CHAPTER-I
INTRODUCTION
Nitrogen is one of the major constituents of life and is also a major component of the atmosphere. As nitrogen in its molecular form is inert, it becomes a limiting factor in the global agricultural output. The atmospheric nitrogen can be useful to plants and animals only in a complex form. Nitrogen in the form of nitrate, nitrite and ammonia is required for assimilation by plants while, nitrogen in heterotrophic form is required by animals, abundance of nitrogen in the atmosphere cannot ensure its biological availability to plant and animal systems. Secondly, the process of denitrification by denitrifying bacteria, decrease the biological availability of assimilable nitrogen in the soil. To complete the nitrogen cycle in soil, the fixation of atmospheric (inert) nitrogen to its biologically available (assimilable) form is a must. The process of nitrogen fixation, therefore, attains significance in the ecology of the planet.

Three major factors contribute significantly to the process of atmospheric nitrogen fixation, which can be divided into two major categories, (a) abiological nitrogen fixation and (b) biological nitrogen fixation.

(a) **ABIOLOGICAL NITROGEN FIXATION:**

Chemical process: Conversion of nitrogen to ammonia by Haber-Bosch process (1), under conditions of high temperature (300-500°C), pressure (300-1000 atmosphere)
and a catalyst is a well known industrial process. The world annual output by this process is estimated to be about 42 millions of metric tonnes of nitrogenous fertilizer (2).

**Electrical discharges:** Significant amounts of nitrogen are fixed by ionizing phenomenon, such as cosmic and UV irradiations, meteor trails, ozonization, thunderstorms and lightning. The ionizing phenomenon provides very high energy needed for nitrogen to react with oxygen or hydroxyl ion of the rain water. Rain water, as a result, is known to contain dissolved nitrogen oxides, including nitrate as a major component. The total amount of nitrogen delivered to earth by rainfall can be estimated to be 45 millions of metric tonnes per annum (3).

(b) **BIOLOGICAL NITROGEN FIXATION:**

Biological nitrogen fixation is one of the major plant activities in its biochemical interest and importance for the maintenance of life on earth. There have been advances in the understanding of the physiology, biochemistry, genetics and regulation of biological nitrogen fixation during the past decade (3-15). The economic and ecological limitations of heavy use of chemical fertilizers have stimulated the research in nitrogen fixation by micro-organisms. About one-half of the total amount of nitrogen fixed annually is contributed by nitrogen fixing organisms (3, 7).
The diversity of biological nitrogen fixing organisms can be resolved into three broad classes:

(a) Asymbiotic or free living
(b) Obligate symbiosis and
(c) Associate symbiosis.

The existence of nitrogen fixing organisms was established as early in 1862 (16), but it was in 1894 that Winogradsky isolated and characterized the anaerobe, *Clostridium pasteurianum*, that could use molecular nitrogen as its sole source of nitrogen (16 a). Since then a wide variety of nitrogen fixing organisms have been reported (17-19). The distribution of nitrogen fixing organisms ranges from obligate aerobes through facultative to obligate anaerobic organisms. New Guinea inhabitants excrete more nitrogen than that ingested by them. The presence of nitrogen fixing *Klebsiella* species in the gut has been shown to be responsible for this (20). Nitrogen fixing *K. pneumoniae* has already been reported in the mammalian intestines (21).

The nitrogen fixing organisms can be represented or grouped as follows:

(A) Asymbiotic or free-living: This group includes some bacteria and algae which can fix nitrogen independently. The group includes aerobic as well as anaerobic nitrogen fixing organisms.
(i) Anaerobic nitrogen fixing organisms: These are: *Clostridium pasteurianum*, *Bacillus polymyxa*, Aerobacter aerogenes and *Klebsiella pneumoniae*; phytosynthetic bacteria: *Rhodospirillum rubrum*; *Chromatiurom vinosum*, *Chlorobiium thiosulfatophilum* and *Rhodopseudomonas palustris*.

(ii) Aerobic nitrogen fixing organisms: These include: *Azotobacter vinelandii*, *Azotobacter agilis* and *Azotobacter chroococcum*. Phytosynthetic Blue green algae: *Nostoc muscorum* and *Anabaena cycindrica*.

(B) Obligate symbiosis: This is exemplified by bacteria of the genus *Rhizobium* that occur in the root nodules of legumes of soybean, peas, alfalfa and clover, and an unidentified microorganism, presumed to be an actinomycete, in various non-leguminous root nodulated angiosperms (22-24). The angiosperm group, which fixes about the same amount of nitrogen as the leguminous plants, is important for forest crops and is not of major significance to agronomic crop production (2, 11).

Neither the rhizobia nor the plants can fix nitrogen independently, but are effective in the symbiotic relationship, even though nitrogen fixation occurs only in bacteroids (18). Three exceptions to this general phenomenon have been demonstrated:
(1) a rhizobia nodule on a non-leguminous plant, *Trema cannabina* (25), (2) a free living nitrogen fixing *R. trifoli* hybrid was obtained by intergenic transfer of nif\(^+\) operon(s) by DNA transformation from *A. chroococcum* to *R. trifoli* (26), and (3) simple factor derived from leguminous as well as non-leguminous plant cells enabled some free living rhizobia strains to express a low level of nitrogen fixing activity (27, 28).

(C) **Associate symbiosis**: An asymbiotic nitrogen fixer forms a loose association with plants. They live around the roots and supply fixed nitrogen to the plants. The non-nitrogen fixing plants provide a suitable environment for the nitrogen fixing organism. This can be important in stimulation of nitrogen fixation in natural ecosystems.

The occurrence of associate symbiosis between nitrogen fixing bacteria and roots of grasses and other species have been reported. Dobereiner (29) has described the association of *Azotobacter pampali* with roots of *Paspalum notatum*, and *Spirillum lipoferum* with roots of *Digitaria decumbens* (30). Associations of nitrogen fixing bacteria and algae with sugarcane, maize and rice roots have already been suggested (31-36). Nitrogen fixing blue green algae, often referred to as
rice paddy organisms' has also been shown to occur in symbiotic association in the maritime lichens and in the root nodules of certain cycadaceae (37, 38). The fixed nitrogen is rapidly transported to the remaining parts of the plant in such systems (39). A nitrogen fixing association between angiosperm Gunnera and Nostoc occurs in the swollen leaf bases of the plant and there is rapid transfer of fixed nitrogen to other parts of the plant (40). The nitrogen fixing Klebsiella sp. have been found to be associated with the leaves of tropical plants of Rubiaceae (41).

