CHAPTER I

INTRODUCTION
The world population will have increased by 2-3 billion by the end of 20th century. To meet the basic requirements of food, an adequate supply of fixed nitrogen is essential. If this is to be achieved by the use of fertilizer nitrogen, an estimated 160 million tons will be required at a cost of more than 40 billion US dollars. This indicates the magnitude of the problem.

The production of nitrogenous fertilizers is an energy intensive process. Natural gas equivalent to approximately $3 \times 10^8$ barrels, or more, of oil is being consumed annually for the synthesis of ammonia by the Haber-Bosch process. With the limited and rapidly depleting supply of fossil fuel coupled with the high costs, the use of fertilizer nitrogen to increase agriculture production is questionable.

It is for this reason that biological nitrogen fixation has been recognised as a high priority research area. According to recent estimates available, 69% or 175 million tons of nitrogen fixed annually world wide, comes from the biological process. The industrial synthesis accounts for only 15% of the total. Agronomically important nitrogen fixing associations account for over 70% of that portion attributed to biological fixation. The biggest advantage of the biological system is that nitrogen is fixed directly
at the site where it is required and without loss. On the other hand, fertilizer nitrogen applied to fields, suffers up to 50% loss due to natural processes of denitrification and leaching.

Strategies are being developed to exploit biological nitrogen fixation in different ways:

a. increasing plant productivity by maximizing the use of and increasing the efficiency of currently important symbiotic associations.

b. an expanded use of free living nitrogen fixers to substitute for fertilizer nitrogen sources in agriculture, and

c. eventually, to extend the ability of nitrogen fixation to the cereals, either directly or by the formation of symbiotic association with nitrogen fixers. The advances in genetic engineering techniques provide a great potential for achieving these goals.

To evaluate each of these strategies, a thorough knowledge of the fundamentals underlying the biological process of nitrogen fixation must be understood in toto. Although the basic process of fixation remains the same, differences are evident in the contributory processes of the diverse types of microbes capable of dinitrogen fixation.

1.0 Nitrogen fixers in the environment

The variety of microbes capable of fixing atmospheric
nitrogen are shown in Table 1.

Table 1: Representative diazotrophs

(A) Symbiotic

<table>
<thead>
<tr>
<th>Associate</th>
<th>Azospirillum</th>
<th>Azotobacter paspali</th>
<th>Grasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>Rhizobium spp.</td>
<td>Legumes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rhizobium</td>
<td>Parasponia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Frankie</td>
<td>Alnus</td>
<td></td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Fungi</td>
<td>Angiosperms and</td>
<td>Gymnosperms</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Azolla</td>
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</tbody>
</table>

The interaction of bacteria with the roots of higher plants and their association which contributes to the nitrogen cycle is well documented. Effective Rhizobium-legume interactions fix nitrogen as long as permissive conditions exist. Cyanobacteria form associations with fungi, liverworts, ferns, angiosperms and gymnosperms. The Azolla - Anabaena azollae association contributes significantly to agriculture, particularly in areas of rice cultivation.
Table 1: (B) Free living

<table>
<thead>
<tr>
<th>Type</th>
<th>Phototrophs</th>
<th>Chemotrophs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>Cyanobacteria (Nostoc; Anabaena)</td>
<td>Thiobacillus ferrooxidans</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Chromatium</td>
<td>Xanthobacter autotrophicus</td>
</tr>
<tr>
<td></td>
<td>Chlorobium</td>
<td>Azotobacter vinelandii</td>
</tr>
<tr>
<td></td>
<td>Rhodospirillum</td>
<td>Bajerinckia indica</td>
</tr>
<tr>
<td></td>
<td>Rhodopseudomonas</td>
<td>Facultative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus polymyxa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Desulfovibrio</td>
<td>Desulfovibrio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desulfotomaculum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clostridium</td>
</tr>
</tbody>
</table>

Woody trees and shrubs have root nodules harbouring Actinomycetes called Frankia\(^1^)\(^1\). Such plants have a wide geographic distribution and often are the first plants to colonize poor soils\(^1^2\), justifying their ecological
importance.

Azospirillum which forms an association with grasses has not yet been thoroughly investigated to determine the amount of nitrogen it can fix\(^1\) and hence a considerable number of questions remain unanswered.

Except for cyanobacteria, the other free living fixers are not of agricultural importance. Their contribution to soil fertility is about 0.1% of that fixed by the symbiotic process\(^3\). These include Azotobacter spp., Clostridium pasteurianum and the sulfur bacteria such as Desulfovibrio. The latter is the only non-photosynthetic nitrogen fixer which occurs naturally in the sea and is important ecologically in the formation of nitrogen-containing marine sediments.

Facultative anaerobes can fix nitrogen only under anaerobic conditions. Klebsiella is the best example of this group. The photosynthetic anaerobes include both sulfur and nonsulfur bacteria such as Chromatium and the purple non-sulfur nitrogen fixing genus Rhodospirillum.

Legumes comprise one of the largest plant families. They include, vegetables, grain and forage legumes. Many of the legume seeds contain 30-45% protein and hence are highly nutritious. In contrast, non-legumes like corn, have only 10-15% protein in their seeds. Though the
eventual aim of agronomic research is to extend the biological process of fixation to the non-legumes, the legumes themselves are of utmost importance as far as present day biological nitrogen fixation is concerned.

2.0 **Rhizobium - Legume Symbiosis**

Biological nitrogen fixation by rhizobia is biochemically a very intricately controlled process. The intricacies start from the very beginning with the recognition between the *Rhizobium* spp and its specific host. This is then followed by the process of infection and nodule development. Once the nodule is biochemically "mature", nitrogen fixation commences and carries on up to senescence which occurs just before or during pod filling. During the active stages of nitrogen fixation, a number of internal and external biochemical factors, as well as environmental factors control the process.

