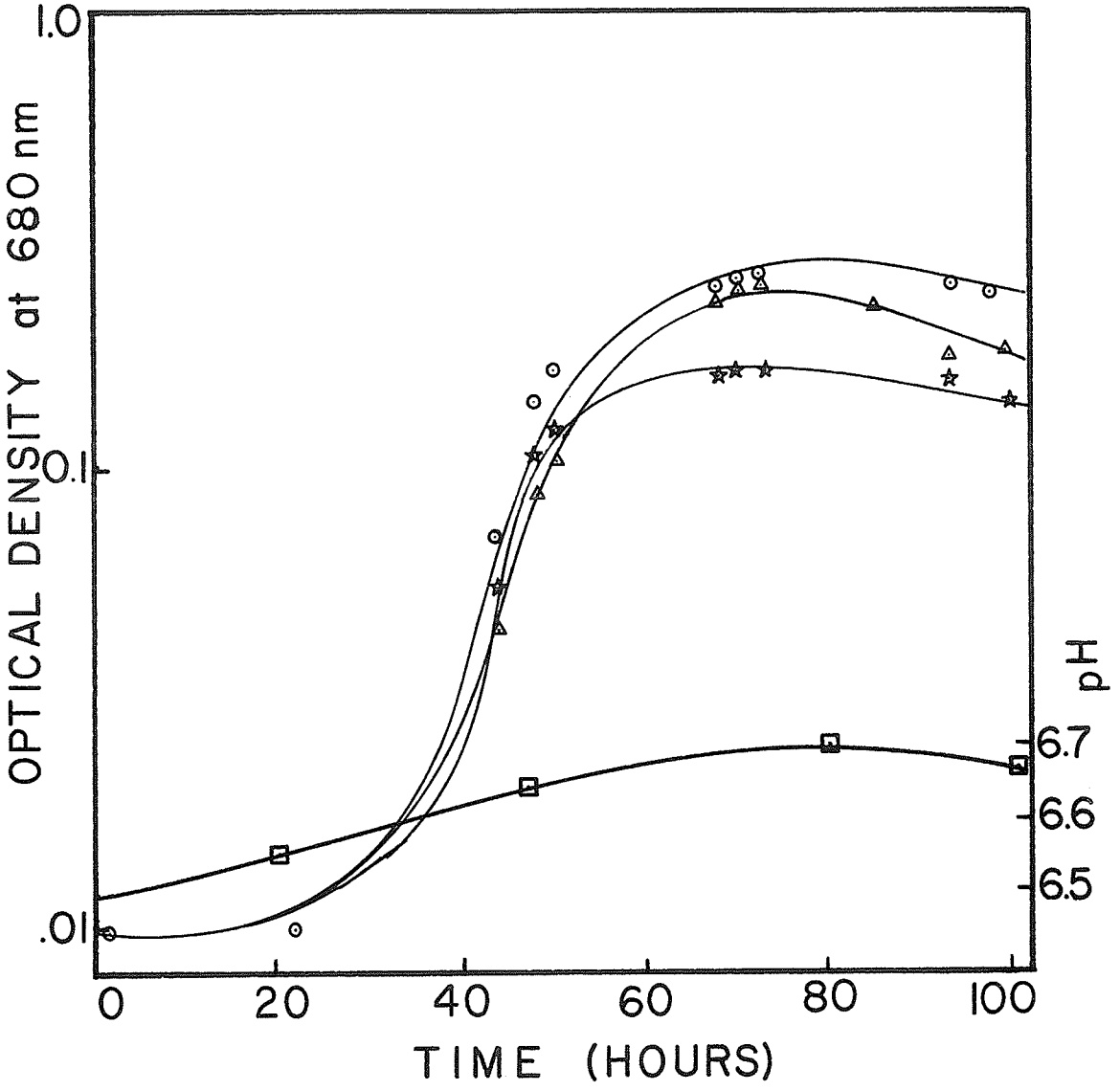
Cell density was measured as described in Materials and Methods.

Inoculum #1 (0.30 at A_{680}) ⊙—⊙

Inoculum #2 (0.12 at A_{680}) ★—★

Inoculum #3 (o.065 at A_{680}) △—△

Change in pH ⊡—⊡


OPTICAL DENSITY at 680 nm
1.0
0.1
.01
0
20
40
60
80
100
TIME (HOURS)
pH
6.7
6.6
6.5

respectively at A_{680} and no apparent differences were observed until the onset of stationary growth. An initial lag phase of 20h was observed and exponential growth began at ~50h. The generation time during the exponential growth phase was calculated to be an average of 8h. Maximum growth was obtained with an inoculum of 0.30 at A_{680} (inoculum #1) which was used in subsequent growth studies. The initial pH of the CS7 medium was 6.5 and a slight increase was observed during the growth phases (Fig. 4).

Effects of Glutamine and Succinate on the Growth of Strain R309

The effects of varying concentrations of glutamine and succinate on the growth of strain R309 were investigated. Only a small difference in the growth rate of strain R309 on CS7 medium containing 2mM glutamine, 1mM or no glutamine was observed (Fig. 5). Maximum growth occurred with 1mM glutamine in the medium. Similar results were obtained with decreasing amounts of Na-succinate (12.5mM, 6.0mM and 3.0 mM) in the liquid CS7 medium (Fig. 6). Maximum growth occurred with 12.5mM Na-succinate in the medium.

Time Profile of Nitrogenase Activity in R. japonicum 122 DES

Nitrogenase activity in strain 122 DES cultured on LNB5 medium was initially detected on the fourth day after inoculation, 5h after flushing with argon and replacing 2ml of the

Fig. 5. Effect of glutamine concentration on the growth rate of Rhizobium sp. R309.

Cell density was measured as described in Materials and Methods.

2 mM glutamine ⊡—⊡

1 mM " ⊙—⊙

no glutamine △—△

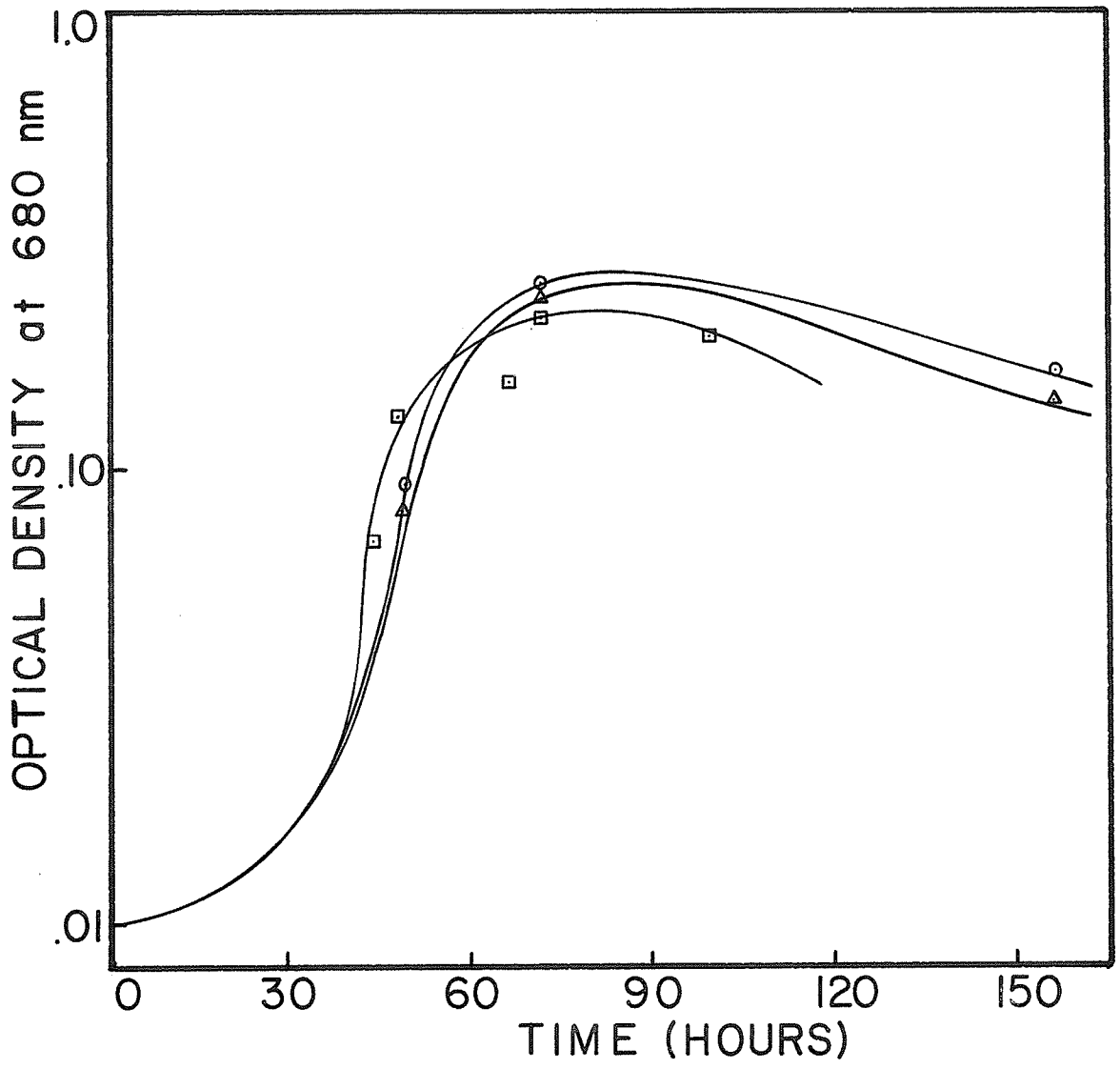

1.0
.10
.01
OPTICAL DENSITY at 680 nm
0
30
60
90
120
150
TIME (HOURS)