Most of the biological nitrogen fixation is attributed to the symbiotic nitrogen fixing systems and the photosynthetic organisms (42, 43). There is no true symbiosis of phytosynthetic bacteria with higher plants. The contribution by blue green algae to the nitrogen status of most natural ecosystems is more important than that by heterotrophic microorganisms, where the nitrogen fixing activity may often be limited by scarcity of the organic substrate (43). In photosynthetic organisms, the energy (ATP) and the reductant needed for nitrogen fixation may be provided by phytosynthesis and therefore, external supply of carbohydrates is not necessary as with other nitrogen fixing bacteria. It has been estimated that 2-27 mg nitrogen is fixed for each gram of carbohydrate oxidized.
The approximate efficiency values in terms of mg nitrogen fixed/g carbohydrate consumed are: *Azotobacter*, 10-20; *Clostridium*, 2-27; *Klebsiella*, 51; and *Bacillus*, 12 (43).

The abundance of heterocystous algae has been regarded as an index of the algal nitrogen fixing potential of a soil (44). Blue green algae may make up to about 70% of the total algal flora (45). In Russia, the species of blue-green algae present has been used as an indicator of soil type (46). Photosynthetic bacteria are also abundant in paddy soils and nitrogen fixation in anaerobic muds was attributed to these (47, 48). Photosynthetic bacteria may undoubtedly be important sources for fixing nitrogen in the environment where they occur (49), but their distribution is restricted by their exacting environmental requirements (50), so that they are probably of much less importance than blue green algae on a worldwide scale.

Russian scientists who had so far emphasized the nitrogen fixing role of *Azotobacter*, with which they inoculate some of their agricultural crop, have now concurred with the view that these organisms do not fix nitrogen quickly enough to play an important role as a provider of combined nitrogen in agriculture. Their main beneficial effect in cultivated soils results from the production of growth substances which stimulate plant yield (51).

The *Rhizobium* - legume symbiosis is estimated to contribute 40 millions of metric tonnes of nitrogen
annually to grain legumes (2). The root nodulated legumes fix 100-300 kg. N/ha/yr. Free living and non-photosynthetic organisms (e.g., *Azotobacter* and *Clostridia*) seem to make a small contribution towards enriching nitrogen content of soils (5-25 Kg. of N/ha/yr) (11). Algae found either in association with plants and fungi, or free-living, have been estimated to fix 25-100 Kg. N/ha/yr in rice paddy field (32-36). A plant-algal association, *Azolla-Anabaena*, is reported to fix 60-140 Kg. N/ha/yr (52,53).

The genus *Rhizobium* contains those bacteria which are able to form morphologically distinct nodules on the roots of members of the *Leguminosae*. It also includes bacteria which no longer possess an invasive characteristic, but have an authentic history of origin from an invasive strain. *Rhizobia* are Gram-negative, rod-shaped bacteria (0.5-0.9 x 1.2-3.0 μm) which often contain prominent granules of poly-β-hydroxybutyrate. The genus is subdivided into two classes of species, (a) the fast growers which have a mean generation time of 2-4 hr and form relatively large (2-4 mm diameter) colonies on agar media within 3-5 days and (b) slow growers which have a mean generation time of 6-8 hr and form small colonies (≪ 1 mm diameter) on agar media after 7-10 days. Strain differences exist amongst species and each strain is defined as a culture not known to have a common clonal history with any other culture.
Table 1 lists various Rhizobium species and the host genera which they preferentially invade. Within an infected nodule the rhizobia enlarge and change shape to form bacteroids. Depending on the host-strain combination, one or more bacteroids are found enclosed within a membranous envelope located within host cells of the inner region of the mature root nodule. Not all cells of this inner region are invaded and an outer cortex of uninvaded host-cells surrounds the bacteroid region (54).

Table 1 Host preference of Rhizobium species

<table>
<thead>
<tr>
<th>Species</th>
<th>Preferred host genus</th>
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<tbody>
<tr>
<td>1. Rhizobium leguminosarum</td>
<td>Pisum, Vicia, Lathyrus, Lens</td>
</tr>
<tr>
<td>2. R. trifolii</td>
<td>Trifolium</td>
</tr>
<tr>
<td>3. R. phaseoli</td>
<td>Phaseolus</td>
</tr>
<tr>
<td>4. R. meliloti</td>
<td>Medicago, Melilotus, Trigonella</td>
</tr>
<tr>
<td>5. R. lupini</td>
<td>Lupinus, Ornithopus</td>
</tr>
<tr>
<td>6. R. japonicum</td>
<td>Glycine max</td>
</tr>
<tr>
<td>7. Coupea type</td>
<td>Vigna, Macroptilium and others</td>
</tr>
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</table>

Species - 1-4 are fast growers and 5-6 are generally slow growers. The 'coupea rhizobia' group contains a diverse range of Rhizobium which cannot be accommodated in group 1-6. They include both slow and fast-growing strains, some of which may even infect non-leguminous angiosperms.
An overall reaction involved in the reduction of nitrogen to ammonia in Rhizobium can be summarized as follows, as described by Bergersen (55).

\[ \text{N}_2 + n\text{ATP} + 6\text{NADPH} + 2\text{H}^+ \rightarrow 2\text{NH}_4^+ + n\text{ADP} + n\text{Pi} + 6\ \text{NADP}^++ 6e^- \]

Biological nitrogen fixation (energy requirement 355 kJ/mol NH$_4^+$) is approximately twice as efficient as the industrial process which has an energy requirement of 680 kJ/mol NH$_3$. The energy input in both biological and industrial systems is needed to overcome the activation energy for the reduction of nitrogen (55).

The basic requirements for nitrogen reduction are (a) the enzyme nitrogenase, (b) a strong reducing agent, ATP and Mg$^{++}$, and (c) low oxygen tension.

A detailed study of the structure and mechanism of nitrogenase in vitro has been possible since Carnahan et al (56), demonstrated that nitrogenase activity in crude cell extracts of Clostridium pasteurianum was stable when oxygen was rigorously excluded. Since then, several groups have contributed to our understanding of nitrogenase (57 - 60). So far, active cell-free extracts from some 15 organisms have been reported. In spite of their different physiological status (free-living anaerobic bacteria;
the legume symbionts; microaerophiles; facultative and strict aerobes; photosynthetic bacteria and blue-green algae), the properties of nitrogenases from different sources show remarkable similarities. The enzyme consists of two metalloproteins, both of which are essential for activity. The larger, MoFe protein, binds to the reducible substrate and might be termed the "true" nitrogenase (61), and the smaller, the Fe protein interacts with ATP and Mg²⁺ in a hydrolytic reaction. Both proteins are irreversibly destroyed by oxygen, the Fe protein most rapidly in vitro, and protection of these proteins from damage by oxygen is one of the overriding principles of the physiology of nitrogen fixation (62,63).

Nitrogen fixation involves the reduction of nitrogen to NH₃ or NH₄⁺ and, as such, requires a source of electrons. As might be expected, the wide differences in the metabolism of nitrogen fixing organisms lead to considerable variation in the electron transport systems within them. However, the requirements of nitrogenases for a reductant make it necessary that electron carrier proteins with sufficiently low (−ve) redox potentials are made available (64). The systems which generate reducing power for nitrogen fixation are quite varied. Different forms of reductants may be used by different
organisms, or even the same organism growing under different conditions (65). Recently, investigations into electron transport pathways involved with nitrogenases in *Azotobacter* species and legume nodule bacteroids have been initiated and some success in reconstructing sequences of electron transport to nitrogenase has been discussed in general reviews of biological nitrogen fixation by Burris (66), Evans and Russell (67), Bergersen (68), Hardy and Burris (69), Dalton and Mortenson (70) and Yates and Eady (64).