2.1 **Infection**

The process of cellular recognition between microorganisms and higher plants is receiving considerable attention. The complementary components of the cell surface responsible for this recognition has been the focus of most biochemical studies. According to the lectin-recognition hypothesis, specific complementary
lectin-polysaccharide interactions serve as the basis of host specificity.

During early stages of the infection process, the bacteria attach via hapten-reversible interactions and later, irreversibly bind to the host cell. This ability of the bacterium to attach to the root hair is controlled by "Roa" (root hair attachment) genes which are located on large plasmids in some rhizobia. Roa- phenotypes are defective in hapten specific attachment. However, recent investigations point out that successful infection requires additional events of cellular recognition. An understanding of this recognition code would provide ways in which the nitrogen fixing associations with crops could be widened.

The lectin hypothesis has been most extensively studied in the R. trifolii - clover symbiosis. Host specific attachments have also been demonstrated in R. japonicum - soybean and R. leguminosarum-pea root systems.

The first point of physical contact between the microbe and its plant host, also termed as Phase I attachment, occurs within minutes after inoculation of R. trifolii cells onto the clover host. Immunochemical analysis has suggested structural relatedness on both symbionts. The bacterial antigen contains receptors
for the multivalent clover lectin, trifoliin A, which is present on clover seeds and seedling roots. The use of a specific inhibitor, 2-deoxy D-glucose, was exploited to demonstrate the specificity of the *R. trifolii* - clover interactions. Consistent with the above results, lectins on pea, alfalfa and soybean roots, accessible for binding to the appropriate rhizobia have been demonstrated.

The characteristic firm binding or anchoring of the bacterial cell to the root hair surface is the Phase II adherence. This phase might be important for the triggering of root hair curling and successful penetration during infection. Electron microscopic studies show a fibrillar structure associated with the bacterium, the function of which remains obscure.

Although attachment of rhizobia to root hairs is essential, several undefined events must occur to initiate root hair infection. This is clear from the fact that very few root hairs to which rhizobia have bound eventually become infected. This could be due to the transient susceptibility of the root hairs to infection or the appearance of a unique determinant on the bacterial polysaccharide. The involvement of root-exudate in eli-
citing faster modulation response by *Rhizobium* spp. in the cowpea system has been demonstrated. A number of such events probably controlled by different gene/s product/s may be playing an equal if not greater role in successful infection.

The presence of combined nitrogen (15 mM nitrate) inhibited all morphogenetic steps involved in modulation of clover seedlings. The details of this regulation is not clear.

Recent reports by Pueppke have shown that soybean lines, which genetically lacked soybean lectin, could be infected. The infection thread formation and subsequent nodule development did take place. He also reported *R. japonicum* strains infecting cowpea, suggesting a non-specificity in the recognition of the host. These observations argue against the lectin hypothesis of Dazzo and Hubbell.

2.2 Nodule development

The development of nodules occur in either of the two patterns known i.e. "meristematic" or "spherical". In the former, there exists an apical or peripheral meristem e.g. *Trifolium* spp., *Vicia* spp., *Medicago* and *Pisum sativum*, while in the latter a central mass of bacteroids containing cells continuously divide e.g. *Glycine max* and *Arachis hypogea*. 
The infection might occur through the infection thread as in case of \textit{Lupinus angustifolius}\textsuperscript{39} and \textit{G.max}\textsuperscript{40} or there might not be any infection thread like structure present at all. In the latter case, the infection appears to be intracellular as in \textit{A.hypogea}\textsuperscript{41}. The same may be true in other legumes but convincing studies are lacking.

Once in the plant cell the bacteria are found to be surrounded by peribacteroid membranes which are of plant origin. These newly infected cells undergo a number of cytological changes\textsuperscript{42,43}. As the infected cells mature, the bacteroids cease dividing, increase in volume\textsuperscript{44,45} and attain different shapes\textsuperscript{45}.

Biochemical studies of the infection process are difficult due to the small quantity of infected tissue available. However, studies indicate that legume nodules have been found to contain high concentration of phytohormones like indoleacetic acid (IAA), as compared to non-nodulated roots\textsuperscript{46}. The presence of appreciable cellulase activity in plants has been suggested to facilitate localized cell wall softening required during infection\textsuperscript{47} and probably intracellular release of rhizobia. Genetic evidence suggest that both plant and rhizobial factors may be involved in rhizobial release.
The modulation process can be divided into three stages viz. early intracellular infection stage, mature nodule and the senescent stage. In meristematic nodules, all the 3 stages may be present simultaneously in different zones making biochemical changes difficult to study though one can separate the zones by dissection. In the case of spherical nodules, the development is synchronous, wherein the developmental stages can be studied easily.

The key indicator for mature nodules is the ability to reduce acetylene. Unless in vitro measurements of nitrogenase components are carried out, the acetylene reduction capacity is referred to as "nitrogenase content". The nitrogenase catalyzed hydrogen evolution activity also reflects the same picture.

Few studies on plant DNA in legume nodules have been carried out. DNA sequences complementary to the G. max leghaemoglobin (Lb) mRNA have been reported. Analysis of the poly (A+) mRNA content of G. max nodules also show the presence of Lb mRNA. The Lb mRNA increased during nodule development and contributed upto 25% of the total mRNA in mature nodules.

Lb appears 2-4 days before nitrogenase activity is detectable and forms upto 40% of the total protein. The haem component of Lb is synthesized by the bacteroids.
Logcki and Verma have termed proteins that are present in nodules but not roots as "nodulins". Upto 20 such low molecular weight nodulins were detected in G. max. Bacteroids have an altered cytochrome make-up compared to aerobically grown cultures of Rhizobium. Recent reports in R. japonicum indicate the presence of cytochrome a, a3, b, c and probably o. The respiratory pathway within bacteroids containing cytochrome P450 operates most efficiently at oxygen concentrations of 0.1μM and appears to be responsible for the high ATP generation. Ubiquinone has been isolated from both bacteroids and cultures of R. japonicum. It functions as a factor in electron transport to oxygen.

