CHAPTER VI

CONCLUSIONS
The fixation of nitrogen by rhizobia and its subsequent channelization into different nitrogenous products is carried out by a co-ordinately regulated array of enzymes. The nitrogen which is eventually exported to the shoot system is either in the form of ureides, amino acids or substituted amides. The form in which the nitrogen is exported is characteristic of a given legume. In soybean, a ureide exporter, the major amino acid exported is asparagine. Investigations were carried out to establish the presence of asparagine synthetase (AS) in both free-living *R. japonicum* 01 (Chapter III section 1) and during the nodulation of soybean by this strain (Chapter III section 2).

*R. japonicum* 01 grown on different nitrogen sources showed higher levels of AS than glutamine synthetase (GS). To rule out the possibility that GS in the crude enzyme preparation was interfering with the AS assay, two approaches were adopted. In the first the sensitivity of AS to L-methionine DL-sulfoximine (MSX) was checked. At a MSX concentration of 1mM the activity of AS was not reduced significantly whereas GS was totally inhibited. In the second, the proteins were resolved on a 7.5% polyacrylamide gel and the enzyme activities of both AS and GS were localized. It was seen that both activities appeared at distinctly different REMs. Both these findings showed that the AS activity was totally distinct from that of GS.
The factors which regulate enzymes involved in ammonia assimilation are numerous. Certain parameters which could probably regulate *R. japonicum* 01 AS, were studied. It was seen that the level of carbon and nitrogen sources used could regulate the levels of AS. Keto acids *in vivo* (2-oxoglutarate and oxaloacetate) were seen to increase the enzyme levels whereas *in vitro* the effect was not significant. Both the adenylates used, ADP and AMP, were seen to inhibit AS activity and would probably be involved in the regulation of AS *in vivo*.

The affinity of AS for hydroxylamine was lower than that for ammonia (apparent Km for hydroxylamine 16.67mM and for ammonia 8.69mM). This lower affinity for hydroxylamine has been reported for AS from other systems also. This hydroxamate forming activity of *R. japonicum* 01 AS was Mn²⁺/Mg²⁺ dependant. In *L. arabinosis* however, Mg²⁺ supported only 70% of the Mn²⁺ dependant activity. The *R. japonicum* 01 enzyme was heat labile, losing its activity within five minutes at 60°C.

Asparagine synthetase has been shown to form asparagine using either ammonia or glutamine as the amino donor. In the case of the *R. japonicum* 01 enzyme, activities with glutamine were higher than those with ammonia, showing that *in vivo* the former substrate would probably be preferred over the latter.
An ATP hydrolysing activity of the enzyme was observed. Earlier, this activity has been used to monitor AS levels. However, the *R. japonicum* 01 AS could hydrolyse ATP irrespective of the presence of the substrates, aspartate and ammonia, but was dependent on the presence of Mn**++.** Hence, it would be likely that the enzyme hydrolysates ATP prior to the binding of aspartate or the amino donor. Such a reaction mechanism has been proposed for the *L. luteus* enzyme whereas the *E. coli* enzyme has been shown to bind ATP after binding aspartate.

An investigation of the *G. max - R. japonicum* 01 symbiosis over a 9 week period was conducted to establish the involvement of AS during nitrogen fixation. The enzyme was present in both the bacteroidal and nodule cytosolic fractions. Earlier reports have established that ammonia assimilatory enzymes are repressed in the bacteroids. However, these high levels of AS could be involved in the secondary reactions of ammonia assimilation and not ammonia assimilation *per se.*

The nodule cytosolic AS levels exhibited a pattern similar to the nitrogenase activity. This goes to show that AS was involved in nitrogen fixation. Of the other ammonia assimilatory enzymes analysed, glutamate dehydrogenase (GDH) showed the highest activity. Both, GS and GOGAT (glutamate synthase) activities were found to be quite low throughout the growth cycle of the plant. Such high levels of GDH have been reported earlier and hence under the experimental
conditions prevailing, GDH could be assimilating a large fraction of the nitrogen fixed by the bacteroids.

Further studies on both AS and GDH, showed that the enzymes were comparatively resistant to heat inactivation and their activities inhibited by adenylates. AS activity was also inhibited by low levels (1mM) of keto acids whereas GDH activity was reduced in the presence of glutamate and increased on addition of aspartic acid. These findings show that the intracellular metabolites could be involved in the regulation of these enzymes in vivo.

Glutamate dehydrogenase from the soybean root nodule cytosolic fraction predominantly showed the reductive aminating reaction which was NADH specific. Both NAD and NADP specific GDH enzymes have been shown to occur in plant systems. This activity was not dependent on the presence of a divalent cation as has been reported for other plant enzymes.

Asparagine synthetase from the nodule cytosolic fraction was found to be Mn$^{++}$/Mg$^{++}$ dependent and Co$^{++}$ supported 50% of this Mn$^{++}$/Mg$^{++}$ dependent activity. With regard to the ammonia affinity of the two enzymes, AS had a higher affinity (apparent $K_m = 6.06\text{mM}$) as compared to GDH (apparent $K_m = 20.0\text{mM}$). Activities of AS with the two physiological amino donors,
ammonium ion and glutamine, showed that both could support activity to the same extent.