The search for electron donors to nitrogenase has been centered around NADH and NADPH.

The possibility of using NADH as a donor for nitrogenase in aerobes was suggested by D'Eustachio and Hardy's success with this coenzyme in reactions with extracts of *C. pasteurianum* (71). The consistent detection of a powerful NAD-specific 3-hydroxybutyrate dehydrogenase in bacteroid extracts led to tests in which bacteroid nitrogenase was provided with NADH, generated by 3-hydroxybutyrate dehydrogenase system (72). Nitrogenase dependent acetylene reduction proceeded provided that an ATP-generating system and a dye such as benzyl viologen was added. Convincing evidences were provided to show that the reduction potential of the NAD - NADH couple of - 320 mV was sufficient for maintenance of nitrogenase
activity of crude extracts of soybean bacteroid or, A. vinelandii. In further investigations (73), the only natural components found to function in place of the dyes were FAD or FMN and an unidentified factor(s) found in nodule extracts. In these experiments, which were conducted with impure enzyme preparations, NADH - dehydrogenase markedly stimulated the transfer of electrons to nitrogenase. The enzyme was apparently needed as a link between NADH and either the dye or the flavin nucleotide. More definitive experiments were conducted by Yates (74), who prepared a homogenous NADH-dehydrogenase from A. chroococcum and clearly established its essentiality in the benzyl viologen-dependent transfer of electrons from NADH to nitrogenase.

Although it has been suggested on the basis of in vitro experiments, that in Rhizobium the reductant is NADPH (73), the evidence provided is not conclusive. It has also been suggested that other aerobic nitrogen-fixer (blue-green algae and Azotobacter) use NADPH as the reductant for nitrogen fixation. (65). The evidence which suggests that NADPH is the most likely source of electrons, is that NADPH supported nitrogenase activity is cell free extracts of A. vinelandii and blue-green algae (74a, 74b).
Benemann and Valentine (75), assumed that the NAD(P)H/NAD(P) ratio must be high to poise the redox potential for nitrogen fixation. Haaker et al (76), invalidated this theory by showing an inverse relationship between NAD(P) H / NAD(P) ratio and nitrogenase activity with increasing pO2 in A. vinelandii. By using the uncoupler 4,5,6,7-tetrachloro-2-trifluoro methylbenzimidazole and the fluorescence probe 9-amino acridine, they showed a direct correlation between nitrogenase activity and the energised state of the cytoplasmic membrane. Since the uncoupler inhibited nitrogenase activity without affecting ATP levels, Haaker et al argued that it affected electron transport. Such results imply that an intact chemostatic gradient across the cytoplasmic membrane is necessary for nitrogenase function. If this is so, then, bearing in mind that Azotobacter can produce ATP anaerobically, if the pH is poised correctly, nitrogenase could be made to function anaerobically in whole cells (77, 78). The need for an intact chemostatic gradient may account for the difficulty in obtaining cell-free extracts from Azotobacter that support nitrogenase with physiological substrates, and the low nitrogenase activities of such extracts when obtained. Such requirements may, in part, answer the criticism of
Evans and Phillips (79). They questioned the significance of experimental results with reconstituted electron transport sequences, on the basis that activities of nitrogenase were low as compared to the rates of nitrogen fixation in vitro. They also pointed out that all the components used were not obtainable in the cell-free extracts, since some of them are known to be associated with particulate fractions (78). A second criticism of Benemann and Valentine's hypothesis is that high NAD(P)H/NAD(P) ratios can also inhibit several key enzymes in *Azotobacter*. It therefore, seems both unlikely and undesirable that the NAD(P)H/NAD(P) ratio should be high in nitrogen fixing systems. Perhaps, association of electron carriers with active enzyme sites imposes constraints on the molecular structures and it changes the redox potential of those carriers in vivo to allow electron donation to nitrogenase (64).

Ferredoxins and flavodoxins are the only known biological electron carriers capable of transferring electrons to the nitrogenase. Ferredoxin was first shown to be an absolute requirement for nitrogen fixation in *C. pasteurianum* by Mortenson (80). Walker and Mortenson (81, 82) showed that it is linked directly between hydrogenase and nitrogenase.
Flavodoxin substituted for ferredoxin in iron-starved *C. pasteurianum* (83). The ubiquitous distribution of these carriers has been shown by their presence in most types of bacteria and plants examined. Some flavodoxins are constitutive while others are induced or derepressed by iron starvation. Both the classes of proteins have been reviewed (84-87). The general account of both the ferredoxin and flavodoxin has been provided by Yates and Eady (64).

If we consider the variety of bacterial types that fix nitrogen, it is not surprising that the electron transport systems to nitrogenase vary between organisms. Anaerobic bacteria, testified by *C. pasteurianum* are considered to use the phosphoroclastic oxidative decarboxylation of pyruvate, as a source of reducing power and energy for nitrogen fixation (88), where electrons are transferred directly by ferredoxin to nitrogenase. The direct link between ferredoxin and nitrogenase was proved by Walker and Mortenson (81), who showed that H₂ supported the rapid reduction of nitrogenase to Fe protein in the presence of purified hydrogenase and ferredoxin. Other enzymic reactions will provide reduced ferredoxin, but these probably make a smaller contribution than pyruvate (89). The situation is more difficult to resolve in aerobic nitrogen fixers, largely because nitrogenase activity in crude cell extracts of aerobic nitrogen-fixing
bacteria, although easily measurable with sodium dithionite as the reductant, is extremely difficult to establish with a physiological reductant. This implies that some part of the electron transport chain to nitrogenase is extremely labile. Despite this difficulty some evidences have been obtained which indicate the path of electron flow in *Azotobacter*. Benemann et al (90) proposed following scheme of electron transfer.

\[ \text{NADPH} \rightarrow \text{ferredoxin} \rightarrow X \rightarrow \text{flavodoxin} \rightarrow \text{nitrogenase} \]

This scheme is supported by evidence which includes the observations that NADPH is the most active substrate, and ferredoxin, flavodoxin; and an unknown factor; were necessary for maximum acetylene-reducing activity. The order of proteins in the scheme is supported by the observation that flavodoxin hydroquinone and not ferredoxin will support nitrogenase activity with purified nitrogenase (91, 92). Reduced ferredoxin will not reduce flavodoxin and therefore "X" becomes an intermediate.