Nitrogenase makes up 10-12% of the bacteroidal protein in mature nodules. Radioimmunoassay of the nitrogenase components showed that traces of the nitrogenase polypeptide C1 (60,000 molecular weight fraction) were present on the 10th day after inoculation whereas the other polypeptide component C2 (34,000 molecular weight fraction), appeared later. In P. sativum, experiments indicated a half life of 2-3 days for both plant and bacteroid proteins, including nitrogenase and Lb. Similar studies have also been carried out in L. luteus.
The bacteroid cell wall is structurally different from that of the bacterium. This was concluded from observations that only a fraction of the bacteroids, when plated onto solid media, formed colonies. Further investigations suggested that the bacteroids were osmotically sensitive due to cell wall changes.

Reports regarding the respiration of nodule tissue have indicated similar respiration rates for both, mature nodules and roots. Alternatively, reports indicate that the respiration potential of nodules is not saturated at normal pO₂ because of the diffusive barrier that exists for gases. In L.angustifolius nodules, this diffusive barrier increased 5 fold between 10-21 days after inoculation. P.sativum and G.max bacteroid suspensions showed maximum oxygen uptake rates at pO₂ lower than that required by aerobically grown cultures. G.max bacteroids have been shown to possess at least two terminal oxidases or "oxidase affinity states", one of which functions most efficiently at a very low concentration of oxygen (0.1 μM dO₂).

Sucrose is generally accepted as being the main carbohydrate source for both roots and nodules. However, in the case of V.faba nodules, glucose and fructose were
the predominant photosynthates and sucrose predominated in the roots. In G.max nodules, in addition to sucrose, myo-inositol and (+) - chiro-inositol formed a large proportion of the photosynthesize.

Sucrose is believed to be hydrolyzed by invertase present in nodules. Subsequent catabolism has not been studied in detail. The requirement of C_4-organic acids by bacteroids is absolute. These TCA intermediates are replenished by an anapleurotic source.

The lipid content in G.max nodules has been reported to reach 20% (w/w), 16% being contributed by the bacteroids. In L.angustifolus nodules, the major fraction of lipids was found to be contained in the peribacteroid membranes.

A number of regulatory factors operate directly or indirectly to influence the nodule maturation. Environmental factors such as salinity, temperature, combined nitrogen supply, light intensities, essential nutrients and ions play an important role in nodule development. In general, the legume and Rhizobium cells have to exchange correct signals at each stage before going on to the next.

Claims have been made for the transfer of macro-
molecules from the bacteroids to the plant cytoplasm but the evidence is not convincing\textsuperscript{55}. However, in the case of Agrobacterium Ti plasmids, their partial integration into plant nuclei has been established\textsuperscript{84,85}. Whether the same occurs in the case of rhizobial plasmids is still unknown.

2.3 Enzymes involved in Nitrogen fixation

2.3.1 Nitrogenase

This is the first enzyme involved in fixing atmospheric nitrogen. Besides being a complex enzyme to study, it is regulated by a very precise and sensitive mechanism. The enzyme consists of two oxygen labile metalloproteins, the MoFe protein, also called dinitrogenase, and the Fe protein, also known as dinitrogenase reductase. Both these proteins are essential for enzyme activity. The two proteins function in a 1:1 ratio, though 2:1 complexes have been detected\textsuperscript{86}. In addition, the enzyme requires MgATP, a source of low potential electrons in the form of ferredoxin or flavodoxin and an anaerobic environment\textsuperscript{87,88}. In addition to nitrogen, the enzyme can reduce a number of triple bonded compounds. In the absence of such compounds it reduces protons to hydrogen. Carbon monoxide can inhibit all the reduction processes of nitrogenase excepting that of protons.

The dinitrogenase from all sources are usually
\( \alpha_2\beta_2 \) tetramers, with a molecular weight in the range of 200-235,000. Most preparations of this protein contain 2 atoms of molybdenum and 20-36 atoms of iron per molecule, depending on the source. The acid labile sulfur is generally less than the iron content. The molybdenum along with the iron and sulfur can be extracted as a Fe-Mo cofactor which can activate the inactive MoFe polypeptides of certain mutants. An alternative fixation pathway in *A. vinelandii* which does not require molybdenum has also been reported.

The known mechanisms of regulation are a) control of the enzyme concentration, b) regulation by nutrients and c) modulation of enzyme activity.

### 2.3.2 Hydrogenase

Hydrogenase is an enzyme which removes electrons from hydrogen or reduces protons. There are three types of hydrogenases -

a) the reversible hydrogenase associated with anaerobic bacteria which acts as an electron sink and associated with the phosphorylative reduction of ferredoxin.

b) the unidirectional hydrogen uptake hydrogenase which was considered until recently to be confined to aerobes.

c) the unique ATP dependent hydrogen evolution associated with nitrogenase.
The first system studied was the hydrogenase from \textit{R. leguminosarum}. The majority of the rhizobial strains examined do not possess an uptake hydrogenase excepting certain strains of \textit{R. japonicum}.

Hydrogenase is an iron sulfur protein catalyzing the activation of hydrogen according to the following reaction:

\[ \text{H}_2 + \text{acceptor (ox)} \rightarrow 2\text{H}^+ + \text{acceptor (red)} \]

Hydrogenase systems in rhizobia are membrane bound and can be classified as unidirectional or hydrogen oxidizing type. Attempts to demonstrate a reversible hydrogenase in \textit{Hup+ bacteroids} has met with little success.

The possible physiological role of hydrogenase has been proposed by Dixon. Hydrogen utilization within nodules may -

a. prevent hydrogen inhibiting nitrogenase
b. afford respiratory protection to the oxygen labile nitrogenase and
c. help in conservation of energy.

Sufficient evidence exists only for the 2nd and 3rd roles stated above. \textit{Hup+ strains} have been shown to increase plant and seed nitrogen but further confirmation of these results is still awaited.