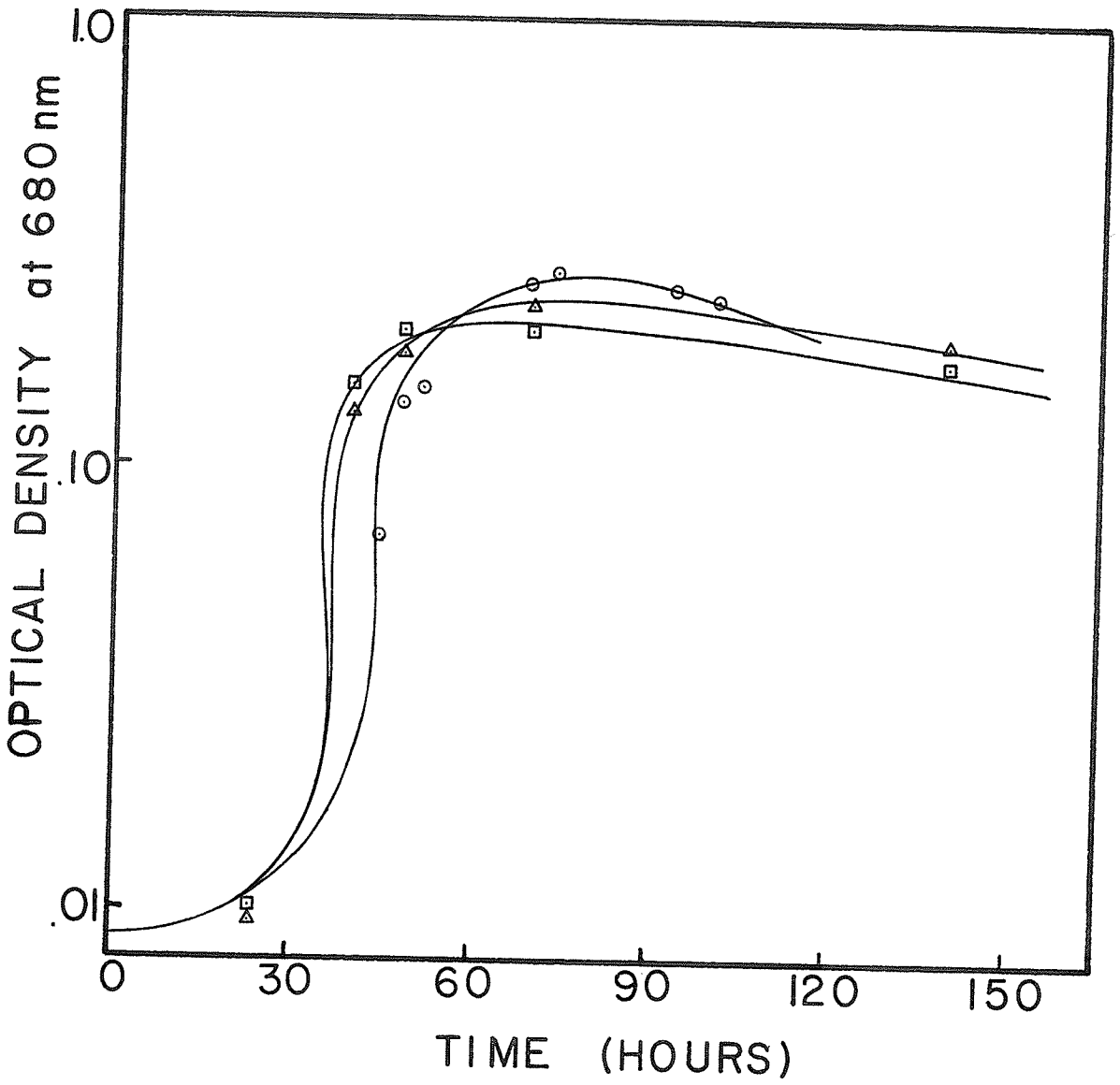
Fig. 6. Effect of Na-succinate concentration on the growth rate of Rhizobium sp. R309.

Cell density was measured as described in Materials and Methods.

12.5 mM Na-succinate ⊙——⊙

6.0 mM " ⊡——⊡

3.0 mM " △——△


OPTICAL DENSITY at 680 nm
1.0
.10
.01
0
30
60
90
120
150
TIME (HOURS)

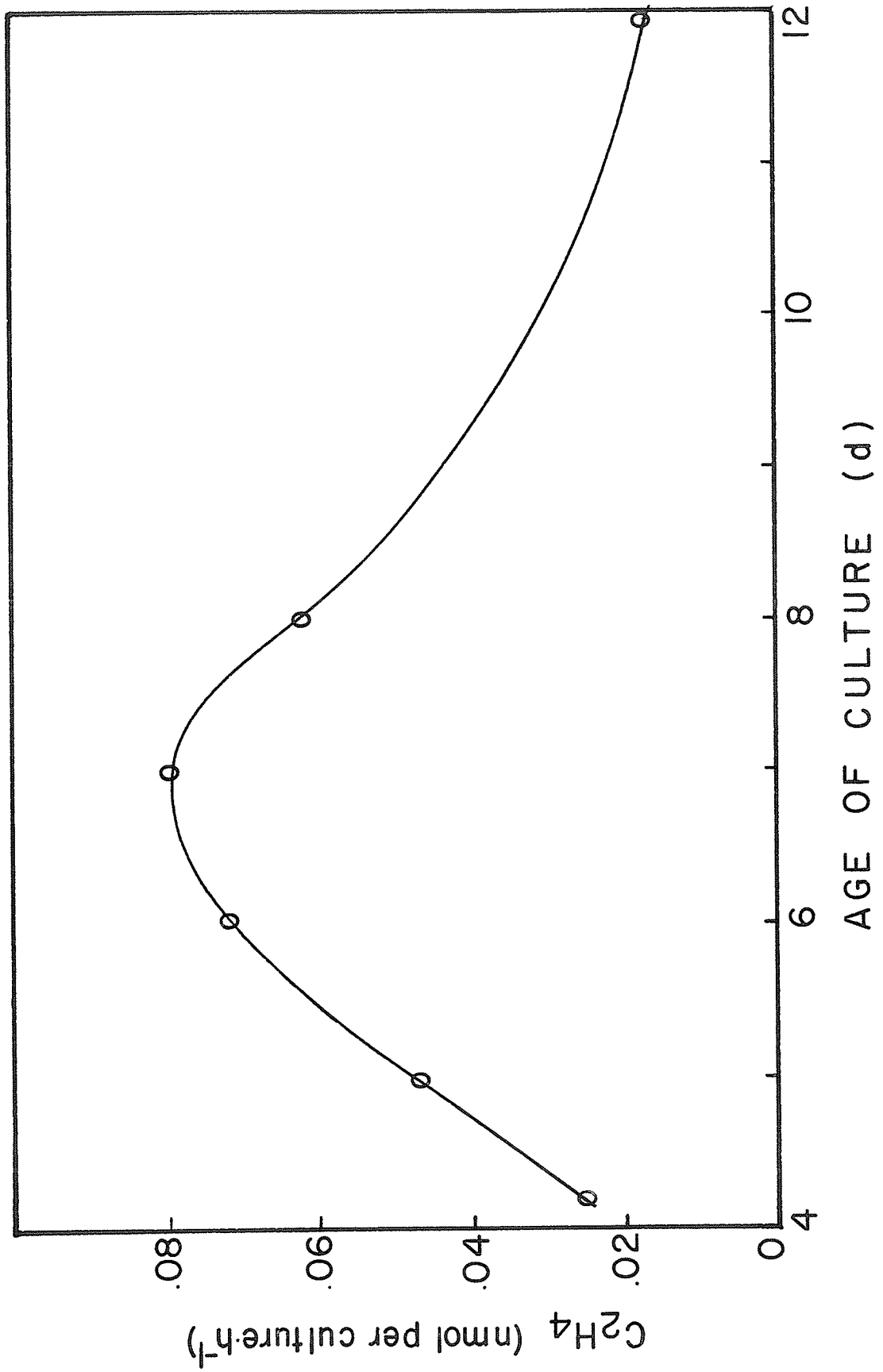
atmosphere with acetylene (1.5ml) and oxygen (0.5ml) (Fig. 7). Nitrogenase activity reached a maximum on day 7 and then began to decline. The assays were done on triplicate samples and the data reported are the average minus the endogenous ethylene in the system determined by an uninoculated control. Increasing the cell density of the inocula resulted in an excess of slime production which was accompanied by an absence of acetylene reduction. Consistent acetylene reduction occurred in an atmosphere of 16.0ml containing 1.5ml acetylene, 0.5ml oxygen (3.1%) and 14.0ml argon. The gas chromatograph was initially serviced and appeared to be in good working condition. A column was prepared with new packing and gave peaks similar to those obtained with the old column. The gas flow rates of H_2:N_2:Air were periodically checked and maintained at the following ratio (ml/min) of 40:40:400.

Nitrogenase Activity in Strains R309 and 32H1

Cultures of R309 were grown on LNB5 medium for 4d and then flushed and gased and nitrogenase activity tested from 4-7 days. Strain R309 exhibited low levels of acetylene reduction (0.014 to 0.030 nmol C_2H_4 produced per culture per hour) over the 3 day period. Acetylene-reducing ability was not consistent amongst all samples tested. The amount of ethylene in the standard was 0.435 nmol/ml or 9.25 ppm. No activity with strain R309 was observed on CS7 medium.

Fig. 7. Time course of acetylene reduction by strain 122 DES cultured on LNB5 medium.

Activity was assayed as described in Materials and Methods. The inoculum for each serum bottle was 1 drop of the same cell suspension. Cultures were flushed and gased at 4 d. Each point is the mean of 3 replicates and values were corrected for endogenous ethylene formation.