Evans and Phillips (93) described several reconstituted electron transfer sequences, in which NADPH (provided by a generating system) transferred electrons to nitrogenase in systems containing ferredoxin-NADP reductase, flavodoxin, and ferredoxin.
from *A. vinelandii* or *R. japonicum* bacteroids. The sequence of transfer in this system is outlined in Fig. 1. The positions of flavodoxin and ferredoxin in the scheme has not been established, and the labile factors in the system have not been identified and located precisely (94). Whether or not the heat stable factor that Yates (95), has shown to stimulate nitrogenase activity in the presence of sodium dithionate, is related to factors referred to by Benemann *et al* (96), remains to be established. In the reconstituted systems involving NADPH, ferredoxin NADP$^-$ reductase from spinach has been utilized by Benemann *et al* (96), and by Wong *et al* (73). Although the existence of weak activities of ferredoxin - NADP reductase in extracts of *Azotobacter* and nodule bacteroids has been detected, the enzyme has not been characterized from these sources, and there is no convincing evidence for its presence in sufficient quantity to be of physiological importance. Active ferredoxin - NADP reductases, however, have been observed in *Bacillus polymyxa* (97) and *Anabaena cylindrica* (98).

An alternative scheme has been forwarded by Haaker *et al* (76), and Haaker and Veeger (99), who also showed that *Azotobacter* flavodoxin hydroquinone would act as an electron donor with crude preparations
Dehydrogenase

\[ \text{SUBSTRATE} \quad \text{i.e. Isocitrate} \]
\[ \text{Malate} \]
\[ \text{Glucose-6-phosphate} \]

Ferredoxin - NADP reductase

Ferredoxin - NADP reductase

FERREDOXIN \hspace{1cm} \text{(from Azotobacter or soybean bacteroids)}

Factor (not characterized)

FLAVODOXIN \hspace{1cm} \text{ (from Azotobacter or soybean bacteroids)}

Nitrogenase

\[ \text{ATP} \]

\[ \text{N}_2 \]

Fig. 1: An outline of a sequence of electron transport from NADP-linked dehydrogenases to nitrogenase. In the investigations of electron transfer in aerobic nitrogen fixers, ferredoxin - NADP reductase from spinach has been added. The order of ferredoxin and flavodoxin in the sequence has not yet been established (from 93).
of nitrogenase and they obtained evidence to suggest that an energised membrane was necessary for electron transport to nitrogenase. It is pertinent to point out that these schemes can not be generalized for electron transport to nitrogenase in aerobes, as Mycobacterium flavum apparently does not possess a flavodoxin (100). Lokau et al (101), have recently investigated physiological electron donors to nitrogenase in Anabaena cylindrica and Anabaena 7120. They found that NADPH, generated in the dark through metabolism of glucose-6-phosphate, 6-phosphogluconate or, isocitrate, or in the light by photosystem I, supported nitrogenase activity.

Nitrogenase is an oxygen sensitive enzyme (63). Thus, aerobic nitrogen fixing systems which utilise oxygen to produce ATP, must, at the same time prevent oxygen from inhibiting or damaging nitrogenase. The fact that oxygen can inhibit nitrogenase in vivo, even in Azotobacter, the most oxygen tolerant of nitrogen fixing aerobes, is demonstrated easily by increasing the \( P_{O_2} \) or the aeration rate of the culture (62, 89).

Various mechanisms by which organisms protect nitrogenase against inhibition by oxygen, have been discussed exhaustively (62, 63, 89, 102).

The simplest concept for protection of nitrogenase from oxygen damage in aerobes is that of respiratory
protection, where the organism respires rapidly enough to maintain an oxygen concentration of zero at the cell surface. It implies that the organism can respire faster in response to increased oxygen without affecting rest of its metabolism. This concept is exemplified by Azotobacter, which has a very high QO₂ values and a branched respiratory chain which is poorly coupled to ATP production at high oxygen concentrations (103).

A second means of protection apparently occurs in Azotobacter. This is called conformational protection which operates when the oxygen level becomes too high for respiratory protection to cope. Yates and Eady (64), have discussed three lines of evidences to support its existence.

1. Nitrogenase can "switch on" after a "switch off" period without any loss of activity or new synthesis.

2. Nitrogenase is relatively stable to oxygen in crude extracts of Azotobacter but is oxygen sensitive when purified.

3. "Nitrogen-limited" Azotobacter, produces the same concentration of ammonia at slow and fast dilution rates which suggests that its activity is controlled within the cell, probably by excess oxygen (104).
From these lines of evidences the "conformational protection" hypothesis results that *Azotobacter* nitrogenase is oxygen-tolerant. Haaker and Veegar (39), analysed the nitrogenase particle of Bulen and Le Comte (105), and discovered that it contained the Shethna Fe protein II (106), in equimolar proportions with the nitrogenase, against damage by oxygen. Sherings et al (107), showed that when *Azotobacter* flavodoxin hydroquinone was used as a reductant for nitrogenase, the presence of Shethna Fe protein II binds to nitrogenase and renders it inactive. Once the Shethna protein was reduced, the complex was dissociated and nitrogenase became active. This association between nitrogenase and the Shethna protein is probably specific because Yates (95), showed that several electron transfer proteins from *Azotobacter* were ineffective in protecting nitrogenase against damage by oxygen. Only crude NADH-dehydrogenase was effective, but once purified, this too was ineffective (102).

A third mechanism of self-protection against oxygen is the slime production. This is a general response of bacteria to excess oxygen (108); prime examples among nitrogen-fixers are *Deoxyxia gummosa* (109); and *M. flavum* (89). However, Wilcockson (110),
investigated the effect of slime production on nitrogenase activity of \textit{K. pneumoniae} strains upon exposure to oxygen, and concluded that slime probably contributed only marginally to oxygen resistance.

The majority of obligate aerobic bacteria that fix nitrogen have been classified as microaerophilic. This means that they can tolerate only very low levels of oxygen when they fix nitrogen although they tolerate oxygen as well as \textit{Azotobacter} when grown in the presence of fixed nitrogen. This group includes, \textit{M. flavum}, \textit{C. autotrophicum}, \textit{Spirillum lipoferum}, \textit{Methylococcus capsulatus}, \textit{Thiobacillus ferrooxidans} and the free living rhizobia (89). These organisms have low QO values and hence can afford little respiratory protection. \textit{M. flavum} may have a form of conformational protection since its nitrogenase is oxygen tolerant in particulate cell extracts (111). It can "switch on" in whole cells without a long lag after a "switch off" phase brought about by a high oxygen solution rate (89).