The expression of hydrogenase requires low oxygen concentrations, low carbon levels and a hydrogen source.
The involvement of cyclic nucleotides in the expression of hydrogenase has also been implicated\textsuperscript{108,109}.

2.4 Ammonia assimilation

Ammonia which is fixed by the bacteroids is excreted into the nodule cytosol fraction\textsuperscript{110}. A very small quantity (\textasciitilde 5\% of the ammonia fixed) is utilized by the bacteroid\textsuperscript{111}. Free living nitrogen fixing rhizobia are also seen to excrete ammonia\textsuperscript{112}. The levels of ammonia assimilatory enzymes in the bacteroids were too low to assimilate all the nitrogen fixed\textsuperscript{113}. The general observation is that rhizobial glutamine synthetase (GS) is repressed under conditions where nitrogenase is derepressed. Conversely, the nodule cytosol has high levels of these ammonia assimilatory enzymes\textsuperscript{114}. The increase in these enzyme activities coincided with the upsurge in levels of Lb and nitrogenase activity\textsuperscript{114}.

Studies with $^{15}$N$_2$ showed that the first organic products were glutamate and glutamine. Asparagine was identified as a sink in serradella nodules which correlated with the high levels of asparagine found in xylem or nodule bleeding sap of several legumes\textsuperscript{115,116}.

2.4.1 Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) has been found to be of near universal occurrence in organisms. In higher plants,
GDH has been found in almost all plant tissues tested so far. In legume nodules, both the bacteroid and plant cytosolic fractions contain the enzyme.\(^{117}\)

The enzyme L-Glutamate:NAD oxidoreductase (deaminating) E.C. 1.4.1.2 catalyses the formation of glutamate using 2-oxoglutarate in the reductive amination reaction. In the deaminating reaction, glutamate is the preferred substrate.\(^{118}\) Higher plants have 2 distinct forms of GDH. The first is a mitochondrial enzyme which is generally NAD linked whereas the second is a NADP linked chloroplast enzyme. Both enzymes have dual coenzyme specificity. However the former shows a higher activity with NADH\(^+\) than NADPH\(^+\). In soybean root nodules the cytosolic GDH showed 7 fold higher activity than the bacteroidal GDH.\(^{119}\)

The pea root enzyme has been reported to have a molecular weight of 208,000.\(^{120}\) Plant NAD linked enzymes are metalloproteins. Both EDTA and \(\beta\)-hydroxyquinoline inhibited the soybean hypocotyl enzyme.\(^{121}\) The pH optima of most GDHs for the aminating reaction ranges from 7.6 to 8.2 and from 8.5 to 9.5 for the deaminating reaction.\(^{118}\)

GDH from plants have a high Km value for ammonia, which ranges from 10 to 80mM.\(^{118}\) Hence, its role in fixation could only exist when ammonia levels were extremely
high. Alternatively the in vitro Km values might not reflect the actual in vivo Km for ammonia or the enzyme might be localized in microenvironments rich in ammonia. The enzyme reaction mechanism for the soybean and safflower cotyledon enzyme species has been proposed to be an ordered reaction \(^{121}\).

2.4.2 Glutamine synthetase

L-\(\text{\textit{G}}\)lutamate:ammonia-\(\text{\textit{L}}\)igase (ADP forming) E.C. 6.3.1.2 or glutamine synthetase (GS) is a key enzyme in both amino acid and purine/pyrimidine biosynthesis. GS is distributed throughout the plant, although activity varies from one tissue to another \(^{122}\). Specific activities are higher in shoots than in roots excepting in nitrogen fixing plants \(^{123}\). The nodule cytosolic GS showed higher activity than the bacteroidal GS. The former is similar to the plant enzyme and constitutes 2\% of the nodule protein \(^{124}\).

GS is a multimeric enzyme with identical subunits. In eukaryotes, the enzyme exists as an octamer with a molecular weight of 350-390,000 whereas in prokaryotes, it exists as a dodecamer of molecular weight 590-600,000.

The enzyme catalyses the formation of glutamine, the first step in ammonia assimilation. The most effective di-valent cation for the catalysis is Mg\(^{++}\). The enzyme reaction mechanism involves the formation of an enzyme bound \(\gamma\)-glutamyl phosphate subsequent to which ammonia binds and the
products formed are released. GS can also catalyse certain other reactions like arsenolysis of glutamine etc. 125.

The enzyme is catalytically active only under conditions of nitrogen limitation. When the ammonia concentration rises, GS levels drop with a concomittant rise in GDH 126,127.

Rhizobia possess two isoenzymes of GS which differ in their pH, thermal stability and effector sensitivity 128. Ludwig 129 has suggested a hypothesis for the involvement of these isoenzymes in different aspects of nitrogen metabolism. However, no supporting evidence for his view exists. Recently, the R. meliloti GSI gene has been cloned into cosmids in an attempt to elucidate its role in symbiotic nitrogen fixation 130.

The regulation of GS has been well worked out 131, particularly in the members of the enterobacteriaceae. The regulation is seen to occur at the following levels -

a. enzyme synthesis 132,133,
b. covalent modification of the enzyme 134 and

2.4.3 Glutamate synthase

This was one of the last ammonia assimilatory enzymes to be reported 138. It was first detected in Klebsiella aerogenes and subsequently, many plants were shown to possess glutamate synthase 139. Originally named glutamine (amide):
2-oxoglutarate aminotransferase (oxidoreductase NADP⁺) hence called GOGAT is now been classified as L-glutamate: NADP⁺ oxidoreductase (transaminating) E.C. 1.4.1.13.

The enzyme is present in most tissues of angiosperms. In nodules, the bulk of the activity is located in the cytosol, low levels being detected in the bacteroids. The plant enzyme is either ferredoxin-dependent or pyridine nucleotide dependent. The bacterial enzyme is highly specific for pyridine nucleotides as the electron donor (NADH/NADPH).