C_2H_4 (nmol per culture·h^{-1})
.08
.06
.04
.02
0
4
6
8
10
12
AGE OF CULTURE (d)

Cultures of 32H1 demonstrated acetylene reduction when grown on CS7 for 4 days and then flushed with argon and 8.1 ml replaced with 5.4 ml O_2 and 2.7 ml acetylene. Samples of the atmospheres were measured at 7d and ethylene produced was in the range of 0.073-0.107 nmol per culture per h. The amount of ethylene in the standard was 0.435 nmol/ml or 9.25 ppm. No activity with strain 32H1 was observed on LNB5 medium.

The cultures of R309 and 32H1 which showed nitrogenase activity contained surface colonies which were pinpoint and characterized by a lack of slime. Increasing the cell density of the inocula resulted in an excess of slime and no observable acetylene reduction.

The Ultrastructure of Strain R309 Cultured on CS7

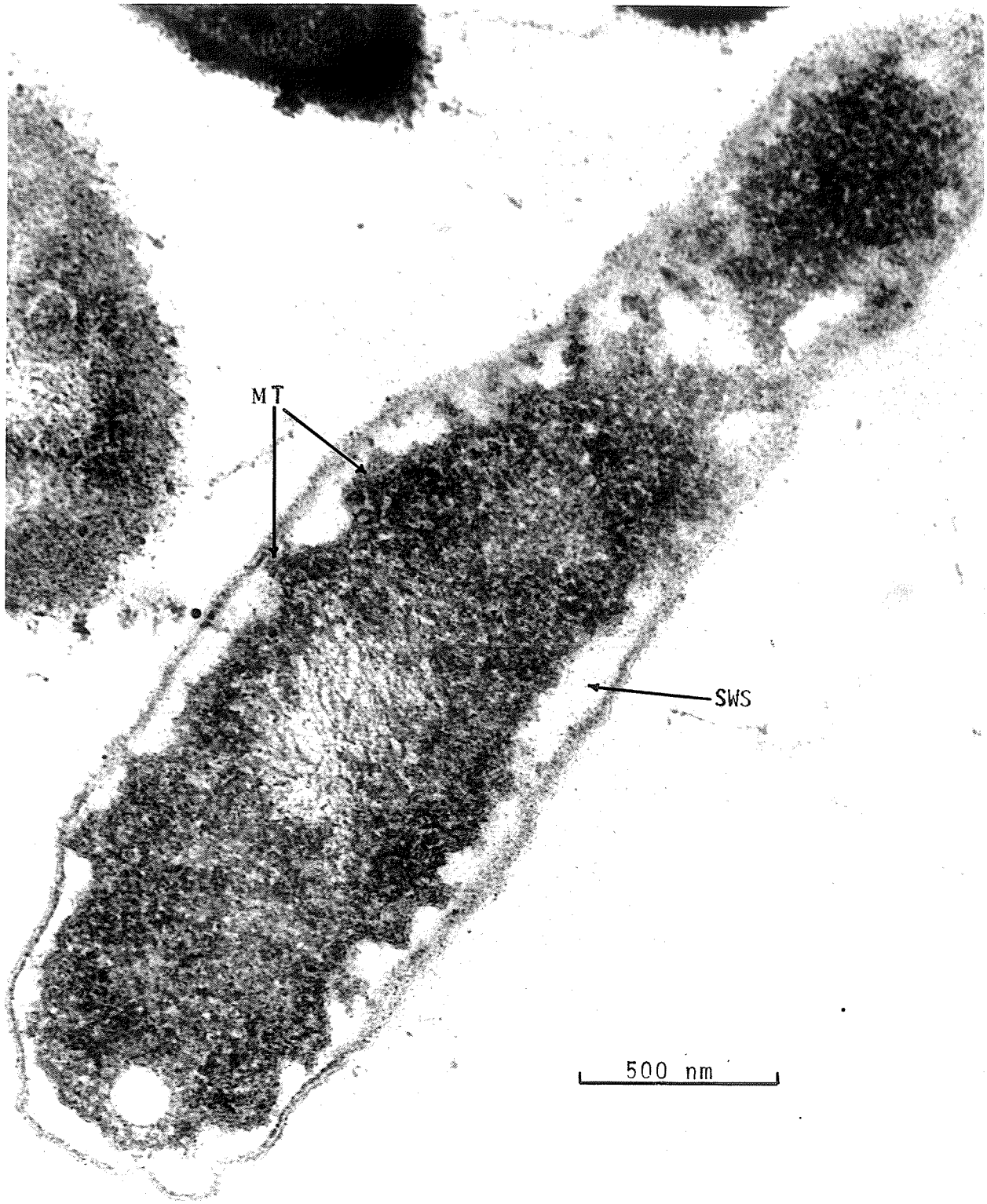
Photomicrographs of strain R309 grown on complete CS7 for 2 days are shown in Fig. 8 and Fig. 9. The cells show a bacteroid-type of structure with a loss of rigidity of the cell wall, a subwall space between the plasma membrane and the cell wall and convolutions of the plasma membrane to form tubules. Photomicrographs of strain R309 grown for 6 days on CS7 without molybdenum are shown in Fig. 10 and Fig. 11. A thick rigid rhizobium-type of cell wall was observed with no changes in the plasma membrane.

Fig. 8. Photomicrograph of Rhizobium sp. R309 cultured on CS7 medium and harvested at 2 d.

The bacteria were grown up as described for the nitrogenase assay in Materials and Methods, but were not flushed or gased. Instead they were harvested by washing and fixed at 2 d as described in Materials and Methods.

Membrane tubule, MT; Subwall space, SWS.

Magnification 66,700 x



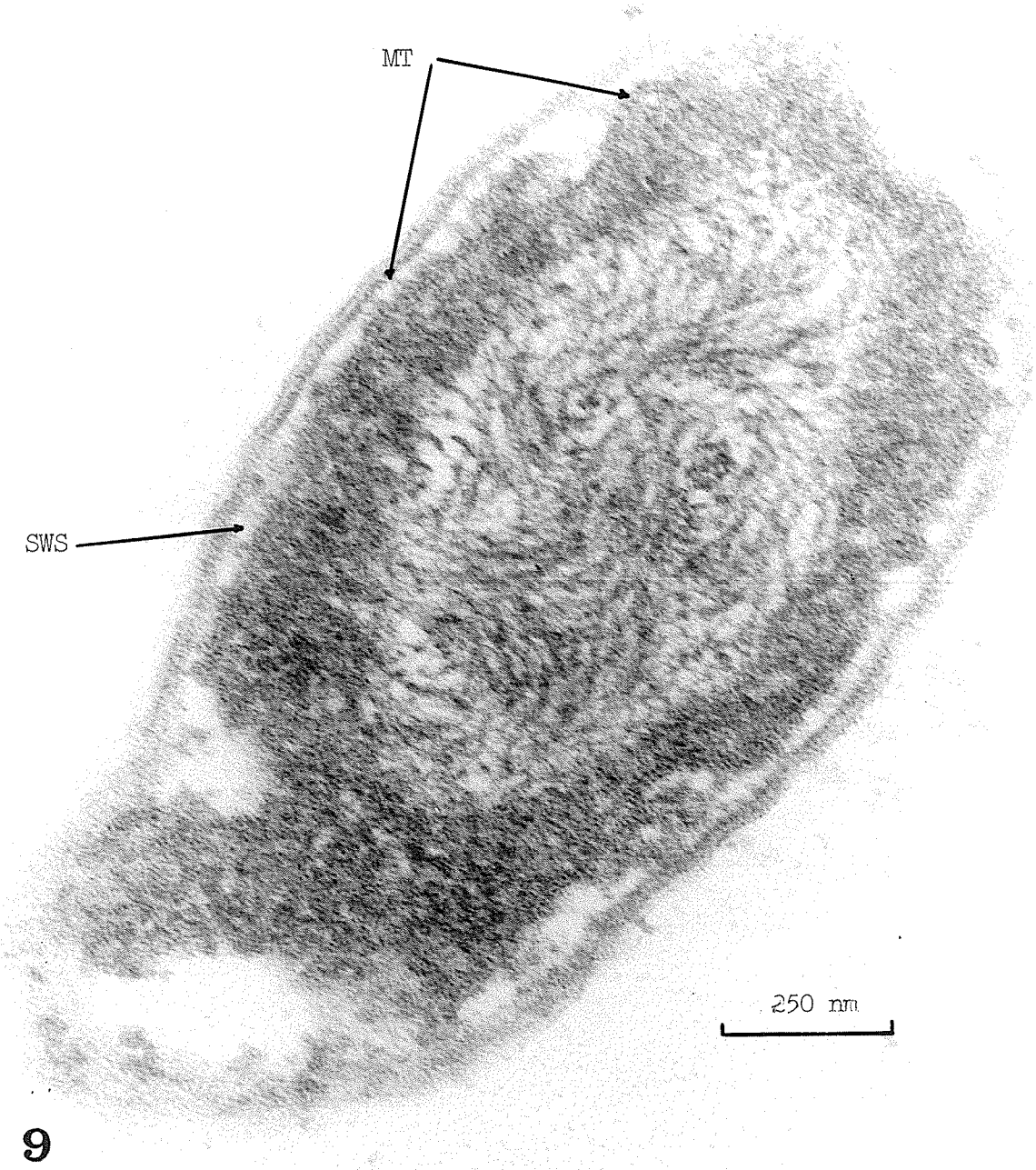
8

Fig. 9. Photomicrograph of Rhizobium sp. R309 cultured on CS7 and harvested at 2 d.

The bacteria were grown up as described for the nitrogenase assay in Materials and Methods, but were not flushed or gased. Instead they were harvested by washing and fixed at 2 d as described in Materials and Methods.

Membrane tubule, MT; Subwall space, SWS.