The most favoured hypothesis to explain oxygen toxicity is that the toxic products of oxygen metabolism, hydrogen peroxide (H$_2$O$_2$), the superoxide radical (O$_2^-$), and hydroxyl radical (OH$^-$), are the toxic agents (112). Aerobic organisms usually contain catalase and superoxide dismutase, which detoxify H$_2$O$_2$, and O$_2^-$ respectively. In general, anaerobes do not contain these enzymes and must avoid oxygen (64).
Leghemoglobin is essential for effective nitrogen fixing legume symbiosis and without it, nitrogen fixation is either slow or absent. The work carried out by Bergersen's group in particular as reviewed by Robson and Postgate (63), has rationalized the role of leghemoglobin as an oxygen transport protein. Leghemoglobin delivers oxygen for aerobic diazotrophy by the bacteroids. Yet, because of its high affinity for oxygen, it prevents the local dissolved oxygen from reaching concentrations damaging to nitrogenase. Physiological experiments with bacteroids as well as kinetic data for oxygen uptake by leghemoglobin and the bacteroid respiratory oxidase are consistent with this view, as is the marked oxygen sensitivity of rhizobial fixation *ex planta* (113). The legume nodule thus emerges as a physiological compartment for protecting nitrogenase from oxygen as well as for supplying bacteroids with photosynthate (63). Leghemoglobin is a sophistication rather than essential, because *Parespánia* (Trema) forms functional nodules without leghemoglobin and its physiological equivalents have not yet been described in *Frankia* or cyanobacterial association. Diffusion barriers partially contribute to oxygen restriction in nodules, and respiratory protection involving hydrogenase probably occurs within the bacteroids (63). Any substrate that enhances the respiration rate may augment respiratory protection.
Ruiz Argneso et al (114), showed that hydrogen through hydrogenase, augmented respiratory protection in _R. japonicum_ bacteroids; Walker and Yates (115, 116), observed that exogenous hydrogen decreased the oxygen sensitivity of _A. chroococcum_ nitrogenase, but only when the carbon source (mannitol) was in short supply. Bothe et al (117), noted that exogenous hydrogen decreased oxygen sensitivity of nitrogenase in _Anabaena variabilis_, and it is reasonable to suppose that hydrogenase and hydrogen augment respiratory protection in other organisms, including _Rhodopseudomonas capsulata_ (118).

Recently Israel et al (119), isolated and purified four different carotenoids from the membranes of the nitrogen fixing bacterium, _Azospirillum braziliense_ strain Cd, grown under aerobic conditions, and one from strain Cd-1, carotenoid synthesis did not occur under microaerobic conditions. In the presence of diphenylamine, which specifically inhibits carotenoid synthesis, the rate of acetylene reduction in strain Cd decreased to 50% of the control. Carotenoid synthesis was inhibited in cells grown in the presence of NH₄Cl at concentrations higher than 10 mM. Carotenoid synthesis started in liquid cultures of strain Cd only after the concentration of NH₄Cl in the medium decreased, and nitrogen fixation became evident at the same time.
In producing carotenoids only under aerobic conditions, _A. braziliense_ resembles _Rhodopseudomonas capsulata_ (120), and the mycelium of _Fusarium aqueductum_ (121), and _Neurospora crassa_ (122). Carotenoids have been shown to act as protecting agents against oxidative damage in microorganisms, owing to their ability to quench singlet oxygen and possibly oxygen radicals (123).

The decreased rates of nitrogen fixation following inhibition of carotenoid synthesis by diphenylamine, the production of carotenoids by _A. braziliense_ strain Cd under aerobic conditions, and the known role of carotenoids in protecting cells from oxidative damage (123), suggest that the carotenoids of _A. braziliense_ may play a role in protecting the sensitive nitrogenase system from oxygen.

The nitrogenase enzyme complex requires ATP as an energy source (124, 125). Nitrogenase activity has a linear response to increasing ATP/ADP ratios, and has a small energy threshold below which it is inactive (126). Ching (127) concludes that ATP (produced aerobically) stimulates nitrogenase activity _in vivo_ as does an increase in the energy charge. These results also suggest participation of adenylate kinase in the production of ATP for maintenance of basal metabolism and nitrogenase activity during times of restricted energy supply (e.g. a shortage of respiratory substrate).
As adenylate kinase uses 2 molecules of ADP (an inhibitor of nitrogenase), to produce one molecule of ATP, it is more efficient at relieving inhibition of nitrogenase than a system which simply converts ADP to ATP pro rata. Organic and inorganic products of nitrogenase activity (i.e. amino acids and ammonia), show no inhibitory effect on the nitrogenase enzyme complex in vitro (129).

An indirect control of nitrogenase activity may arise from the partitioning of electrons between nitrogen reduction and ATP-dependent hydrogen production. A consistent feature of many experiments in which hydrogen evolution from nodules is measured is that, as phytosynthate supply to the nodule increases, either by manipulation of the environment (e.g. light intensity), or the plant (e.g. depodding), the rate of hydrogen evolution increases relative to the rate of nitrogen fixation (129-131). This may represent an increase in ATP-dependent hydrogen evolution, possibly removing excess reducing power, or it may be a manifestation of smaller hydrogenase activity which when active, recaptures reducing power lost in hydrogen production (131). Recently, Nelson and Child (132), have isolated R. leguminosarum strains with high relative efficiency of nitrogen fixation. Two groups could be recognized: One possessing significant uptake hydrogenase activity,
the other lacking hydrogenase activity, but with low hydrogen evolution. Although the two groups did not differ with respect to plant dry weight, or nitrogen content, the identification of the latter group may be of some significance for optimizing the efficiency of nitrogen fixation (132).

Although purified nitrogenase preparations are not inhibited by NH$_4^+$, the addition of NH$_4^+$ to nitrogen fixing cultures of some organisms results in rapid inhibition of nitrogenase activity. This phenomenon is well documented in case of phototrophic bacteria (133). In *Rhodopseudomonas capsulata* (134), and *R. palustris* (135), nitrogenase activity is completely inhibited within a few minutes of the addition of NH$_4^+$ added. In *A. vinelandii* the situation is more complex. The addition of NH$_4^+$ produces a rapid reversible decrease in nitrogenase activity (136 - 138). A rapid decrease in nitrogenase activity may be due to depletion of the ATP pool caused by a temporary increase in glutamine synthetase (GS) activity. It has been shown that the addition of NH$_4^+$ to cultures of *A. cylindrica* produces a transient 40% decrease in the ATP pool (139). This suggests that NH$_4^+$ does not inactivate the nitrogenase and, consistent with this view, when nitrogenase is isolated from NH$_4^+$-inhibited *R. palustris*, the enzyme is active (135). When measured in extracts of *A. vinelandii* at different times after
addition of NH\textsubscript{4}\textsuperscript{+}, a gradual decrease in activity was observed (137); L'vov et al (140) isolated a particulate nitrogenase preparation from A. vinelandii, which was susceptible to NH\textsubscript{4}\textsuperscript{+} - inhibition as suggested by Nambiar and Shethna (138).