The lupine nodule cytosolic enzyme is a monomer of molecular weight 235,000 and has FMN as a prosthetic group. Both bacterial and plant enzymes have a pH optima between 7.4 - 7.8. The affinity of legume nodule GOGAT for 2-oxoglutarate is higher in legume nodules (30-50 µM) than the enzyme of non-nodulated roots (0.4-1.0mM). The same holds true for glutamine, 0.4-0.6mM for the enzyme of nodulated roots and 1-2mM for the enzyme of non-nodulated roots. This suggests that the nodule enzyme is distinct from its counterpart in the root. A number of mono and divalent cations, amino acids and nucleotides influence the enzyme activity. The enzyme is in general unstable, losing 20% of its activity per week.
2.5 Asparagine metabolism

It had been shown that asparagine could be synthesized when precursors like ammonia, aspartate, fumarate, malate and succinate were supplied to Phaseolus multiflorus leaves. Succinate has been found to be the most effective carbon source. Hence the conclusion was that asparagine biosynthesis must be compartmentalized and hence not accessible to externally applied aspartate. Subsequent efforts to localize the system have not been successful.

In root nodules, evidence exists for the synthesis of oxaloacetic acid from phosphoenol pyruvate by PEP carboxylase. This not only supplies the carbon skeleton for asparagine biosynthesis but also reassimilates the carbon-dioxide evolved due to nodule metabolism.

In Lemma plants, between 20-50% of the total nitrogen uptake passes through the asparagine pool. It was concluded that asparagine was formed directly from glutamine by the transfer of the amide group. Asparagine synthesis has also been detected in germinating seeds. In this case, glutamate is converted to asparagine and liberates 6 carbon atoms for respiration. Cyanide incorporation into asparagine has also been demonstrated in plants, bacteria and fungi but the possibility of this pathway is doubtful since the generation of cyanide is extremely limited in these systems.
2.5.1 Asparagine synthetase (AS)

L-Aspartate: ammonia ligase (AMP-forming) E.C. 6.3.1.1 can use either ammonia or glutamine as the amino donor for the synthesis of asparagine.

\[
\text{Aspartic acid} + \text{glutamine} + \text{ATP} \xrightarrow{\text{Mg}^{++}} \text{Asparagine} + \text{glutamate} + \text{AMP} + \text{PPi}
\]

\[
\text{Aspartic acid} + \text{ammonia} \xrightarrow{\text{Mg}^{++}} \text{Asparagine} + \text{H}_2\text{O} + \text{AMP} + \text{PPi}
\]

AS is a particularly unstable enzyme and hence precautions during extraction are essential\textsuperscript{148}. Inclusion of substrates also stabilize the enzyme activity\textsuperscript{147,115,155}. The presence of an AS inhibitor in mung bean seedlings has been detected\textsuperscript{148}. Studies with actinomycin D and cycloheximide have shown that both transcription and translation are required for the appearance of AS activity in germinating cotyledons\textsuperscript{148,152}.

Both Lupin nodule and maize roots possess AS activity\textsuperscript{154-156} but the compartmentalization or localization in any specific organ has not been established. AS from different plants show a lower Km for glutamine (0.04-1mM) than for ammonia (2-3.2mM) suggesting that glutamine is the normal substrate. The \textit{L. albus} enzyme could use D-glutamate, \textit{γ}-methylene glutamine and \textit{N}-acetyl glutamine as substrates instead of \textit{L}-glutamine\textsuperscript{156}. The reaction was inhibited by inhibitors of glutamine-amide transfer reactions, namely azaserine and albizzine\textsuperscript{157}.
The best studied enzyme is from *L. luteus* which is an oligomer of 160,000 and forms a dimer in the presence of Mg$^{++}$ and ATP giving the catalytically active form. The mouse cell and *E. coli* AS$^O$ are small enzyme species with molecular weights of 105,000 and 82,000 respectively.

Kinetic analysis show that the enzyme reaction has a ping pong mechanism in which aspartate, glutamine and MgATP bind to different enzyme forms not interconvertible through reversible steps. It is suggested that ATP or glutamine bind to the enzyme first, followed by a release of PPi and the formation of an enzyme bound adenylate. Aspartate then binds to the active site. Contrary to this, aspartate binds first in case of the mouse and *E. coli* enzymes.

The presence of an asparaginase has interfered with studies regarding levels of AS during nitrogen fixation. In *vivo*, such a condition might not arise due to compartmentalization of these two enzymes. Measurements of enzyme activity can only be made if the two enzymes are separated by fractionation or by the use of a specific inhibitor for asparaginase. Such investigations would throw light on the role of AS and asparagine in nodule metabolism.
2.6 Bioenergetics

The biological reduction of nitrogen catalyzed by dinitrogenase reductase and dinitrogenase requires energy in the form of ATP and a low potential reductant such as reduced ferredoxin. The free energy released upon ATP hydrolysis is probably sufficient to overcome the energy of activation barrier for the reaction. In vitro estimates for ATP hydrolyzed/e⁻ transferred ranges from 2 to over 20. This ratio is dependent on a number of variables like ATP concentration etc. The generally agreed stoichiometry for the reaction is:

\[
\text{N}_2 + 6 \text{Fd (red)} + 12 \text{ATP} + 12 \text{H}_2\text{O} \rightarrow 2\text{NH}_4^+ + 6\text{Fd (ox)} + 12 \text{ADP} + 12 \text{Pi} + 4\text{H}^+
\]

\[\Delta G = -136 \text{ kcal/mol} \quad \text{N}_2 \text{ reduced.}\]

The input energy is equivalent to 174 kcal (6 Fd + 12ATP) which corresponds to complete oxidation of 0.58 moles of glucose, assuming that the coupling efficiency is 40%. In terms of apparent costs, biological nitrogen fixation costs less than biological reduction of nitrate or the industrial process of ammonia synthesis.