Magnification 99,600 x

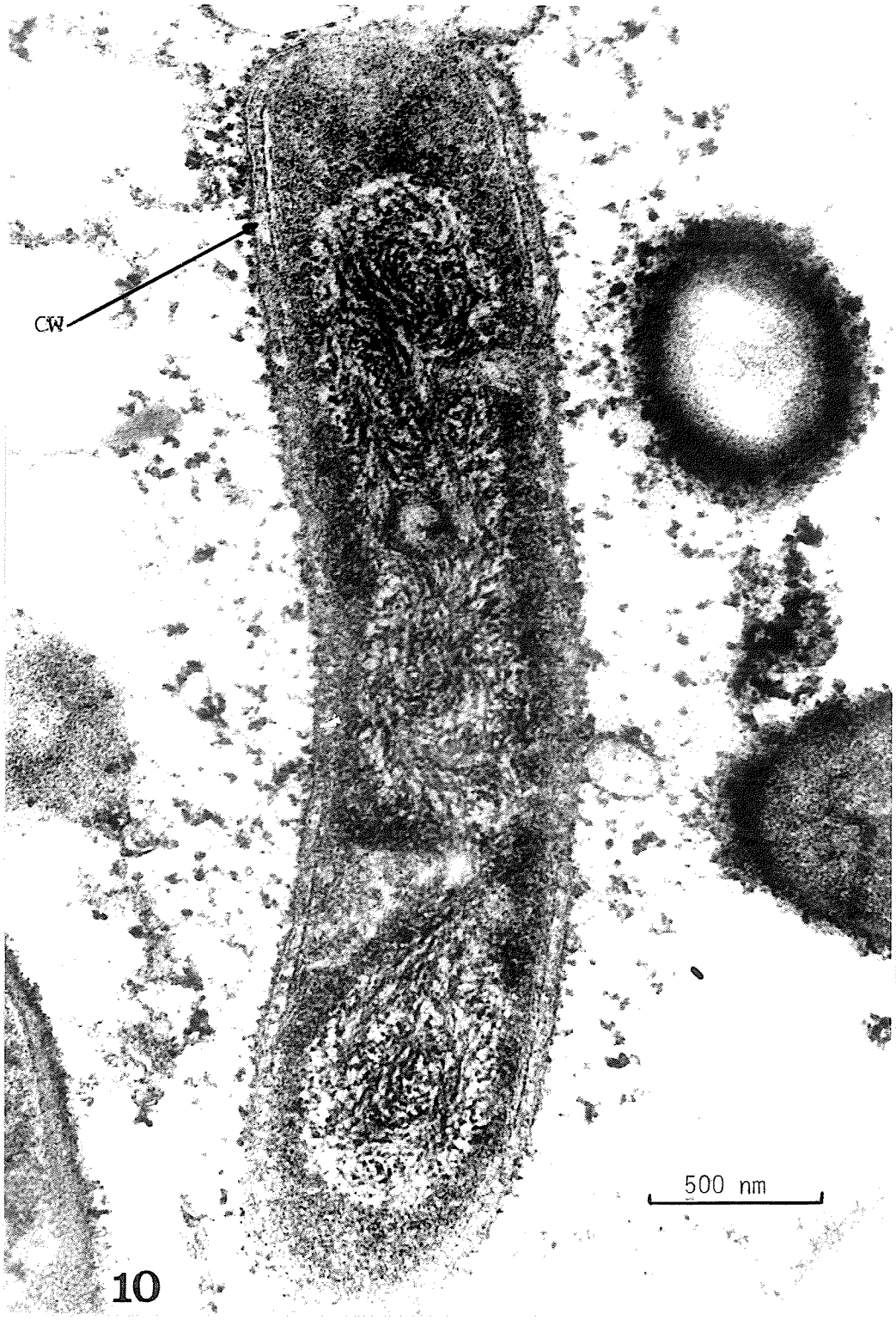


9

Fig. 10. Photomicrograph of Rhizobium sp. R309 cultured on CS7 medium without molybdenum and harvested at 6 d.

The bacteria were grown up as described for the nitrogenase assay in Materials and Methods, but were not flushed or gased. Instead they were harvested by washing and fixed at 6 d as described in Materials and Methods.

Cell wall, CW. Magnification 54,000 x

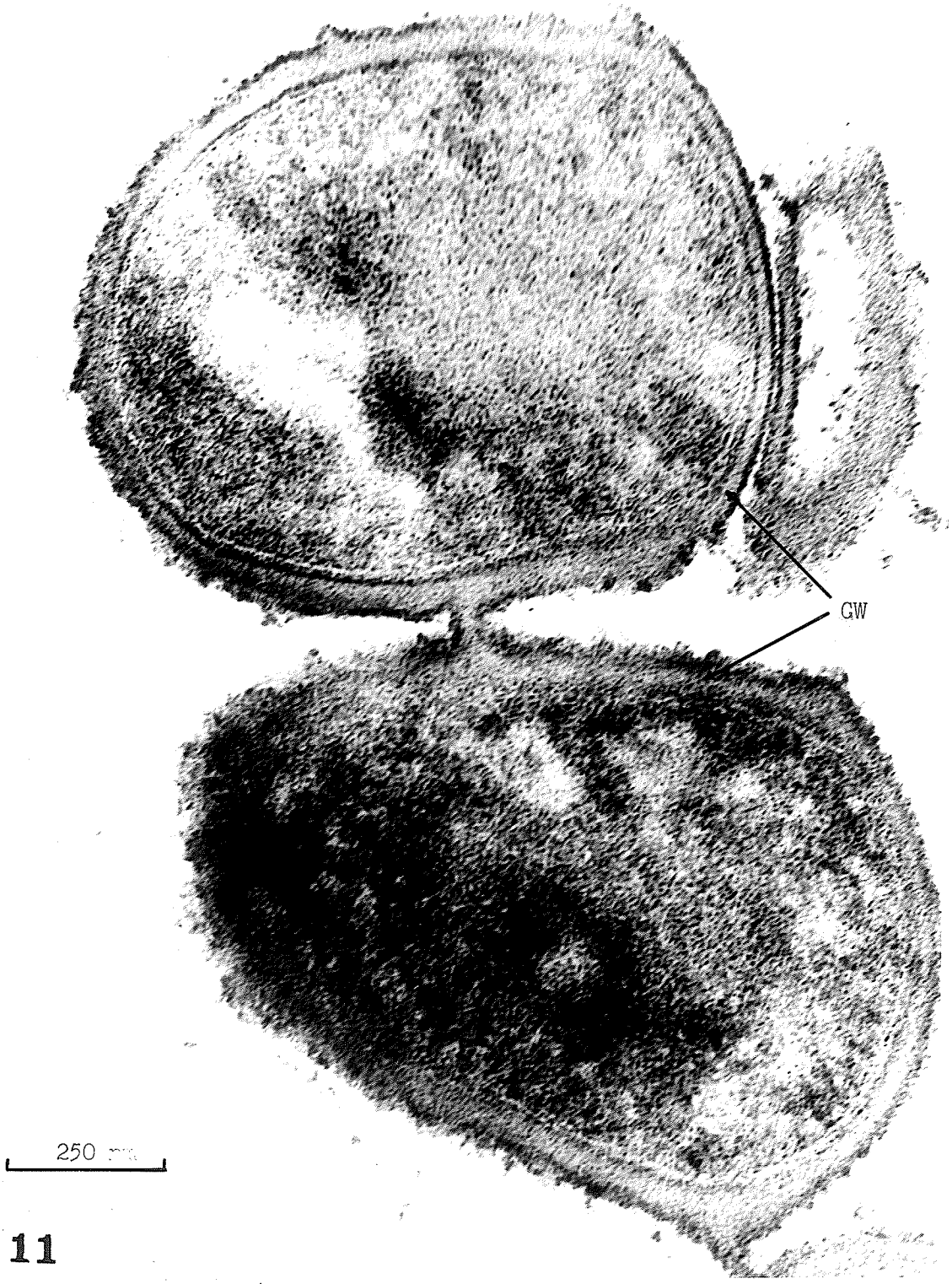


10

Fig. 11. Photomicrograph of Rhizobium sp. R309 cultured on CS7 medium without molybdenum and harvested at 6 d.

The bacteria were grown up as described for the nitrogenase assay in Materials and Methods, but were not flushed or gased. Instead they were harvested by washing and fixed at 6 d as described in Materials and Methods.

Cell wall, CW. Magnification 99,600 x



11

Harvesting Procedure for Whole Cell Assays

Cultures for assays of glutamine synthetase were harvested at room temp with CTAB to prevent a change in the state of adenylylation (Bender et al., 1977). Approximately 1.0g of cells was obtained from the growth on four Roux bottles.

Cultures of strain 122 DES produced an overabundance of slime making harvesting more difficult. The cells had to be loosened from the agar with a glass rod before pre-washing with CTAB in PBS. It was then necessary to remove the slime in order to collect a pellet of cells.