Bishop et al (141), demonstrated a repression of GS, accompanied by increased adenylylation, in the presence of NH\textsubscript{4}\textsuperscript{+} in free-living R. japonicum, although Scowcroft et al (142), failed to demonstrate this type of control in cultured nitrogen fixing 'cowpea rhizobia'. Bergersen and Turner (143), and Ludwig and Signer (144), have reported regulatory effects of GS on nitrogenase activity in 'cowpea rhizobia', strain 32H1, which has no GS activity and also lacks nitrogenase activity (144). Bergersen and Turner (143), noted that changes in the adenylylation state of GS occur prior to changes in nitrogenase activity in cultured nitrogen fixing 'cowpea rhizobia', especially when an imposed state of repression of nitrogenase is progressively alleviated. Repression of GS by ammonia has been reported in cultures of nitrogen fixing Rhizobium sp. (144-147), though not in bacteroids. The activity and extent of adenylylation of bacteroid GS was unaltered by ammonium treatment of either bacteroid suspensions, or of intact nodulated soybean plant, even though nitrogenase activity was reduced significantly in the latter case (141).
If NH$_4^+$ regulates GS adenylylation and hence nitrogenase synthesis in bacteroids, very precise control must exist. Since nitrogenase produces ammonium ions, which may repress GS in vivo, the NH$_4^+$ and active GS concentrations in the bacteroid would have to be closely maintained. Active GS would be necessary for the continued synthesis of nitrogenase and hence ammonia production, but assimilation of ammonia in the bacteroid would increase the glutamine concentration and hence reduce the concentration of active GS. However, Upchurch and Elkan (148), have described derivatives of R. japonicum which can simultaneously derepress nitrogenase while repressing synthesis of ammonia assimilatory enzymes.

Ammonia holds a central position in the growth of microorganisms on inorganic sources of nitrogen. Ammonia is probably used by most microorganisms capable of growth on inorganic nitrogen, and is the product of the reduction of molecular nitrogen.

Until recently there was only one major ammonia assimilatory pathway to consider: that is via the reductive amination of L-ketoglutarate (L-KG) to give glutamate, as catalyzed by the enzyme glutamate dehydrogenase (GDH). Although ammonia was
known to be assimilated into glutamine via glutamine synthetase (GS), this was not considered important in the formation of $\alpha$-ammonia nitrogen, because assimilation was into the amide position. Since then, Tempest, Meers and Brown (149), have discovered a new enzyme in bacteria that can catalyze the transfer of the amide -amino group glutamine to $\alpha$-KG, resulting in the production of two molecules of glutamate. This enzyme is known as glutamate synthase, and is often referred to by its acronym GOGAT.

The presence of GDH in the bacteria is well established (150-158). Due to a high Km for ammonia it appears unlikely that this enzyme functions efficiently in ammonia assimilation, except when the environmental ammonia concentration is high.

The enzyme GS, catalyses the irreversible reaction by which glutamine is formed from glutamate and ammonia, in the presence of divalent cation (Mg$^{++}$ or Mn$^{++}$) and ATP. The activity of GS is regulated in three different ways: by control of enzyme synthesis, by cumulative feed-back inhibition, and by a complicated system of chemical modification in the enzyme structure, which have subtle effects on enzyme activity. Several workers have found variations in the activity of GS in extracts of organisms grown on different nitrogen-containing substrates. Thus, when the organisms were grown on ammino acids or in the
presence of excess ammonia, low levels of enzyme activity were found in *Escherichia coli* (159), *Bacillus subtilis* (156-162). Meers and Tempest used a chemostat to define the factor responsible for high GS activity in cultures of *Aerobacter aerogenes* (163). They concluded that at least with several gram-negative bacteria, the ammonia concentration had a profound effect on GS activity in vivo. Also in this process, the synthesis of glutamine is followed by the reductive transfer of the amide group to ΑKG in the presence of GOGAT, leading to the formation of glutamate. The relationship between this pathway and that mediated by GDH is shown in Fig. 2. The net results of the two pathways are the same, except that the route involving glutamine requires expenditure of energy in the form of ATP. Presumably this energy expenditure is the "price that organisms pay" in order to assimilate low concentration of ammonia (149). It is interesting to note that this pathway was almost absent in glucose-limited organisms, where ammonia was present in quantities sufficient for GDH to function but energy supply was restricted. Under these glucose-limited conditions, GDH was formed in increased quantities. These early results obtained with *A. aerogenes*, suggested that alternative pathways to ammonia incorporation were used, depending on the growth
conditions. Such a view is close to the suggestions made by Umbarger (164), who was of the opinion that, under conditions of nitrogen limitation, the ATP-driven conversion of ammonia to an amide group could act as a "pump", which could scavenge the last traces of ammonia from the environment. It is relevant to note that the Km of bacterial GS for ammonia is usually low (156, 163). This observation is consistent with Umbarger's "scavenging" role for this enzyme under conditions wherein GDH (by virtue of its high Km for ammonia) would be inadequate. This alternative pathway of glutamate synthesis was first reported in _A. aerogenes_ by Tempest et al (149), and subsequently by Meers et al (165). Since then, this pathway has been shown to operate in many other bacterial species (112, 151, 156, 162, 166, 170).

The work of Nagatani et al (171) is of particular interest since it clearly demonstrated that a number of nitrogen-fixing species can utilize the GS/GOGATamination route. These results have later been confirmed by Dainty (167), and Drozd et al (172). Brown et al (156, 169), have demonstrated that marine pseudomonads grown on nitrate as their sole nitrogen source, synthesize glutamate via glutamine, whether grown in carbon or nitrogen limited environments. These species synthesized GDH when grown on excess ammonia as their
Fig. 2: Pathways of ammonia assimilation in the enteric bacteria for the production of glutamate and glutamine, and some of the roles of these compounds in intermediary metabolism. (from 15).
nitrogen source. The importance of glutamine route in the physiology of marine bacteria was confirmed by Brown et al (169). It was observed that there was a lag when organisms were transferred from a high-ammonia to a nitrate-containing medium, but there was no lag when the organisms were transferred from a low-ammonia to nitrate-containing medium. The lag in the former case was interpreted as due to the time required to synthesize the two enzymes, viz., GS and GOGAT. The conversion of molecular nitrogen or nitrate to ammonia requires the expenditure of energy by the cell and furthermore, ammonia is a repressor of both the conversions. Therefore, for organisms to continue either to fix nitrogen or reduce nitrate, a mechanism must exist that will efficiently remove free ammonia from the intracellular pool as soon as it is formed. Presumably, the GS/GOGAT pathway of ammonia assimilation performs this function, because the low Km of the enzyme GS enables the organisms to assimilate ammonia before it accumulates at levels where it would inhibit growth.