Estimating the overall cost of the biological process would mean an estimation of all individual associated processes. At present, such values are available.
for a very few processes like carbon-dioxide efflux. However this approach has helped evaluate budgets for nitrogen fixing plants. Nevertheless, experiments that have been conducted for this evaluation\textsuperscript{164} are —

a. estimates based on comparative growth rates.

b. estimates based on respiration measurements.

c. acetylene reduction assays.

d. estimates based on differences in yield.

The limitations here are, that \textit{each} method used depends on different techniques and is based on different assumptions. Secondly, they do not distinguish between the cost associated with nitrogen fixation, growth and maintenance. Approaches have now been directed wherein parameters like fixed nitrogen addition or the use of \textit{fix}-rhizobial strains are varied during the study\textsuperscript{165,166}.

2.6.1 \underline{Estimates of ATP requirement in free living forms} —

The free living bacteria have been exploited as a model system for the studies of nitrogen fixation costs. Such studies using \textit{Clostridium pasteurianum}\textsuperscript{167} showed a 43% decrease in cell yield under nitrogen fixing conditions, yielding a calculated value of 20 moles ATP utilized/mole nitrogen fixed. This accounted for 28% of the total ATP
synthesized. Similar studies in *K. pneumoniae* gave estimates of 29 moles ATP/mole nitrogen reduced. Using *K. pneumoniae* mutants defective in assimilating ammonia, Anderson and Shanmugam have shown that the minimum cost would be between 21-25 moles ATP/mole nitrogen fixed. This excluded costs for cell growth and ammonia assimilation. After deducting reducing costs for carbon utilization, the values fell to 14-16 moles ATP/mole nitrogen fixed. This is in good agreement with theoretical values based on *in vitro* measurements. A very low value has been obtained for similar experiments in *Azotobacter chroococcum* but this has not been explained satisfactorily.

2.6.2 Estimates of ATP requirement in symbiotic relationships

Efforts have also been directed towards determining the cost of symbiotic nitrogen fixation. Experiments which take into account growth rate, total carbon content, and carbon exchange rates show, that symbiotic nitrogen fixation requires higher energy than the assimilation of combined nitrogen. Correlating respiratory activity to \(^{15}\)N\(_{2}\) incorporation in whole plants, showed that 4-6 and 7-10 g C were consumed per g nitrogen fixed for nodules and nodulated roots respectively. This agrees well with the theoretical values available. Studies for evaluating the yields gave inconsistent results due to variations in environmental conditions. Hence definite agronomic costs
for nitrogen fixation and ammonia or nitrate assimilation will have to be determined for each location.

2.6.3 Factors affecting efficiency -

Factors known to affect the efficiency or apparent costs for nitrogen fixation are as follows -

a. interactions between photosynthesis and nitrogen fixation$^{180,181}$.

b. PEP carboxylase activity in root nodules$^{181,182}$.

c. hydrogen evolution by nitrogenase and an efficient uptake hydrogenase$^{183,184}$.

d. export of nitrogenous products from the nodules$^{185}$.

e. sites and relative rate of nitrate reduction in roots and nodules.

2.6.4 Enzymological aspects - (ATPase)

The most complex enzyme systems are the ones associated with the generation of energy. These include the electron transport proteins and a proton ATPase complex ($H^+\text{ ATPase E.C. 3.6.1.3 ATP phosphohydrolase}$). The role of this ATPase is to catalyze the formation of ATP using the electrochemical gradient. Functionally 4 different classes of ATPases are present.

a. those associated with ion transport.

b. those associated with ATP synthesis.

c. those associated with macromolecular transport.
The H\textsuperscript{+} ATPase is known as the ATP-synthetic or mitochondrial type and resides in bacteria, mitochondria, chloroplast and chromaffin granules. The enzyme functions reversibly, either in the ATP hydrolysing direction for transport\textsuperscript{186} or in the ATP synthesizing direction as a synthesizer of ATP from ADP and Pi\textsuperscript{187}.

The mitochondrial ATPase is a complex enzyme consisting of 2 separable moieties. The water soluble structure or head piece is called the F\textsubscript{1} ATPase and is hydrophilic whereas the F\textsubscript{0} portion is a hydrophobic entity. F\textsubscript{1} is involved in ATP hydrolysis and F\textsubscript{0} in H\textsuperscript{+} translocation. The molecular weight of the former is approximately 3,80,000 whereas that of the latter is unknown. The F\textsubscript{1} ATPase consists of 5 different polypeptides \(\alpha, \beta, \gamma, \delta\) and \(\varepsilon\).ler.

The subunit stoichiometry of this ATPase is similar in almost all systems studied so far\textsuperscript{187,188}. The composition of the F\textsubscript{0} counterpart is less clear but evidence of a 4 polypeptide makeup is available. In addition, inhibitor binding proteins have also been detected on gel electrophoresis. They include factor B, factor 6, DSCP, DCCD binding protein and an uncoupler binding protein.

The mechanism of H\textsuperscript{+} ATPase involves a number of
complex interactions of which at least two are as yet unknown. These include how $H^+$ translocation is coupled to transport and how $H^+$ translocation is coupled to ATP synthesis. Mechanisms of the other events involving the enzyme functions are available. These include the reaction pathway, catalytic sites and the adenine nucleotide binding sites. However, the details of the physicochemical process carried out by the enzyme are not absolutely clear.

The study of regulation of mitochondrial $H^+$ ATPase has been confined to 3 different types of molecules: Anions, nucleoside triphosphates and a small peptide inhibitor. Of these, the peptide inhibitor has been studied in detail. It exists in bacteria, mitochondria and chloroplasts and has a molecular weight in the range of 5-20,000. It inhibits ATP hydrolysis but is ineffective against the ATP synthesis reaction.