These studies indicate that both AS and GDH are involved in ammonia assimilation in the soybean root nodules.

Though the oxygen concentration in the root nodule is regulated\textsuperscript{14}, the energy required for nitrogen fixation is probably generated by oxidative phosphorylation. In an attempt to justify this hypothesis, an investigation of ATPase from both \textit{R. japonicum} 01 grown under microaerophilic conditions (Chapter IV section 1) and the \textit{G. max - R. japonicum} symbiosis (Chapter IV section 2) was undertaken. Microaerophilic conditions were used since the bacteroids in the root nodules are present in an environment of low pO\textsubscript{2}\textsuperscript{14}. The ATPase was found to be released from the membrane during the extraction procedure employed. Buffers free of metal ions have been shown to solubilize this membrane bound enzyme\textsuperscript{15} and hence the same could have occurred in the case of \textit{R. japonicum} 01 enzyme. This was further supported by similar substrate specificities that were exhibited by both the soluble and membrane solubilized enzymes.

The partially purified enzyme showed that a divalent cation was essential for its activity. Mg\textsuperscript{++} supported the highest activity followed by Ca\textsuperscript{++}. The enzyme preparation also exhibited phosphatase activity but this activity was negligible as compared to the ATPase activity. As reported
for other ATPases, GTP and ATP were hydrolysed to the same extent. However, higher concentrations of ATP inhibited the ATPase activity showing that MgATP and not ATP, as in the case of other bacterial ATPases, is the substrate for the enzyme.

A number of ATPase species have been shown to be cold labile. The bacterial enzyme was found to lose its activity when stored below 10°C for >12 h. This cold inactivation was reversible when the enzyme preparation was held at temperatures above 10°C. The rate of reactivation was dependent on the reactivation temperatures employed. The other well documented property of ATPases, trypsin activation, was not exhibited by the R. japonicum 01 enzyme. This indicates the probable absence of a peptide inhibitor. The ATPase activity was seen to be activated by phospholipids (phosphatidyl-inositol and phosphatidylethanolamine). Such a property has also been exhibited by the E.coli enzyme.

To substantiate the hypothesis mentioned earlier, investigations were also carried out in the G.max - R. japonicum 01 symbiosis. ATPase levels from different fractions of the soybean root nodules were monitored over the nodulation period. The bacteroidal and mitochondrial enzymes showed high activities around the 5th to the 7th week after germination. This high enzyme activity coincided with the high rates of
nitrogen fixation as estimated by the acetylene reduction capacity of the root nodules. Since the enzyme preparations used for these assays were crude homogenates, the phosphatase activity under conditions similar to those used for ATPase was estimated. Negligible phosphatase activity was observed in all the samples analysed. Therefore, ATPase estimates were assumed to reflect actual enzyme levels. The ATPase activity is probably sufficient to provide the ATP required for nitrogen fixation.

Both the bacteroidal and nodule cytosolic enzymes were Mg\(^{++}\)/Ca\(^{++}\) dependent and the monovalent ions, K\(^{+}\) and Na\(^{+}\), supported less than half the optimal ATPase activity. Though ATPases have been shown to be Mg\(^{++}\) dependent and Na\(^{+}\)/K\(^{+}\) activated\(^{20}\), Mg\(^{++}\) activated enzyme species have also been reported\(^{21}\). The ATPase inhibitors used, quercetin C, ouabain and DCCD, inhibited both the bacteroid membrane solubilized and bacteroid cytosolic enzymes. This supports the assumption that the latter was the solubilized fraction of the membrane bound enzyme. The mitochondrial ATPase, on the other hand was not inhibited at all. The low concentrations of the inhibitors used could be responsible for these results.

Plant ATPases have been implicated in ion transport\(^{22}\). The high ATPase activities of the root nodule cytosolic fraction could thus represent an increased metabolic activity.
of the plant cells. Since these elevated enzyme activities coincide with the nitrogenase activity, the plant cells during the period of nodulation would be undergoing a high rate of metabolism.

This study implies that the requirement of large amount of ATP for nitrogen fixation could be met by oxidative phosphorylation. The elevated levels of ATPase from all the fractions analyzed lends support to this view.

During the genetic transformation of rhizobia, the cells undergo the development of a competence stage wherein the maximum number of transformants are obtained. The development of this competence stage is dependent on the presence of a medium rich in amino acids. An investigation (Chapter V) of the amino acid uptake prior to the development of competence in *R. japonicum* D211 showed that proline, glycine, alanine and cystine were taken up in significant quantities as compared to the other amino acids. An earlier study in *R. trifolii* has shown that glycine supplementation of the medium drastically enhanced the number of transformants. A similar study in *R. japonicum* D211 showed that proline enhanced the development of competence by an hour. The exact mechanism of this enhancement cannot be specified.

A number of competence specific protein species were
seen to be present in competent cells. These were found to be absent in noncompetent cells. Such membrane bound and cytosolic proteins have been implicated in genetic transformation. Since these proteins are competence specific, they must be involved in transformation. However, allocating appropriate roles to these proteins in *R.japonicum* genetic transformation processes is beyond the scope of the present investigation. This would form the basis of an interesting study in establishing the molecular events that take place during genetic transformation in *R.japonicum* D211.
REFERENCES