Although growing plants need to assimilate nitrogen, ultimately in the fully reduced form, excessive concentrations of ammonia or ammonium ions are toxic to plants. The toxicity of ammonia to higher plant tissues has been demonstrated repeatedly, even though the exact biochemical cause of toxicity is not still clear (173-176).
Plants possess mechanisms which not only mediate normal rates of assimilation in the presence of non-toxic amounts of ammonia, but also permit the disposal of elevated levels of ammonia. There are several ways in which plants may be able to assimilate and dispose off high concentrations of ammonia. In many cases plants may detoxify excessive ammonia by simply accelerating the pace, at which they carry out nitrogen assimilation by the usual pathways. In other cases, the normal assimilation pathway may possibly be supplemented by additional ammonia utilizing reactions, initiated only when the plant is subjected to excessive levels of ammonia (177). Exceptionally large amounts of ammonia have to be disposed off when there is either a large exogenous source of ammonia, or, alternatively, an internal source within a plant tissue is present. Large amounts of ammonia may be endogenously generated during rapid nitrogen fixation or when proteins are being degraded. In the latter case there is usually a deamination of aminoacids, the carbon skeletons then being used as respiratory substrates (177).

It is necessary to learn the mechanisms by which ammonia is efficiently metabolized, so that it can be used for growth, or atleast converted to an innocuous form, rather than accumulating to concentrations that
become toxic to plant cells. Studies in which $^{15}\text{N}_2$ was fed to detached nodules, have demonstrated that ammonia is the first stable product of fixation by nitrogenase (178). Bergerson (179), showed that the bacteroid fraction of nodules is without doubt the site of nitrogen fixation. Thus in order to metabolize and prevent excessive accumulation of ammonia generated by bacteroidal nitrogenase, the host plant must have a very efficient assimilation mechanism to make productive use of all the ammonia generated.

The two major ammonium assimilatory enzyme systems are those catalyzed by GDH and GS/GOGAT. Miflin and Lea (180), have discussed the relative significance of the two pathways. They concluded that the assimilation of ammonia into amino acids occurs by GS and GOGAT, and it is unlikely that GDH plays a major role in ammonia assimilation, except in situations of ammonia excess. Evidence in favour of this assertion is provided by the characteristics of the two enzyme systems, labeling studies with $^{15}\text{N}$ and $^{13}\text{N}$ and by the data obtained from inhibitor studies.
Having a $K_m$ (NH$_4^+$) upward of 5 mM (181), GDH can probably assimilate ammonia only when the intracellular ammonia concentration is usually high. A relatively prominent role of GDH at high ammonia levels is further suggested by the finding, that considerable increase in the overall level of this enzyme are often observed, when plants are exposed to excessive concentration of ammonia (182, 183). If the GS/GOGAT route is operating, the nitrogen of nitrate, the amide-nitrogen of glutamine, and the amino-nitrogen of glutamate, should be in equilibrium. Lewis and Pate (184), have fed these substrates appropriately labeled with $^{15}$N to pea leaves, and have shown that, for a range of time after feeding, there is no significant difference in the labeling pattern of amino acids derived from glutamine-amide $^{15}$N, $^{15}$NO$_3^-$ or $^{15}$N glutamate. Further, the kinetics of $^{15}$NO$_3^-$ and $^{15}$NH$_4^+$ assimilation in rice roots (105), and the studies of $^{13}$N incorporation in blue-green algae (106), also support the operation of the GS/GOGAT pathway. In the latter case, the use of $^{13}$N has enabled incorporation of nitrogen over periods as short as 15 seconds and the results established unequivocally that the sequence of fixation and assimilation is, $N_2 \rightarrow NH_3 \rightarrow$ amide nitrogen of glutamine $\rightarrow \alpha$-amino nitrogen of glutamate.
Methionine sulfoximine (FISO) inhibits GS and azaserine, and 6-diozo-5-oxo L-norvaline (DON) are the inhibitors of GOGAT (106, 107). The results from experiments employing $^{13}$N and also from total pool analysis show that nitrogen fixation is blocked by MSO at the level of ammonia, confirming the role of GS rather than of any dehydrogenase, as the primary assimilation reaction (106, 107).

In experiments by Stewart and Rhodes (188), who used MSO and azaserine with NO$_3^-$ grown Lemna, both ammonia and $\alpha$-KG increased significantly in the presence of MSO. The initial rate of ammonia accumulation was in accordance with the calculated rate of nitrate reduction.

GS was isolated in essentially homogenous form from sheep brain (189). Studies in several laboratories in the late 1940's and early 1950's established the reaction catalyzed by GS as:

$$\text{L-glutamate} + \text{ATP} + \text{NH}_3 \rightarrow \text{L-Glutamine} + \text{ADP} + \text{Pi}$$

A divalent cation (Mg$^{++}$, Mn$^{++}$ or Co$^{++}$) is required when GS is incubated with L-glutamate, ammonium ions, and ATP (in the presence of Mg$^{++}$, at pH 7.0 and 37°C), the reaction reaches equilibrium when about 90% of the glutamate added is converted to glutamine. When hydroxylamine is
converted to $\gamma$-glutamyl hydroxamate (189). Although GS activity is generally measured at this optimal pH range (7.0-7.4) in the presence of L-glutamate, ATP, Mg$^{++}$, and with either ammonium ions or hydroxylamine, the pH optimum of the reaction may vary from about pH 4.8 to 8.5, depending on the nature and concentration of the divalent cation present (190, 191).

Stadtman and his associates have vigorously studied the biophysical and enzymatic properties of the GS purified from *E. coli* (192). The enzyme has a molecular weight of 600,000 and contains twelve identical subunits. The subunits are arranged in two hexagonal units layered with a 4.5 nm spacing between them. In addition, a divalent cation Mg$^{++}$ or Mn$^{++}$ is required for stability.

The studies by Ginsburg and Stadtman (192), and Wohlueter et al (193), have shown that the enzymatic activity of GS is regulated by three different mechanisms:

1. By the interaction of a relaxed (inactive) and a taut (active) form in response to variations in concentrations of divalent cations.