Glutamine Synthetase Assay System

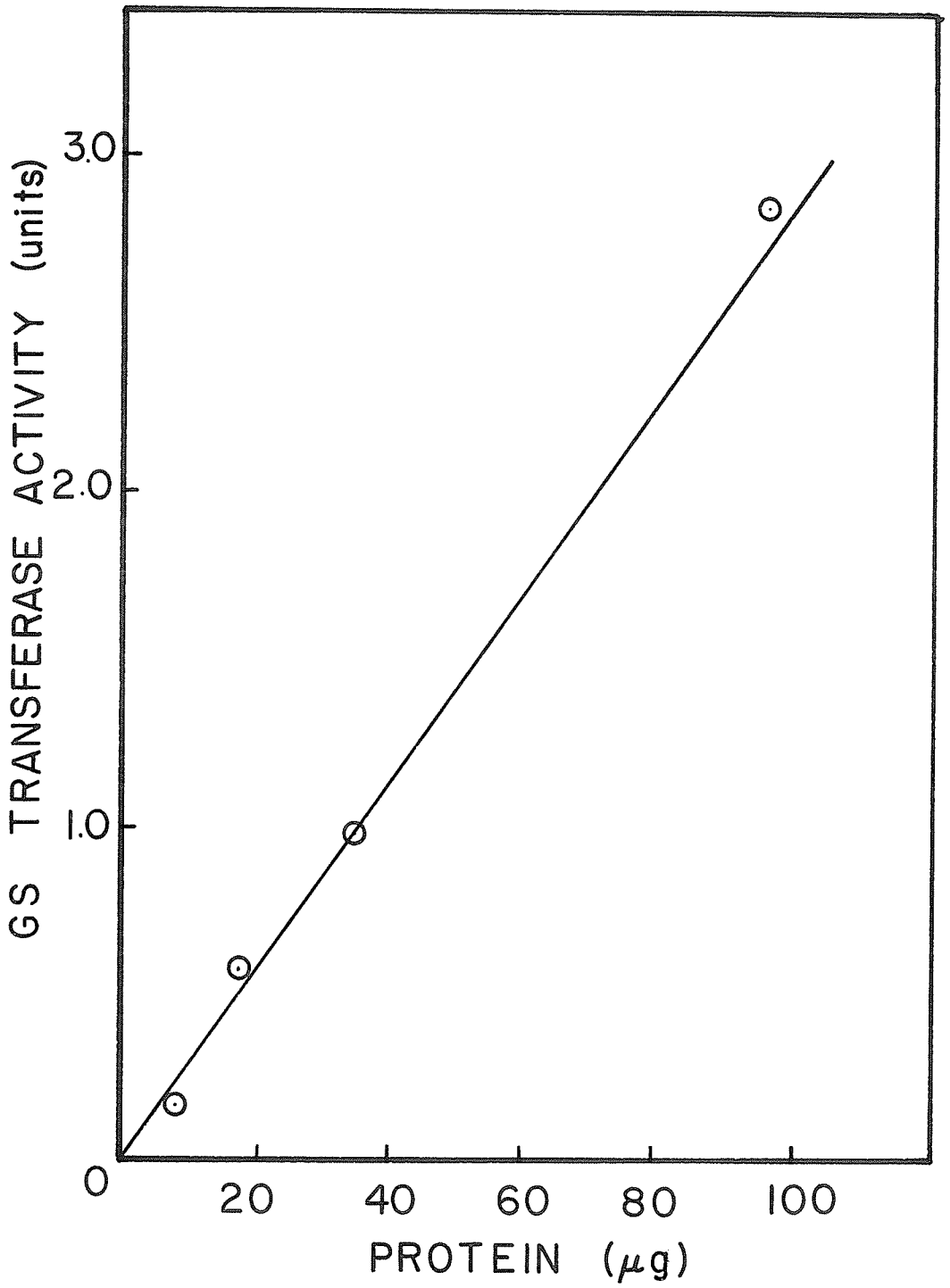
Standard curves were prepared with 1.0-5.0 µmol of γ-glutamyl hydroxamate in the assay system. Cell suspensions containing 50-100 µg of protein in 0.05 ml of 1% KCl were used for the glutamine synthetase (GS) assays. The reaction mixture for the transferase assay of GS was adjusted to pH 7.15 and the forward assay reaction mixture to pH 7.7.

The transferase activity of glutamine synthetase assayed on whole cells of B30 grown at 28°C for 7d showed a linear relationship with the protein concentration (Fig. 12). Standard curves for the transferase and forward activity of GS repeatedly gave similar results with the same γ-glutamyl hydroxamate concentrations.

Fig. 12. Effect of protein concentration on the glutamine synthetase transferase activity of whole cells.

Activity was assayed as described in Materials and Methods.

One unit of enzyme catalyzes the formation of 1 μmole of γ-glutamyl hydroxamate per min at 540 nm.


GS TRANSFERASE ACTIVITY (units)
3.0
2.0
1.0
0
20
40
60
80
100
PROTEIN (μg)

Temperature Profile of Glutamine Synthetase Activity

Cultures of strain R309 grown on CS7 media at 15, 20, 28 and 37°C were harvested at 4, 5, and 6d and assayed for total GS activity (Fig. 13). The media was preincubated at the appropriate temperature prior to inoculation. The lowest activity of strain R309 occurred at 20°C and the highest activity occurred in cells grown at 28°C. These temperatures were subsequently used as variables in the glutamine synthetase assays.

Glutamine Synthetase Activity Profile for Strains 122 DES, R309, 32H1 and B30

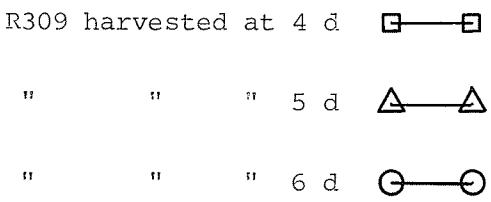
Cultures of strain 122 DES were grown at 28°C and were assayed for total glutamine synthetase activity at 4, 5, 6 and 7 days. The GS activity included the adenylylated and deadenylylated forms of the enzyme and was maximum at day 6 (Fig. 14). At 4d the activity of strain 122 DES was low and comparable to strains 32H1 and B30 grown at 28°C (Table 1).

Cultures of strain R309 were grown at 20°C and 28°C and were assayed for total glutamine synthetase activity at 4, 5, 6, 7, 8 and 10 days (Fig. 15). A significant level of GS activity was observed at the start of the assay on 4d cultures grown at 20°C and 28°C and maximum activity occurred at 7d. In the cultures grown at 28°C the activity at the start of the assay was approximately two-fold greater than initial activity at 20°C and increased only slightly during the incubation time

Fig. 13. Effect of temperature on the glutamine synthetase transferase activity of *Rhizobium sp.* R309.

Activity was assayed as described in *Materials and Methods*. Between 0.05 and 0.10 mg of protein were used per assay. Cells were harvested at 4, 5, and 6 d.

One unit of enzyme catalyzes the formation of 1.0 micromole of γ-glutamyl hydroxamate per min at 540 nm.



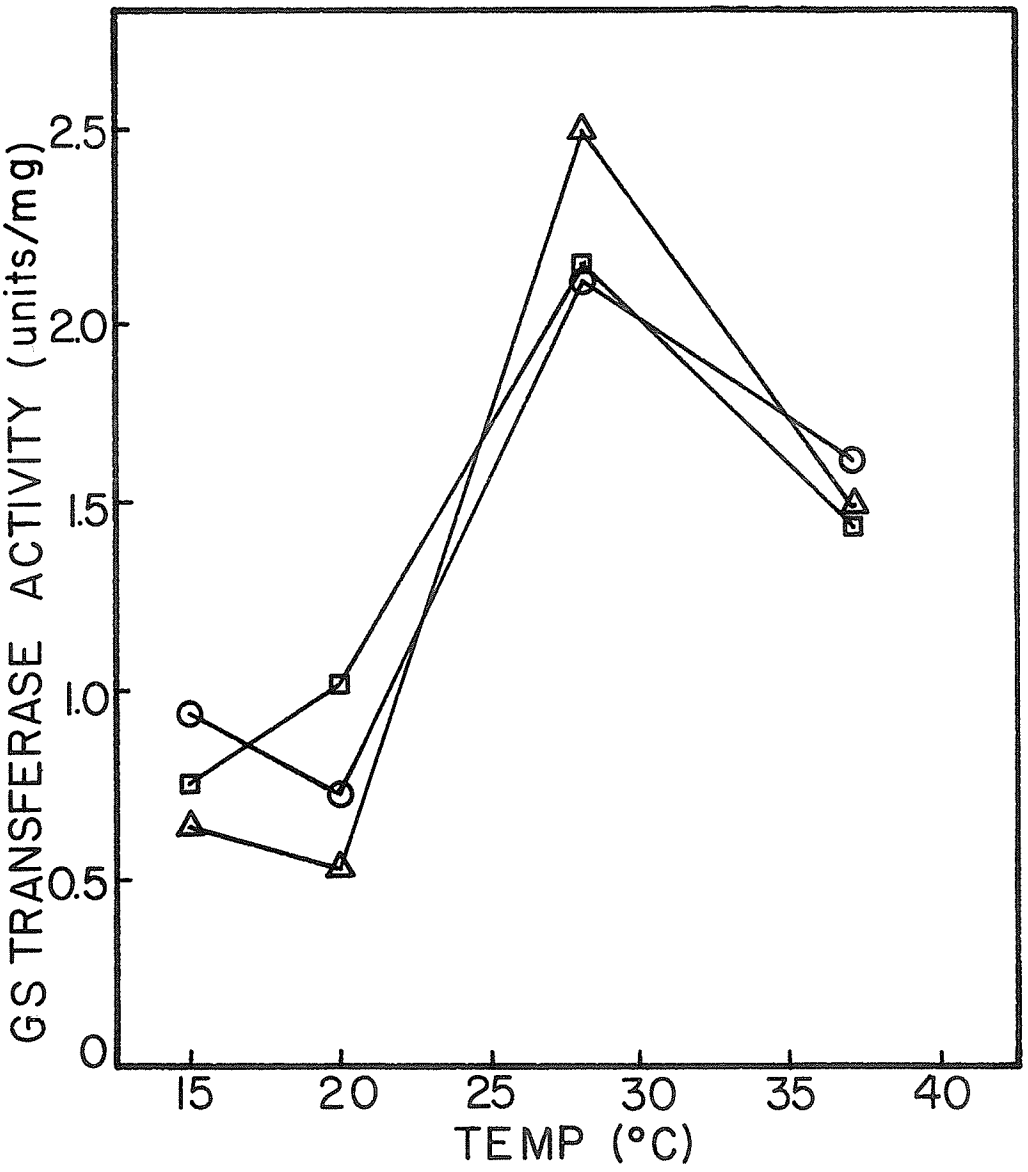

GS TRANSFERASE ACTIVITY (units/mg)
0
0.5
1.0
1.5
2.0
2.5
15
20
25
30
35
40
TEMP (°C)

Fig. 14. Pattern of total glutamine synthetase activity in strain 122 DES grown on LNB5 media at 28°C.