2.7 Nitrogen transport

The first stable product of nitrogen fixation is ammonia which is released from the bacteroids into the plant tissue. The ammonia thus has to pass through three membranes. It is not known whether the pH gradient plays a role in its transport and in which form the ammonia is transported.
Xylem sap analysis has given clues as to the form in which nitrogen is transported. Based on this, plants fall into 2 categories namely ureide exporters and amide exporters. In the former group, allantoin and allantoic acid comprise 50% or more of the nitrogen exported whereas in the latter case, asparagine, glutamine or substituted amides are the exported products.

Glutamine is the primary product of nitrogen fixation. Secondary products such as asparagine and ureides are synthesised using glutamine as an amino donor. The synthesis and transport of ureides in soybean and cowpea have been investigated in great detail. Detailed studies regarding formation of citrullin, canavanine and substituted amides are not available, though these compounds comprise major exports in the xylem of certain nitrogen fixing systems.

There seems to be a better economy of both energy and carbon utilization in nodules which produce ureides rather than amides. 8.5 moles of ATP are utilized for every mole of ammonia fixed as ureides as compared to 15 moles ATP per mole of ammonia fixed as asparagine. On the other hand, amino acids require less energy for incorporation into protein since ureides first have to be converted into amino acids.
A sparse network of vascular tissue exists in nodules. Thus, materials to be exchanged between cells and vascular tissue have to traverse a number of cells. Certain nodules contain specialized "transfer cells" which show high rates of transport for nitrogenous compounds. Bleeding xylem saps have shown between 1-6 mg nitrogen/ml, reflecting the high levels of nitrogen transported from the nodules. The transpiration rate and solubility of nitrogenous products in the xylem sap are also thought to govern the export of nitrogen by the xylem.

2.8 Nodule senescence

Nodule senescence is considered to have a 3 fold practical importance.

a. the nitrogen availability which depends on the life span of the nodule.

b. release of host specific rhizobia back into the rhizosphere.

c. nodule decay may result in underground transfer of combined nitrogen to other plants.

The longevity of nodules is governed by the growth habits of the plant i.e. annuals, biennials or perennials. Premature senescence in certain cases correspond to ineffective plant-Rhizobium combinations, or a sudden reduction of photosynthate. In the latter case, haem
levels decreased by 40% after 2 days of darkness and by the third day, nodules became green. The levels of adenylates and the ATP/ADP ratio also declined.

Combined nitrogen in the form of NaNO₃ reduces acetylene reduction activity, nodule growth and results in a premature degeneration of bacteroid-containing tissue. High temperature is seen to have similar effects. With regard to the water content, both drought and water logging of soils seriously affect nodule longevity.

Nodule senescence is detected by a decline in acetylene reduction, nitrogenase catalyzed hydrogen evolution and Lb content. A number of changes regarding the nitrogen metabolizing enzymes and nitrogen export, occur. The lytic enzymes take over the breakdown of the nodule structure with a concomittant decline in pH. The bacteroids get replaced by rod forms and eventually a rise in the bacterial count in the rhizosphere is seen.

At present two views regarding nodule senescence exist - a) a decline in carbohydrate supply and b) reduction in the ratio of carbohydrate/combined nitrogen. Though the latter view seems more likely, convincing data is not available. The role of hormones such as cytokinins has also been suggested.
3.0 Genetics

3.1 Genetics of free living diazotrophs

The majority of studies on regulation of the nif genes have been carried out in *Klebsiella pneumoniae*, a facultative anaerobe. Mutants of *K. pneumoniae* have been used as physical probes and functionally compared with nif genes in diazotrophs like *Rhizobium*, cyanobacteria, *Azospirillum* and *Azotobacter*.

In *K. pneumoniae*, the nif complex consists of 17 genes and is located near the his biosynthetic genes. Expression of the nif genes is controlled by the ntr gene products and the proteins encoded by nifL and nifA. The nifA product activates while that of nifL represses the transcription of the other nif transcriptional units. The ntr system consists of an operon of 3 genes - ntrA (*glnF*) ntrB (*glnI*) and ntrC (*glnG*) \(^{216,217}\). This operon is autogenously regulated by ntrB and ntrC in response to the magnitude of fixed nitrogen \(^{218}\).

Varying the growth conditions by increasing or decreasing the concentrations of ammonia or oxygen, prevented nif expression in different ways \(^{219}\). Derepressed cultures in the absence of molybdenum do not synthesize significant amounts of nitrogenase \(^{220}\). This control might be encoded by the nifD and nifK gene products \(^{221}\). Though nitrogenase is active at 37°C, its synthesis at that temperature
is repressed. Such a regulation is brought about by the nifA and nifL gene products. The regulation by ATP levels might also be mediated via gene products of nifL and nifA.

In *A. chroococcum*, genes homologous to the *K. pneumoniae* nifHDK genes are contiguous which suggests a cluster similar to the one present in *K. pneumoniae*. However, a thorough study in *Azotobacter* is awaited.

The following are the functions of the nif gene products as known to date:

- **nifHDK**: Nitrogenase structural genes.
- **nifQBVNE**: FeMo cofactor, structure and function.
- **nifFJ**: Electron transfer to dinitrogenase reductase.
- **nifMS**: Activation or processing of dinitrogenase reductase.
- **nifLA**: Regulatory proteins.
- **nifUXY**: Unknown.

### 3.2 Rhizobium genetics

In fast growing rhizobia i.e. *R. leguminosarum*, *R. phaseoli* and *R. trifolii*, genes for nodulation and nitrogen fixation are found to be located on large plasmids (>200Kb in size). These plasmid-linked phenotypes could be transferred from one strain to another at high frequencies. Such transconjugants were seen to acquire
the transferred plasmid. The loss of the plasmid or deletion therein caused a loss of nodulation and/or nitrogen fixation\textsuperscript{227}. Thirdly, mRNA from bacteroids hybridized strongly with the \textit{sym} plasmid of the \textit{Rhizobium} strains whereas mRNA from free-living rhizobia did not\textsuperscript{228}. Fourthly, sequences homologous to \textit{K. pneumoniae nifH} and \textit{nifD} were located on the plasmid\textsuperscript{229}.