2. By cumulative feedback inhibition by various and products of glutamine metabolism.

3. By covalent alterations of the enzyme by the reversible adenylylation of a specific tyrosyl residue on each subunit.
The adenylylated form of the enzyme is more sensitive than the unmodified form to feedback inhibition by the products of glutamine metabolism. The ATP dependent modification apparently occurs in a variety of gram-negative bacteria. The attachment and removal of the covalently bound AMP residues from GS is accomplished by the action of an adenylyltransferase (ATase), a monomer having a molecular weight of 130,000 (192). There are two factors that determine whether adenylylation or deadenylylation predominates: the levels of several metabolites and the interaction of ATase with a small regulatory protein, (P II). The levels of α-KG and glutamine have antagonistic effects on the activity of ATase. When the relative concentrations of α-KG are high, deadenylylation activity of the ATase is increased; when the relative level of glutamine is high, the adenylylation reaction is favoured. In addition, ATP stimulates that ATase to deadenylate. The small regulatory protein (P II), that interacts with ATase is a tetramer composed of four identical subunits (mol wt = 11,000; 194). It exists in two states. Unmodified PII (PII A) stimulates the adenylylation of GS by ATase, and uridylated P II (PIID) enhances the deadenylylated reaction. The
Fig. 3: Biosynthetic capacity of GS in E. coli (from 1-5'). Adenylylation and deadenylylation of GS results from the interaction of P_{HA} or P_{HD} with ATase and GS as described in the text. These reactions are stimulated, (+), or inhibited, (●), by the levels of various metabolites and by Mg^{2+} or Mn^{2+}. The interconversion of P_{HA} and P_{HD} by UTase and UR enzyme responds to the levels of these same metabolites. The UTase is also activated by either Mg^{2+} or Mn^{2+} while the UR enzyme is activated only by Mn^{2+}. Nonadenylated GS (E_0), which has maximum biosynthetic activity, requires Mg^{2+} to catalyze the formation of glutamine. As the adenylylation state of the enzyme increases, the Mg^{2+}-dependent biosynthetic capacity decreases and the enzyme exhibits some biosynthetic activity when Mn^{2+} replaces Mg^{2+}. Fully adenylylated GS (E_{12}) will catalyze the biosynthetic reaction at a low rate in the presence of Mn^{2+} but not Mg^{2+}. Adenylylated GS is more sensitive to inhibition (●) by various endproducts of glutamine metabolism than is the nonadenylated form of the enzyme.
modified form of PII is the result of a covalent attachment of a UMP residue to PIIA by the action of uridylyl transferase (UTase). The activity of this enzyme is also modulated by the concentrations of αKG and glutamine as indicated in the Fig. 3. The uridylyl residue is removed by a uridylyl-removing enzyme (UR), which may be the same protein as the UTase. The adenylylated state of the GS protein is affected by the relative levels of glutamine and αKG, not only in experiments with cell extracts (195), but also in whole cells (196).

The GS in enteric bacteria grown in the presence of excess ammonia tends to be more adenylylated than in nitrogen-limited cultures (193, 196–201). The absolute level of GS protein in these cells is inversely related to the availability of nitrogen in glucose containing minimal medium (193, 194, 196, 197, 202–206). In a nitrogen limited culture, the level of GS is five to tenfold higher than that observed in cells grown with excess ammonia, and represents several percent of the total cell protein. However, the absolute intracellular level of GS can vary over 100-fold in other growth media. Thus, cells grown in nutrient broth (200, 207), in glucose minimal medium supplemented with certain aminoacids (205, 207), in
minimal medium with certain other carbon sources (206), contain extremely low levels of highly adenylylated GS. Bender and Magasanik (206), have suggested that the extremely low rate of GS synthesis in cells grown in such medium does not result simply from repression by ammonia but rather reflects the ratio of glutamine to α-KG. This notion follows from the proposal (208, 209) of Magasanik and co-workers, that the absolute levels of GS in wild type cells reflects the adenylylation state of the enzyme. Earlier experiments by Senior (196), using cells from ammonia-limited chemostat cultures strongly suggested, that the adenylylation state of GS in E. coli reflects the intracellular ratio of α-KG to glutamine. The work of Stadtman and his co-workers (192-195, 210), helped to make the above prediction. They recently showed (211-213) that the state of adenylylation of GS is determined by the concentrations of many metabolites that affect the activities of the converter enzymes, ATase, UR and UTase. Most important of these are α-KG and glutamine which, in opposition to one another, exert reciprocal effects on the modification and demodification reactions. α-KG inhibits the adenylylation but stimulates the deadenylylation of
GS, whereas glutamine has opposite effects on these reactions. Also, $\alpha$-KG stimulates the deuridylylation of PII D. It is therefore evident that the ratio of $\alpha$-KG to glutamine is of primary importance in regulating the state of GS adenylylation.

Following the discovery of a regulatory role for GS in *K. pneumoniae* (214), a similar role for it was investigated in *Rhizobium*. Evidences have accumulated that GS does play a part in the regulation of nitrogen fixation in *Rhizobium* (202, 215, 216), although this investigation has been complicated by the finding of Darrow and Knotts (217), that *R. japonicum* contains two forms of GS, GS I and GS II. Recent studies have shown that another slow-growing strain, *Rhizobium* sp. 32 HI, also contains two forms of GS. GS I, which is reversibly adenylylated and repressed only two-fold by ammonium, plays a major role in nitrogenase regulation, whereas GS II may be required for purine biosynthesis (218). Fuchs and Keister (219), have identified two distinct GS in *Agrobacterium* and in several strains of fast growing *Rhizobium*, and concluded that the possession of GS II appears to be a specific attribute of members of the *Rhizobiaceae* (220).
PRESENT INVESTIGATION

The present investigation deals with some of the biochemical aspects of symbiotic relationship between *Rhizobium meliloti* and *Trigonella foenum-graecum*.

The regulation of ammonia assimilatory enzymes viz., glutamate dehydrogenase (GDH), glutamine synthetase (GS), and glutamate synthase (GOGAT), was studied in *R. meliloti*. The effect of nitrogen and carbon sources in the culture medium was seen on the activities of GDH, GS, and GOGAT.

GS plays a major role in the nitrogen metabolism. The importance of the enzyme in the regulation of nitrogen metabolism of *Rhizobium*, has been complicated by the discovery of two forms of GS, GS I and GS II. Out of these two forms, GS I plays a major role in the regulation of nitrogenase. Thus it was of interest to study the regulation of GS I in *R. meliloti*. Data described here include the regulatory aspects of GS I from *R. meliloti*, with regard to its response towards divalent ions, nucleotide triphosphates, and end-products of glutamine metabolism.

Since, nitrogenase is an oxygen sensitive enzyme, oxygen which is required to produce ATP, must be prevented from inactivating the nitrogenase. Organisms generally contain catalase and peroxidase enzymes which
detoxify hydrogen peroxide \( (H_2O_2) \), and superoxide dismutase which detoxify oxygen radical \( (O_2^-) \). \( H_2O_2 \) and \( O_2^- \) are toxic products of the oxygen metabolism.

The probable role played by these oxidative enzymes, concomitant with the ontogeny of *Trigonella foenum-graecum* root nodules, and the corresponding changes in the nitrogenase levels have been studied. The interrelationship between the oxidative enzymes, maintenance of reducing power, and nitrogen fixation have also been stressed.

The study was made to ascertained the probable source of reducing power required by nitrogenase. The present study deals with the biochemical changes associated with the development of nodules during the growth cycle of *Trigonella foenum-graecum* plants. The probable role played by dehydrogenase enzymes like malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, concomitant with the development of nodules and corresponding changes in the nitrogenase levels, have been emphasized. The interrelationship between the dehydrogenase enzymes, generation of reducing power and keto acids, detoxification of excess ammonia produced by bacterioidal nitrogenase, and nitrogen fixation have been examined.
Nitrogenase synthesis is repressed by ammonia if accumulated in higher amounts. Ammonia generated by the bacteroidal nitrogenase is secreted out into the nodule cytosol. To prevent the excess accumulation of ammonia, the host plant must have very efficient ammonia assimilatory mechanisms to make productive use of all the ammonia secreted. An attempt has been made in the present study to ascertain the probable pathway for ammonia assimilation in root nodules of *Trigonella foenum-graecum*.
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