Activity was assayed as described for the transferase assay in Materials and Methods. About 0.12 mg of protein were used per assay.

One unit of enzyme catalyzes the formation of 1.0 micromole of γ-glutamyl hydroxamate per min at 540 nm.

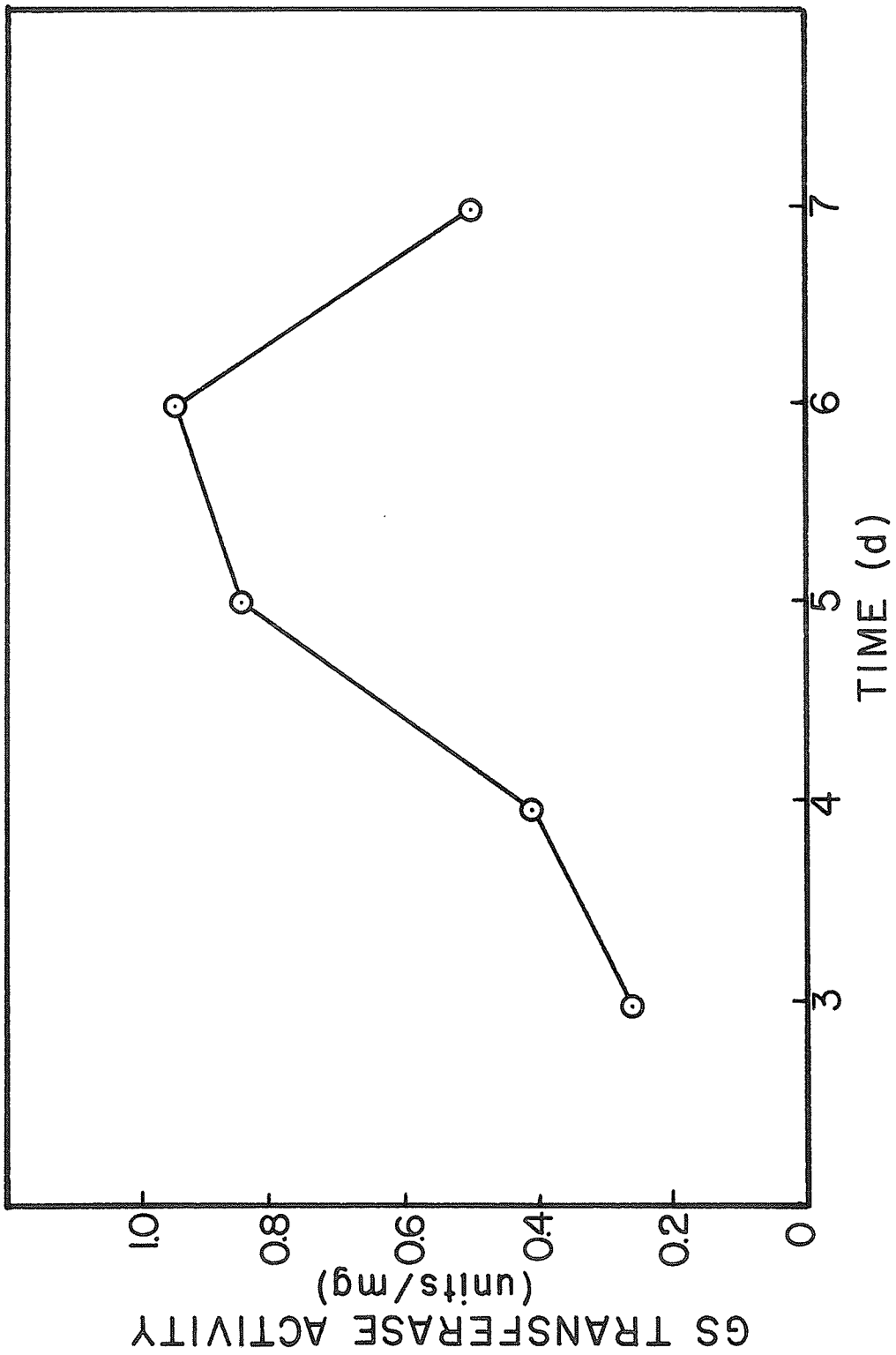

GS TRANSFERASE ACTIVITY
(units/mg)
0
0.2
0.4
0.6
0.8
1.0
3
4
5
6
7
TIME (d)

TABLE 1

Total Glutamine Synthetase Activity in *Rhizobium spp.*, 122DES, R309, 32H1 and B30.

Rhizobium sp.	Glutamine Synthetase Activity *			
	4 d cells		7 d cells	
	20° C	28° C	20° C	28° C
122 DES	- **	0.27	- **	0.73
R309	1.00	2.19	1.99	2.88
32H1	1.33	0.00	1.15	0.48
B30	1.01	0.35	1.19	0.43

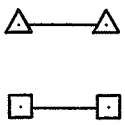
* specific activity is defined as μmoles of γ-glutamyl hydroxamate formed / mg of protein / min.

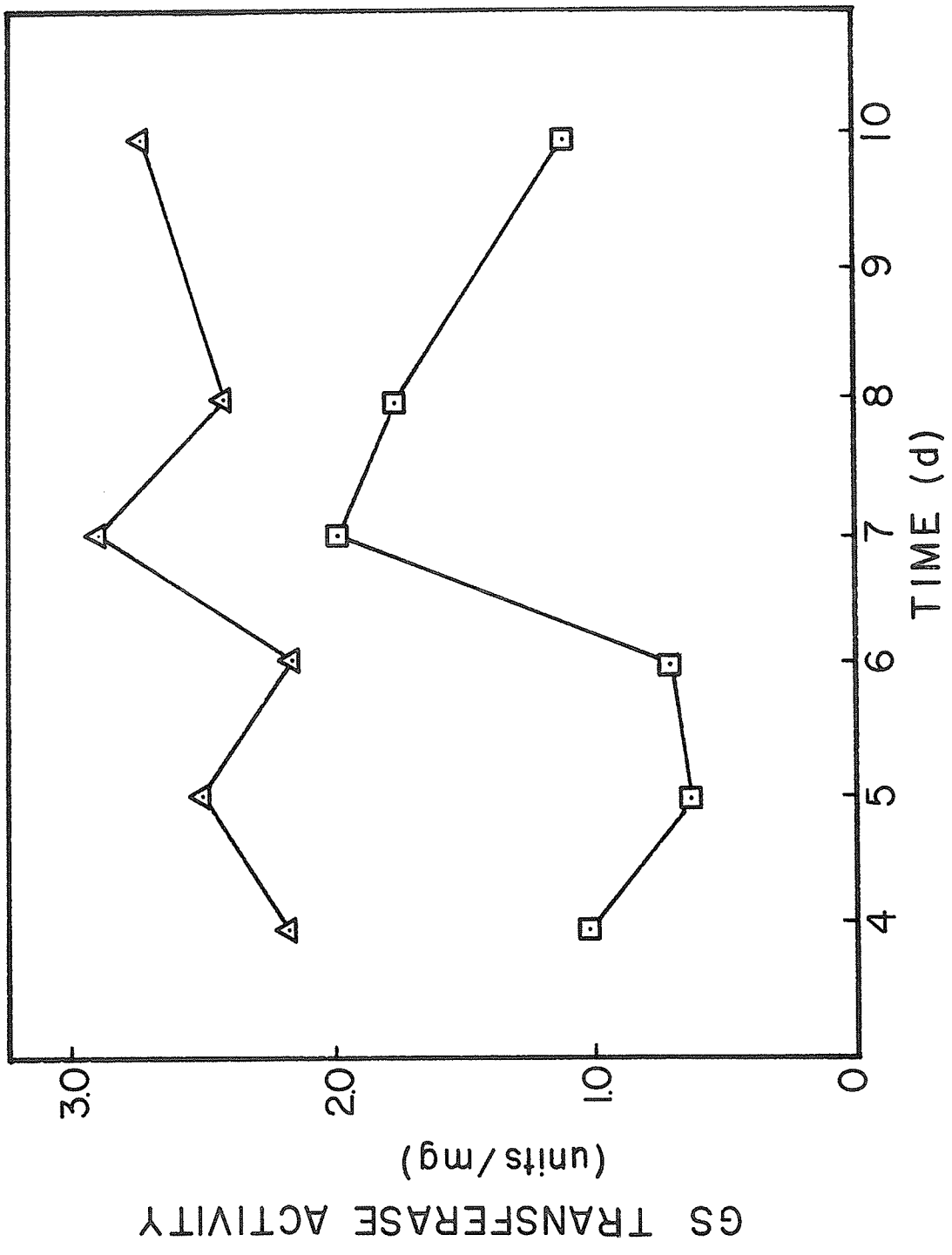
** not assayed for.

Fig. 15. Pattern of total glutamine synthetase activity in strain R309 grown on CS7 media at 20°C and 28°C.

Activity was assayed as described for the transferase assay in Materials and Methods. About 0.09 mg of protein were used per assay.

One unit of enzyme catalyzes the formation of 1.0 micromole of γ-glutamyl hydroxamate per min at 540 nm.




GS TRANSFERASE ACTIVITY
(units/mg)
3.0
2.0
1.0
0
4
5
6
7
8
9
10
TIME (d)

showing some fluctuation in the level of activity.

Cultures of strain 32H1 grown at 20°C and 28°C were assayed for total glutamine synthetase activity from 4-10 days (Fig. 16). Glutamine synthetase activity of 4 and 5d cultures was not detectable at 28°C and 20°C respectively. In cells grown at 20°C maximum activity occurred at 7d whereas cells grown at 28°C showed no increase in GS activity for up to 8d.

Cultures of strain B30 grown at 20°C and 28°C were assayed from 4-10 days (Fig. 17). A high level of GS activity was observed in cells grown at 20°C for 4d and did not increase significantly during the course of the experiment, whereas a low activity was observed in cells grown at 28°C and did not increase significantly during the course of the experiment. The level of GS activity in cells grown at 20°C was approximately three-fold greater than in cells grown at 28°C.