In the case of \textit{R. meliloti}, another fast grower, the \textit{nod} and \textit{fix} phenotypes were coded by megaplasmids\textsuperscript{227}. This \textit{sym} megaplasmid has been mobilized into \textit{nod}\textsuperscript{-} mutants\textsuperscript{230} and into \textit{Agrobacterium tumefaciens}\textsuperscript{231}. In the latter case, exconjugants formed ineffective nodules on \textit{M. sativa}. In \textit{R. meliloti}, the \textit{nifHDK} genes are encoded by the megaplasmid \textit{p R m e 41b} and the organization of the genes resemble that of the \textit{K. pneumoniae} cluster\textsuperscript{232}. The \textit{fix} genes are present on both the chromosome and megaplasmid. However, little regarding their function in symbiotic nitrogen fixation is known\textsuperscript{233}.

The situation in slow growing \textit{Rhizobium} species such as \textit{R. japonicum} is different. Though \textit{nod}\textsuperscript{-} and \textit{fix}\textsuperscript{-} mutants are available\textsuperscript{234}, genetic analysis has proved difficult and inefficient\textsuperscript{235}. As yet, no evidence exists regarding the plasmid borne nature of \textit{nif} and \textit{nod} genes\textsuperscript{236}. However, cloning techniques have proved more useful than classical
genetic analysis. The nitrogenase structural genes from *R. japonicum* 110 have been cloned and nifH is found to be unlinked with nifD and nifK\(^\text{237}\). Modified methods in site directed mutagenesis are being used to exploit the cloned DNA to generate *R. japonicum* mutants\(^\text{238}\).

### 3.3 Transformation

The discovery of genetic transformation in bacteria by Griffith in 1928 was one of the important landmarks in the history of genetics\(^\text{239}\). Subsequently the recognition of DNA as the hereditary material was confirmed\(^\text{240}\). It remains a process in which the physical or chemical alterations in the DNA structure can be correlated with its biological activity.

Genetic transformation in *Rhizobium* was first reported by Balassa\(^\text{241}\). However, those observations are now viewed with doubt, since sonication which is now known to shear DNA, was used for preparing the donor DNA\(^\text{242}\). Work on genetic transformation in *Haemophilus, Diplococcus, Bacillus* etc. evolved methods for studying this process in detail.

Workers have shown that rhizobial recipient could distinguish between heterologous and homologous DNA\(^\text{243,244}\). Subsequently, a detailed study in cowpea rhizobia and *R. japonicum* followed\(^\text{243,245}\). Studies on DNA uptake and mapping auxotrophic markers in *R. japonicum* have also been carried out\(^\text{245-247}\).
The uptake of donor DNA during genetic transformation takes place only during a competence stage. The development of this competence stage varies with the rhizobial species under study\textsuperscript{244-246}. In \textit{R. phaseoli}, maximum competence occurs every 2-3 hours, coinciding with the cell division. In \textit{R. japonicum}, the cells developed competence during the late logarithmic phase\textsuperscript{248}, whereas in \textit{R. trifolii} it is related to the polysaccharide synthesis\textsuperscript{249}.

A competence factor (CF) has been isolated from \textit{R. japonicum} and was seen to be proteinic in nature\textsuperscript{250}. The appearance of this CF coincided with the development of competence. The physiological role of this CF is still obscure.

**PRESENT INVESTIGATION**

The assimilation of ammonia during symbiotic nitrogen fixation involves a number of enzymes. Though glutamate/glutamine are the first assimilated products in most \textit{Rhizobium}-legume symbiotic systems, a number of other amino acids have also been detected. Asparagine which forms the major amino acid exported by \textit{G. max} root nodules is synthesized by AS.

To determine the involvement of AS in free living \textit{R. japonicum} a study was carried out using various nitrogen sources. Another enzyme known to play an important function in nitrogen metabolism, GS, was also studied simultaneously.
GS levels served as the basis for the comparison of AS activity with a well studied ammonia assimilatory enzyme. The factors that may regulate AS activity, in vivo, were investigated. The ability of the enzyme to synthesize asparagine from either ammonia or glutamine, as the amino donors, was investigated.

During the symbiotic association of *R. japonicum* - *G. max*, the levels of AS were monitored over the nitrogen fixing period. An analysis of the other ammonia assimilatory enzymes, GDH, GS and GOGAT were also carried out. These studies were conducted in both the bacteroidal and nodule cytosolic fractions. The regulation and the ability to synthesize asparagine from the amino donors, glutamine and ammonia, were analysed. A similar investigation was conducted for the nodule cytosolic GDH.

The minimum energy required to reduce one mole of nitrogen to ammonia is between 12-20 moles of ATP. The most efficient mechanism of ATP generation in the nodule is the process of oxidative phosphorylation. An important enzyme involved in this process is ATPase. The enzyme was studied from both the asymbiotic and symbiotic stages of *R. japonicum*. The properties of *R. japonicum* ATPase were studied and compared with those of other systems.
In the symbiotic association of *R. japonicum - G. max* an attempt was made to find out if this energy generating system would be functional. For this purpose ATPase from the bacteroidal, mitochondrial and nodule cytosolic fraction was studied. Further, this study was conducted over a nine week period of the symbiotic association, so that the activities of ATPase from different fractions could be correlated with the nitrogen fixing process in the nodule.

Studies on genetic transformation in *R. japonicum* have indicated that a competent stage has to develop for DNA to be taken up by the recipient cell. For the development of competence, a medium rich in amino acids is essential. Hence a study was carried out to find out precisely which amino acid/s were essential for the development of competence.

During the competence stage, certain specific proteins have been reported to be preferentially synthesized in *Streptococcus sanguis* and *S. pneumoniae*. The presence of such specifically synthesized new protein species during the competence phase of *R. japonicum* was investigated. The location, membrane bound or cytosolic, of these proteins was also studied.
REFERENCES