Cultures of B30 were also grown at 28°C on CS7 with the glutamine deleted and assayed for total glutamine synthetase activity at 4-10 days (Fig. 17). The GS activitiy of B30 on complete media was the same as shown in Fig. 17. At 4d the GS activity of glutamine-deprived cells was slightly higher than cells grown with glutamine and showed a gradual decline during the course of the experiment.

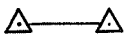
Relative States of Adenylylation of GS in Strain R309

The amount of unadenylylated enzyme present in cultures of R309 grown at 28°C and harvested after 4-10d is listed in

Fig. 16. Pattern of total glutamine synthetase activity in strain 32H1 grown on CS7 media at 20°C and 28°C.

Activity was assayed as described for the transferase assay in Materials and Methods. Between 0.05-0.10 mg of protein were used per assay.

One unit of enzyme catalyzes the formation of 1.0 micromole of γ-glutamyl hydroxamate per min at 540 nm.

32H1 grown at 28°C 

" " " 20°C 

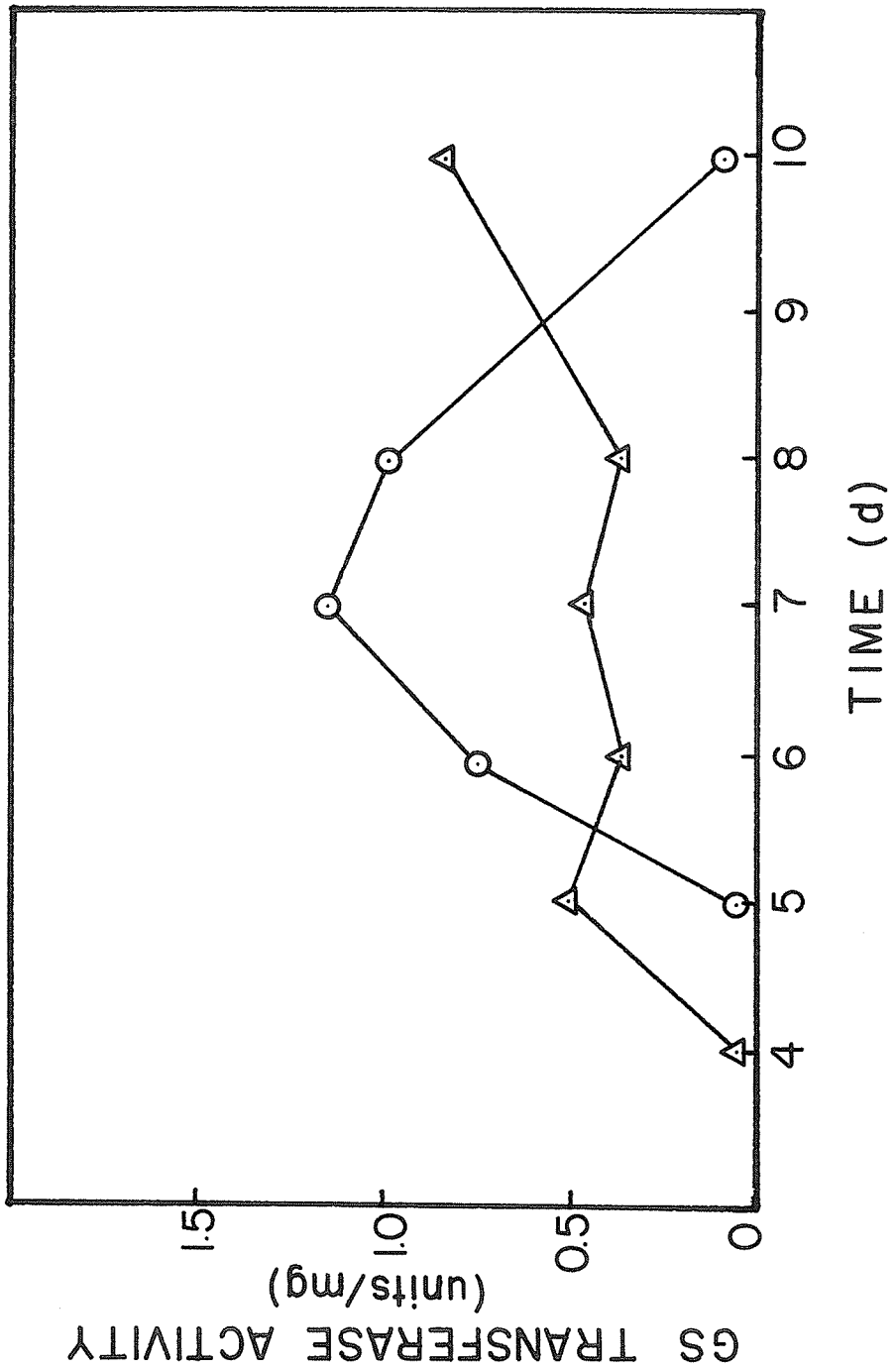
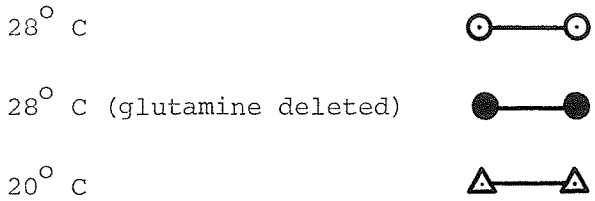

GS TRANSFERASE ACTIVITY
(units/mg)
0
0.5
1.0
1.5
4
5
6
7
8
9
10
TIME (d)

Fig. 17. Total glutamine synthetase activity in strain B30 grown on CS7 media.

About 0.08 and 0.20 mg of protein were used per assay of the 20° C and 28° C grown cells respectively. One unit of enzyme catalyzes the formation of 1.0 μmole of γ-glutamyl hydroxamate per min at 540 nm.



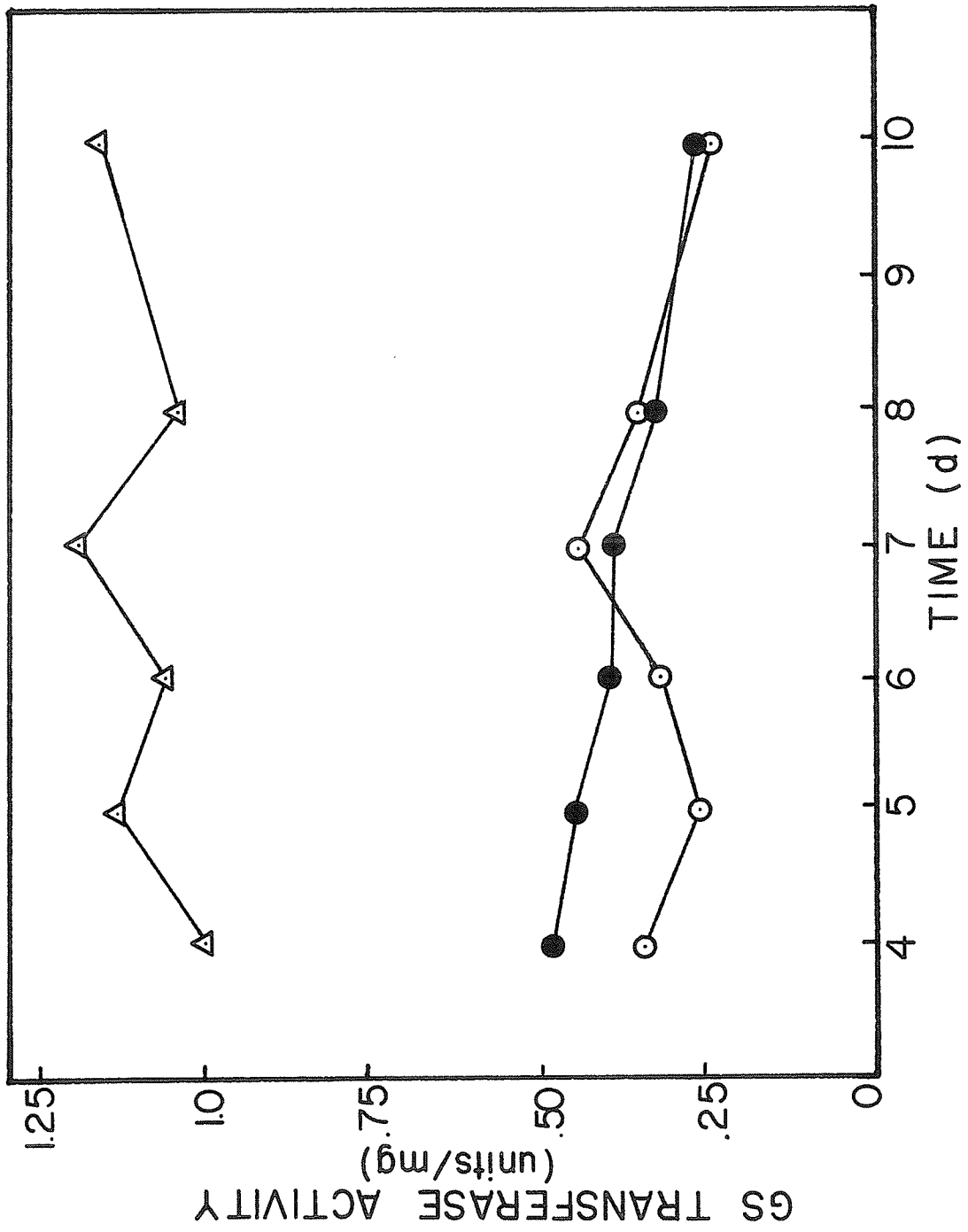

GS TRANSFERASE ACTIVITY
(units/mg)
1.25
1.0
.75
.50
.25
0
4
5
6
7
8
9
10
TIME (d)

TABLE 2

Relative States of Adenylylation of Glutamine Synthetase of *Rhizobium sp.* R309 grown at 28° C.

Time of Incubation (days)	Glutamine Synthetase Activity *		
	$-Mg^{2+}$ **	$+Mg^{2+}$ ***	% Mg/Mn
4	0.72	0.075	10.4
5	1.53	0.199	13.0
6	0.453	0.028	6.18
7	1.22	0.159	13.0
8	0.88	0.213	24.2
10	0.729	0.121	16.6

* specific activity is defined as μmoles of γ-glutamyl hydroxamate formed / mg of protein / min.

** transferase assay

*** forward assay

Table 2. The ratio of unadenylylated enzyme to total GS present was calculated by determining the specific activity of the forward assay and dividing by the specific activity of the transferase assay. The results indicate that only 10-24% of the glutamine synthetase activity is due to the unadenylylated enzyme. In other words, 75-90% of the glutamine synthetase is in the adenylylated or inactive form. The highest percentage of adenylylated enzyme occurs at 7d.

Relative States of Adenylylation of GS in Strain B30

The relative state of adenylylation of glutamine synthetase in B30 is shown in Table 3. At 20°C, the highest percentage of unadenylylated enzyme was 67.3 and occurred on day 7 whereas at 28°C it was 64.1% at day 6. There was no forward activity observed at 4d and 10d at 28°C indicating that the enzyme was completely adenylylated. The results indicate that temperature had little effect on the adenylylation of glutamine synthetase.

The effect of glutamine on the relative adenylylation of glutamine synthetase in cultures of B30 is shown in Table 4. Cells grown on complete CS7 (1 mM glutamine) showed the highest percentage of unadenylylated enzyme at day 6 (64.1%). In comparison, cells grown on CS7 with glutamine deleted showed the highest percentage of unadenylylated enzyme on day 7 (50.9%).

However, the level of active GS in glutamine-deprived cells does not differ from that of cells grown on CS7.

TABLE 3

The Relative Adenylylation of Glutamine Synthetase of Rhizobium sp. B30

Time of Incubation (days)	Glutamine Synthetase Activity *					
	28° C			20° C		
	$-Mg^{2+}$ **	$+Mg^{2+}$ ***	% Mg/Mn	$-Mg^{2+}$	$+Mg^{2+}$	% Mg/Mn
4	0.345	0.0	0	1.01	0.113	11.2
5	0.268	0.075	28.0	1.13	0.318	28.1
6	0.315	0.202	64.1	1.06	0.326	30.8
7	0.427	0.105	24.6	1.19	0.801	67.3
8	0.340	0.043	12.7	1.04	0.581	55.6
10	0.255	0.0	0	1.16	0.286	24.7

* specific activity is defined as μmoles of γ-glutamyl hydroxamate formed / mg of protein / min.

** transferase assay

*** forward assay

TABLE 4

Effect of Glutamine on the Relative Adenylylation of Glutamine Synthetase from *Rhizobium* sp. B30

Time of Incubation (days)	Glutamine Synthetase Activity *					
	(-) glutamine			(+) glutamine		
	$-Mg^{2+}$**	$+Mg^{2+}$***	% Mg/Mn	$-Mg^{2+}$	$+Mg^{2+}$	% Mg/Mn
4	0.468	0.076	16.2	0.345	0.0	0.0
5	0.446	0.098	22.0	0.268	0.075	28.0
6	0.400	0.109	27.3	0.315	0.202	64.1
7	0.389	0.198	50.9	0.427	0.105	24.6
8	0.340	0.059	17.4	0.340	0.043	12.7
10	0.265	0.145	54.7	0.255	0.0	0.0

* specific activity is defined as μmoles of γ-glutamyl hydroxamate formed / mg of protein / min.

** transferase assay

*** forward assay

DISCUSSION

DISCUSSION

In the assays for glutamine synthetase (GS) activity in Rhizobium spp. strains 122 DES, R309, 32H1 and B30, fluctuation in the levels of activity was revealed not only among the four test strains but also in replicate samples of the same strain in some cases. It would seem that undetermined factors influence the activity of GS and should be investigated. Recently, Stadtman et al. (1979) evaluated the assay systems used for the determination of GS activity and emphasized that the isoactivity pH for each organism or strain must be determined. Goldberg and Hanau (1979) also examined one of the procedures for determining GS activity and found that assays conducted at pH's other than the isoactivity point will overestimate the GS levels of cultures containing predominantly adenylylated enzyme and underestimate the GS level of cultures containing predominantly unadenylylated enzyme. In this investigation the pH of the assay system used was adopted from Bender et al. (1977) and has also been used by Ludwig (1978) for the measurement of GS activity in rhizobia. The method of harvesting whole cells of the test strains with CTAB, described by Bender et al. (1977), was used to prevent a change in the state of adenylylation of glutamine synthetase. All of the samples of one test strain were incubated on different days so that the harvesting could be synchronized and assays performed on all samples (4-10d cells) with the same assay system. Often levels of unadenylylated GS in the forward assay system were too low to be measured in strains 122 DES, R309 and

32H1 which could suggest that the pH was too low for an accurate measurement of the GS activity. Strain B30 showed the highest levels of unadenylylated enzyme - up to 67% - (Tables 3 and 4) which seems to indicate that B30 may have an isoactivity point closer to pH 7.7 than the other strains tested.

Strain B30 was chosen for assaying the effect of protein on the transferase activity of GS (Fig. 12) because it exhibited high activity. Because of the marked similarity between the standard curves of the forward activity and the transferase activity of GS, the effect of protein concn on the forward reaction was not determined.

The temperature optima of GS activity showed strain differences. Even though the test strains of rhizobia belong to the slow-growing group which form nodules on tropical zone leguminous plants, two of the test strains, 32H1 and B30, showed a higher level of GS activity at 20°C (Fig. 16 and Fig. 17). Strain R309, on the other hand, demonstrated its temperature optima to be 28°C (Fig. 13) and showed almost a two-fold increase in the level of activity at 28°C over that at 20°C (Fig. 15).

Maximum GS activity of strain 122 DES occurred at 6 days (Fig. 14) while maximum GS activity of strains R309, 32H1 and B30 occurred at 7 days (Fig. 15, 16 and 17). Even though data on nitrogenase was not provided on all test strains due to technical problems in the acetylene reduction tests, results show that a peak in GS activity occurs at the same time as optimum nitrogenase activity (Fig. 7).

The acetylene reduction assay system was used for the detection of nitrogenase activity in strains 122 DES and 32H1. Maximum nitrogenase activity in strain 122 DES occurred at seven days (Fig. 7) which agrees with the results of Gibson and coworkers (1976) who also found optimum nitrogenase activity at seven days. In the Lupin plant host, nitrogenase activity is not measurable until the twelvth day during nodule development (Robertson et al., 1975).

The test assay system, which was modified by the addition of the chelating agent Fe-citrate-EDTA and by the lowering of the agar concn to 1.0%, failed to increase the yield of ethylene. Acetylene reduction was attained with 122 DES at an oxygen atmosphere of 3.1% and with 32H1 at 27.0%. The assay system failed to detect nitrogenase activity in R309 and varying the oxygen atmosphere from 1.6% - 30.0% did not resolve the problem. This study indicates that nutritional and other requirements need to be determined for the expression of nitrogenase activity of each strain.

Another factor involved in the detection of acetylene reduction was the observation that the surface growth was composed of two colonial variants as has been reported by others (Kuykendall and Elkan, 1976; Upchurch and Elkan, 1977). Cultures showing a preponderance of small, translucent clones frequently showed nitrogenase activity while cultures with a preponderance of large, slimy clones were consistently negative. Such variants were first considered contaminants and were checked for purity by phase-contrast microscopy. Subculturing the single clones resulted in revertants. However Kuykendall and Elkan (1976) found that the addition of Tween

40 to the media permitted the separation of the colonial variants.

It has been found that an organic source of nitrogen is required for nitrogen-fixation in rhizobia (Pagan et al., 1975). Growth studies on strain R309 showed that increasing (2mM) or deleting glutamine from the liquid CS7 media (Fig. 5) had no effect on the growth rate contrary to the report of Gibson and coworkers (1976). It has also been reported that succinate (12.5 mM) or another carboxylic acid is required for optimum nitrogenase activity (Gibson et al., 1976). There was no noticeable effect on growth yield after reducing the concn of Na-succinate to 6.0 and 3.0 mM (Fig. 6) although Gibson et al. (1976) found a decrease in dry weight with decreasing (5.0 and 2.5 mM) and an increasing concn (25 mM).

It was surprising to find a change to the bacteroid-type of cell in the photomicrographs of two day cultures of R309 (Fig. 8 and Fig. 9). Bacteroid-like structures were also observed in electron micrographs of strain R309 harvested during optimum nitrogenase activity (personal observation). Nitrogenase activity has been reported by Gibson et al. (1976) and Ludwig and Signer (1977) as appearing as early as three days but not measurable at two days. The earliest samples tested in this investigation were four day cultures and nitrogenase activity was measurable. The six day cultures deprived of molybdenum, which has been shown to prevent the syntheses of nitrogenase (Brill et al., 1974), did not demonstrate any changes similar to those in the two day cultures (Fig. 10 and Fig. 11).

The optimum activity of the glutamine synthetase and nitrogenase enzyme systems occurred at ca. seven days but changes to the bacteroid-type of cell occurred at two days. This indicates that the nitrogenase enzyme is present early on in development but that the expression of nitrogenase does not occur until later.

Induction of nitrogenase by glutamine synthetase has yet to be confirmed. Goldberg and Hanau (1979) suggest that there may be no obvious correlation between the average state of adenylylation of GS and the expression of a number of enzymes involved in nitrogen metabolism. However, they observed as have others (Magasanik *et al.*, 1974), that a strong correlation exists between the intracellular levels of glutamine synthetase and the activities of these other enzymes.

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